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Influence of different chemical agents (H_2O_2, *t*-BHP and MMS) on the activity of antioxidant enzymes in human HepG2 and hamster V79 cells: relationship to cytotoxicity and genotoxicity

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We investigated activities of antioxidant enzymes (AEs), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in human HepG2 and hamster V79 cells treated with a scale of concentrations of hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BHP) and methyl methanesulfonate (MMS). Cytotoxicity and genotoxicity of these substances were evaluated simultaneously. We have found out that H_2O_2, *t*-BHP and MMS predictably induce significant concentration-dependent increase of DNA lesions in both cell lines. Cytotoxicity detected in V79 cells with help of PE test was in a good conformity with the level of DNA damage. MTT test has proved unsuitable, except for MMS-treated V79 cells. Compared with human cells HepG2, hamster cells V79 manifested approximately similar levels of SOD and CAT but ten times higher activity of GPx. Across all concentrations tested the most significant increase of activity of the enzyme CAT was found in H_2O_2- and *t*-BHP-treated HepG2 cells, of the enzyme SOD in *t*-BHP- and MMS-treated V79 cells, and of the enzyme GPx in H_2O_2-treated V79 cells. We suggest that stimulation of enzyme activity by the relevant chemical compounds may result from transcriptional or post-transcriptional regulation of the expression of the genes CAT, SOD and GPx. Several authors suggest that moderate levels of toxic reactants can induce increase of AEs activities, while very high levels of reactants can induce their decrease, as a consequence of damage of the molecular machinery required to induce AEs. Based on a great amount of experiments, which were done and described within this paper, we can say that the above mentioned principle does not apply in general. Only the reactions of *t*-BHP affected HepG2 cells were consistent with this idea.

Key words: human hepatoma HepG2 cells, Chinese hamster lung V79 cells, antioxidant enzymes, DNA damage, cytotoxicity

Oxidative stress occurs in a cell or in a tissue if the concentration of the reactive oxygen species (ROS) and free radicals exceeds their antioxidant capability [1]. As the highly reactive forms of oxygen from both exogenous and endogenous sources may be involved in the pathogenesis of many diseases [2] as well as in the process of aging [3], the acquisition of new knowledge about antioxidant defense mechanisms of cells is very important. Research of molecular bases of antioxidant responses may benefit from the use of established cultures of mammalian cells *in vitro*. We investigated relationships between the extent of DNA lesions induced by genotoxic agents with different mechanism of action (hydrogen peroxide – H_2O_2, *tert*-butyl hydroperoxide – *t*-BHP and methyl methanesulfonate – MMS) and the activity of the most important antioxidant enzymes (superoxide dismutase – SOD, glutathione peroxidase – GPx and catalase – CAT) in HepG2 and V79 cells cultured *in vitro*. Genotoxic effects of H_2O_2, *t*-BHP and MMS were investigated by the single cell gel electrophoresis and cytotoxic effects were evaluated by plating efficiency (PE) test and MTT assay.

Hydrogen peroxide (H_2O_2) occurs naturally at low levels in the air and water, in human and plant tissues and bacteria, and in some food and beverages. Exposure of humans to exogenous H_2O_2 can occur through inhalation, ingestion or skin contact mainly through hair dyeing/bleaching and tooth bleaching materials, pharmaceutical preparations and through ingestion in food. 3% solutions are used as a sanitizing mouthwash [4]. H_2O_2 is a common reactive oxygen intermediate produced endogenously by several physiological processes, such as inflammatory respiratory burst or oxidative phosphorylation.

It is a natural source of oxidative damage in cells, causing a spectrum of DNA lesions (including single and double strand breaks), damage of lipids, proteins and other macromolecules. Halliwell [5] concluded that the DNA-damaging effects of oxidative stress are connected either with the metal ion-catalyzed conversion of ROS into the highly reactive hydroxyl radicals (·OH) or with a series of metabolic events that initiate the activation of DNA-backbone cleaving nucleases within the cell. Daroui et al. [6] described H_2O_2-induced topoisomerase I that mediates DNA damage and cell death. Piperakis et al. [7] suggested that H_2O_2 activates caspase 3 (a cystein protease) that is one of the effectors of apoptosis and necrosis.

Tert-butyl hydroperoxide (*t*-BHP) is an organic peroxide used in a variety of oxidation processes which are common in the chemical industry. Organic peroxides can initiate explosive polymerization in materials with unsaturated chemical bonds and, like their inorganic counterparts, they are powerful bleaching agents. *Tert*-butyl hydroperoxide rapidly penetrates into mammalian cells and can be metabolized to free radical intermediates by cytochrome P450 or hemoglobin. These can subsequently initiate lipid peroxidation, affect cell integrity, and form covalent bonds with cellular molecules [8]. Alternatively, *t*-BHP can be rapidly converted by glutathione peroxidase to *t*-butyl alcohol and glutathione disulfide (GSSG), which is then converted to reduced glutathione (GSH) by GSSG reductase, resulting in pyridine nucleotide oxidation (NADP). Loss of GSH and oxidation of pyridine nucleotide are associated with altered Ca^{2+} homeostasis, which is considered to be a critical event in blebs on plasma membrane, an early sign of *t*-BHP-induced toxicity (taken from Hwang et al. [9]). Involvement of iron-catalyzed radical reaction in the induction of cytotoxicity by *t*-BHP described Ochi and Miyaura [10]. Guidarelli et al. [11] reported that at least some of the *t*-BHP-derived radical species are generated at the level of mitochondria *via* a Ca^{2+}-dependent mechanism. This mechanism leading to DNA strand breaks involves mitochondrial formation of H_2O_2 and is causally linked to the impairment of the ability of mitochondria to transport electrons from cytochrome *b* to cytochrome c_1. The loss of calcium homeostasis, a rapid increase of free cytosolic calcium concentration and activation of endonucleases contribute to the onset of cytotoxicity in *t*-BHP-treated cells. Barry and Eastman [12] demonstrated that the Ca^{2+}/Mg^{2+}-dependent endonuclease is the only endonuclease involved in apoptosis. It can be stated that *t*-BHP induces membrane alterations, GSH depletion, damages to cell proteins, arachidonic acid cascade mobilization, and mostly DNA damage [13].

As the third DNA-damaging agent we selected alkylation substance MMS, which has been used as an experimental model compound for several decades to elucidate the mutagenic mechanisms of alkylation compounds. As the main representatives with mutagenic activity there are thought to be O^6-methyl guanine and O^4-methyl thymidine, but it can be expected that other methylated bases (first of all N^3-methyl adenine) may also exhibit promutagenic activity. Fortini et al. [14] suggested that the DNA polymerase β mediates single nucleotide base excision repair (BER), which is the favorite pathway for repair of N-methyl purines, while oxidation-induced single strand DNA breaks, arising from oxidized abasic sites, are the substrate for long-patch BER. Alkylation of cellular macromolecules by reactive electrophiles contributes to chemical toxicity and oxidative stress of cells. Induction of oxidative stress by alkylation agents is frequently caused by depletion of intracellular antioxidant glutathione (GSH) that represents a nucleophilic tripeptide [15]. It has been postulated that the loss of glutathione-D-transferase may disturb cellular antioxidant defenses, with the consequent accumulation of reactive oxygen species generated as byproducts of normal cellular function. MMS belongs to simple alkylation agents that can be formed endogenously from cellular precursors, but they may also originate from exogenous sources such as diet, tobacco smoke or environmental pollution. Some of these sources may be so common that most humans are exposed to them in small amounts and it is difficult to distinguish between their exogenous and endogenous origin [16]. Examples are *N*-nitroso compounds occuring in smoked meat.

In our experiments we used two cell lines (i) human carcinoma cell line HepG2 derived from the liver tissue of fifteen years old male with differentiated hepatocellular carcinoma. These cells can synthesize hepatic plasma proteins and express specific drug metabolizing enzyme activities comparable to normal hepatocytes; (ii) near-diploid V79 fibroblastoid cells obtained from lung of male Chinese hamster, which are well characterized, grow fast, have a small number of chromosomes, and provide us with reproducible results. Though both HepG2 and V79 cells are used in experimental laboratories frequently, no detailed inquiries of the activities of the three basic antioxidative enzymes (SOD, GPx and CAT) have been done till now. This especially applies to the cells treated with different genotoxins. Our paper is focused to filling this gap.

Material and methods

Cell cultures. Human hepatoma HepG2 cells were obtained from Professor A.R. Collins (University of Oslo, Oslo, Norway) and near-diploid Chinese hamster lung V79 fibroblasts from Professor A. Abbondandolo (National Institute for Cancer Research, Genova, Italy). HepG2 cells were cultivated in William's E medium and V79 cells in DMEM medium. Both media were supplemented with fetal calf serum (10%) and antibiotics, penicillin (200 U/ml)/streptomycin (100 μg/ml) and kanamycin (100 μg/ml). Cells were growing at 37°C in a humidified atmosphere of 5% CO_2 on plastic Petri dishes (∅=10 cm).

Chemicals and their commercial suppliers. Hydrogen peroxide (H_2O_2; Sigma, Sigma-Aldrich Co., Steinheim, Germany; 10 M) was stored at 4°C and diluted in phosphate-buffered saline (PBS, Mg^{2+}- and Ca^{2+}-free) or complete DMEM medium to final concentrations of 0.025-2 mM

immediately before the treatment of HepG2 or V79 cells, respectively.

Tert-butyl hydroperoxide (*t*-BHP; Aldrich, Sigma-Aldrich Co., USA; Mw of *t*-BHP = 90.12, it is a 70% water solution). *T*-BHP was disolved in DMSO and diluted in serum-free medium (William's E or DMEM) to final concentrations of 17.5-2000 μM.

Methyl methanesulfonate (MMS; Sigma, Sigma-Aldrich Co., USA; 10 M) was diluted in PBS with Ca^{2+} and Mg^{2+} immediately before use. Final concentrations of 0.2-4 mM were used for the treatment of both cell lines.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), normal melting point (NMP) agarose and Triton X-100 were purchased from Sigma, Sigma-Aldrich Co., Steinheim, Germany; DMEM medium, fetal calf serum (FCS), kanamycin, penicillin/streptomycin and low melting point (LMP) agarose from Invitrogen™ Gibco® Life technologies Ltd., UK; William's E medium from PAN-Biotech GmbH, Germany; methylene blue from Fluka, Sigma-Aldrich Co., Buchs, Switzerland and phosphate-buffered saline (PBS; Mg^{2+}- and Ca^{2+}-free) from OXOID LIMITED, Basingstoke, UK.

Treatment of cells. The culture medium was sucked out off the monolayers of HepG2 and V79 cells growing exponentially in William's E or DMEM medium on Petri dishes or plastic 96-well plates at 37°C, and cells were treated with genotoxins as follows:

HepG2 cells were affected by H_2O_2 (0.025-1 mM) in PBS (Mg^{2+}- and Ca^{2+}-free) on ice for 5 min. V79 cells were exposed to H_2O_2 in complete DMEM medium for 30 min at 37°C (concentrations 0.1-2 mM).

Exposition to *t*-BHP (17.5-2000 μM) was in both cell lines done in serum-free media either for 60 min (HepG2 cells) or 30 min (V79 cells).

Treatment with MMS (0.2-4 mM) was performed in both cell lines in complete phosphate-buffered saline (PBS, with Ca^{2+} and Mg^{2+}) at 37°C for 30 min.

Cytotoxicity. *Plating efficiency (PE) test.* Exponentially growing V79 cells were treated according to the experimental protocol. Thereafter, they were trypsinized, soluted, and 300-500 cells/60 mm Petri dish were seeded (in threes) for determination of colony-forming ability. Cells were incubated in CO_2 incubator at 37°C for 7 days and then several drops of 1% methylene blue solution were added into each of the three Petri dishes for visualization of colonies. Cytotoxicity evaluation with help of colony-forming ability is a very sensitive test. PE is a measure of the number of colonies originating from single cells.

MTT assay. MTT assay is a colorimetric assay which measures the activity of enzymes to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a yellow tetrazolium salt. During incubation of cells with MTT, yellow tetrazolium salt is reduced to purple formazan in the mitochondria of living cells. This reduction occurs only when reductase enzymes are active; therefore it is often used as a measure of cell viability. We used the procedure described by Mosmann [17] with minor modifications. In brief, HepG2 or V79 cells were seeded in 96-well culture plates at the density of 2.5×10^4 HepG2 cells/well or 5×10^3 V79 cells/well in a volume of 200 μl. After 24-48 h incubation, the cells were treated with different concentrations of the genotoxins tested. After the treatment, the medium was removed and cells were incubated with 50 μl of MTT dye solution (1 mg/ml in PBS, Mg^{2+}- and Ca^{2+}-free) suspended in 100 μl of complete medium for 4 h at 37°C. Then, the medium was removed, DMSO was added to each well and plates were continuously shaken for 30 min. Photometric evaluation (at 540 nm excitation and 690 nm emission wavelengths) was carried out using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The viability of cells was calculated by following formula: $A_{\text{treated cells}}/A_{\text{control cells}}\times100\%$.

Single cell gel electrophoresis (SCGE; comet assay). The procedure of SCGE described by Singh et al. [18] and modified by Slamenova et al. [19] was used with minor adjustments. In brief, microscopic slides were coated with 1% NMP agarose in distilled water and were subsequently dried. The tested HepG2 or V79 cells (control cells or cells treated as mentioned in the section Treatment of cells) were placed on pre-coated microscopic slides (at a density of $2.5\text{-}3\times10^4$ cells/50 μl of 0.75% LMP agarose) and covered with a cover slip. After solidification of the gels, the cover slips were removed and slides were placed in lysis mixture (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH=10, 1% Triton X-100) for 1 h at 4°C. The samples were consequently transferred to an electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH>13) for 40 min at 4°C for unwinding and then subjected to electrophoresis at 25 V (current adjusted to 0.3 A) for 30 min at 4°C. Finally, slides were neutralized with 400 mM Tris-HCl (pH=7.5) twice for 10 min and stained with fluorescent dye (EtBr, 5 μg/ml). For evaluation of DNA damage as % DNA in the tail, at least 100 EtBr-stained nucleoids were scored for each slide with a Zeiss fluorescent microscope and the automated computerized image analyser Metafer 3.6 system (Meta Systems GmbH, Altlussheim, Germany).

Measurement of SOD, GPx and CAT activities. Human hepatoma HepG2 or hamster lung V79 cells exposed to different concentrations of genotoxins (as specified in chapter Treatment of cells) and the control cells (1×10^7 cells/aliquot) were solved 1:1 in 0.1% Triton X-100 for determination of superoxide dismutase (SOD, EC 1.15.1.1), glutathione peroxidase (GPx, EC 1.11.1.9) and catalase (CAT, EC 1.11.1.6) activities. To determine the activity of SOD, we used 5×10^4 HepG2 or 3×10^4 V79 cells and the commercial RANSOD kit (Randox Laboratories Ltd., UK). The method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The activity is measured by the degree of reaction inhibition. The enzyme activities of the three most discussed types of

SOD (Cu/Zn-SOD, Mn-SOD and Fe-SOD) were measured together. For GPx determination according to the method of Paglia and Valentine [20], we used 5×10^4 HepG2 or 3×10^4 V79 cells and cumene hydroperoxide as a substrate. Catalase activity was determined according to Goth [21]. Samples of 5×10^5 cells were incubated with H_2O_2, as a substrate, at 37°C for 60 s. The enzymatic reaction was stopped with ammonium molybdate and the yellow complex of molybdate + H_2O_2 was measured at 405 nm. Specific activity of CAT was expressed as U/mg protein. One unit of CAT activity was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2/min. Similarly the activities of SOD and GPx were expressed as U/mg of protein. The protein concentrations were determined using the Bradford method.

In untreated cells the activity of SOD represents approx. 1200 U/mg of protein (HepG2 cells) and approx. 1274 U/mg of protein (V79 cells), the activity of GPx represent approx. 4 U/mg of protein (HepG2 cells) and 36 U/mg of protein (V79 cells), and the activity of CAT represent approx. 442 U/mg of protein (HepG2 cells) and approx. 400 U/mg of protein (V79 cells). These values were changed (more or less) in those control cells which were incubated in conditions used for the application of H_2O_2, *t*-BHP and MMS (5 min on ice in PBS buffer, 30 or 60 min in serum-free medium at 37°C, 30 min in PBS buffer at 37°C). All activities of SOD, GPx and CAT in the treated cells were therefore recalculated towards corresponding values in control groups representing value 1.

Statistical analysis. The results are presented as means from at least three sets of independent experiments (4 parallels in each experiment) ± standard deviation (SD). The differences between defined groups were tested for statistical significance using Student's *t*-test ($^{*,\#}p<0.05$; **, $^{\#\#}p<0.01$; ***, $^{\#\#\#}p<0.001$).

Results

H_2O_2 increases the level of DNA lesions in HepG2 and V79 cells. In this study we investigated DNA-damaging effects of H_2O_2 by the single cell gel electrophoresis (SCGE). The maximum level of DNA lesions was achieved in HepG2 cells at 0.4 mM H_2O_2 (Figure 1A) and in V79 cells at 1.4 mM H_2O_2 (Figure 2A). The reason of these differences rests in different manner of H_2O_2 application. HepG2 cells were treated with H_2O_2 for 5 min in PBS on ice and V79 cells (in which the ability to form colonies was measured) were treated with H_2O_2 in complete medium at 37°C. Both methods are satisfactory, however, 5 min treatment of cells on ice is more effective. Cytotoxic effects of H_2O_2 were studied in HepG2 cells by MTT assay and in V79 cells by both MTT assay and PE test. Results are presented in inserts in Figures 1A and 2A.

H_2O_2 induces significant increase of the enzyme CAT in HepG2 cells and the enzyme GPx in V79 cells. Activity of AEs was assayed in both cell lines at around equal genotoxicity of H_2O_2. Figures 1B and 2B represent activities of antioxidant enzymes SOD, GPx and CAT, expressed as multiples of untreated controls. In HepG2 cells exposed to graded concentrations of H_2O_2 we observed low, but statistically significant stimulation of SOD activity at concentration 0.1 mM, stimulation of GPx activity at concentration 0.4 mM and stimulation of CAT activity at all concentrations tested (0.05-0.4 mM, Figure 1B). In V79 cells all tested concentration of H_2O_2 (i.e. 0.2-1.6 mM) increased only the activity of the enzyme GPx (Figure

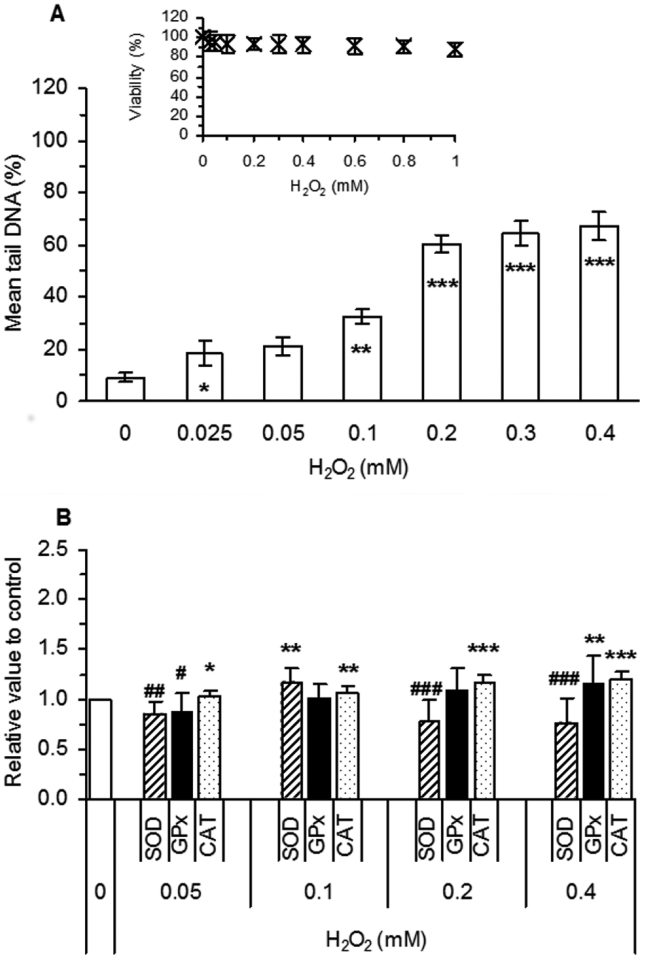

Figure 1. The influence of H_2O_2 on HepG2 cells. Part A – The influence of various H_2O_2 concentrations on the level of DNA strand breaks measured by the comet assay technique. The data represent means ± SD from 3 experiments. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ indicate significant differences compared to the control. Insert in Figure 1A shows the results of MTT test. Part B – The activities of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in HepG2 cells treated with H_2O_2. White bar represents the activity of enzymes in cells without treatment (expressed as relative value of 1). Individual enzymes are presented as stripped (SOD), black (GPx) and stippled (CAT) bars, expressed as relative values to 1. The data represent means ± SD of 3-5 independent experiments. *, $^{\#}p<0.05$; **, $^{\#\#}p<0.01$; ***, $^{\#\#\#}p<0.001$ indicate significant increases or decreases compared to the control, respectively.

2B). Other concentrations of H_2O_2 either did not change or decreased the activity of enzymes in both cell types in comparison with controls.

***Tert*-BHP increases the level of DNA lesions in HepG2 and V79 cells.** DNA-damaging effects of *t*-BHP was assayed by the comet assay and cytotoxic effects by MTT assay (HepG2 cells) and by MTT assay and PE test (V79 cells) equally as we did in the case of H_2O_2. With regard to a very different generation time of HepG2 (36 h) and V79 cells (12-15 h) the treatment of cells with *t*-BHP in serum-free medium has taken 60 min in HepG2 cells and 30 min in V79 cells. Results showed a gradual, concentration dependent increase of DNA lesions in HepG2 cells (Figure 3A). In V79 cells, which are very sensitive to toxic effects of *t*-BHP (see results of PE test, Figure 4A, insert) there was the increase of DNA lesions less pronounced, but statistically significant (Figure 4A).

***Tert*-BHP induces significant increase of the enzyme CAT in HepG2 cells and the enzyme SOD in V79 cells.** HepG2 cells exposed to graded concentrations of *t*-BHP manifested

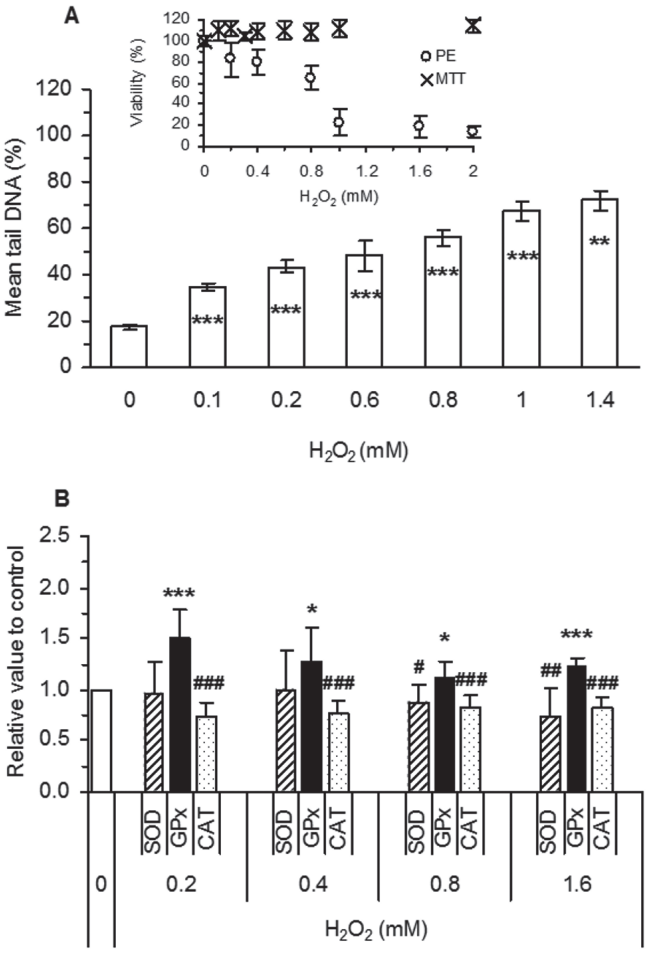

Figure 2. The influence of H_2O_2 on V79 cells. Part A – The influence of various H_2O_2 concentrations on the level of DNA strand breaks measured by the comet assay technique. The data represent means ± SD from 3 experiments. $^{**}p<0.01$; $^{***}p<0.001$ indicate significant differences compared to the control. Insert in Figure 2A shows the results of MTT test: × and PE test: ○. Part B – The activities of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in V79 cells treated with H_2O_2. White bar represents the activity of enzymes in cells without treatment (expressed as relative value of 1). Individual enzymes are presented as stripped (SOD), black (GPx) and stippled (CAT) bars, expressed as relative values to 1. The data represent means ± SD of 3-5 independent experiments. *, $^{\#}p<0.05$; $^{\#\#}p<0.01$; ***, $^{\#\#\#}p<0.001$ indicate significant increases or decreases compared to the control, respectively.

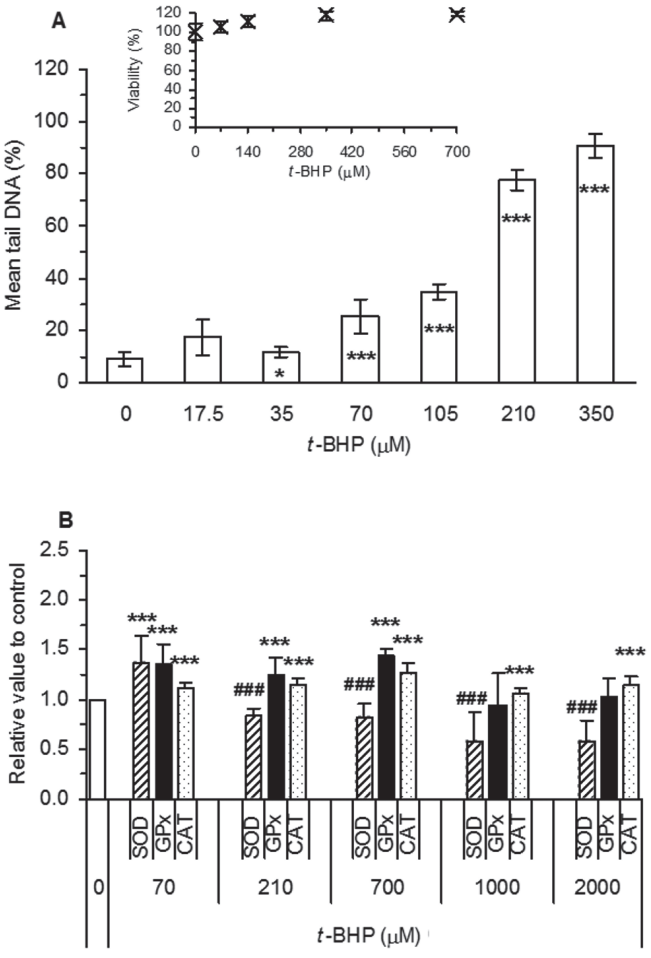

Figure 3. The influence of *t*-BHP on HepG2 cells. Part A – The influence of various *t*-BHP concentrations on the level of DNA strand breaks measured by the comet assay technique. The data represent means ± SD from 3 experiments. $^{*}p<0.05$; $^{***}p<0.001$ indicate significant differences compared to the control. Insert in Figure 3A shows the results of MTT test. Part B – The activities of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in HepG2 cells treated with *t*-BHP. White bar represents the activity of enzymes in cells without treatment (expressed as relative value of 1). Individual enzymes are presented as stripped (SOD), black (GPx) and stippled (CAT) bars, expressed as relative values to 1. The data represent means ± SD of 3-5 independent experiments. ***, $^{\#\#\#}p<0.001$ indicate significant increases or decreases compared to the control, respectively.

statistically significant increase of SOD activity only at concentration 70 μM (Figure 3B). Higher concentrations of *t*-BHP caused rather strong decrease of SOD activity. The activity of GPx in HepG2 cells was increased at concentrations <700 μM (Figure 3B), and increase in CAT activity was detected at all concentrations (70-2000 μM, Figure 3B). In contrast *t*-BHP-treated V79 cells manifested increased activity of SOD at all concentrations tested (70-560 μM), while the activities of GPx and CAT were reduced mainly at lower concentrations of *t*-BHP (Figure 4B).

MMS increases the level of DNA lesions in HepG2 and V79 cells. Similarly to the case of H_2O_2 and *t*-BHP, we evaluated DNA-damaging effect of MMS by SCGE method and cytotoxicity of MMS by PE and MTT tests. Results are presented in the Figures 5A and 6A. While MTT test did not show any cytotoxic effect of MMS in HepG2 cells (neither at concentrations which significantly damage DNA, Figure 5A, insert), V79 cells manifested reduced viability in PE test and at higher MMS concentrations also in MTT test (Figure 6A, insert).

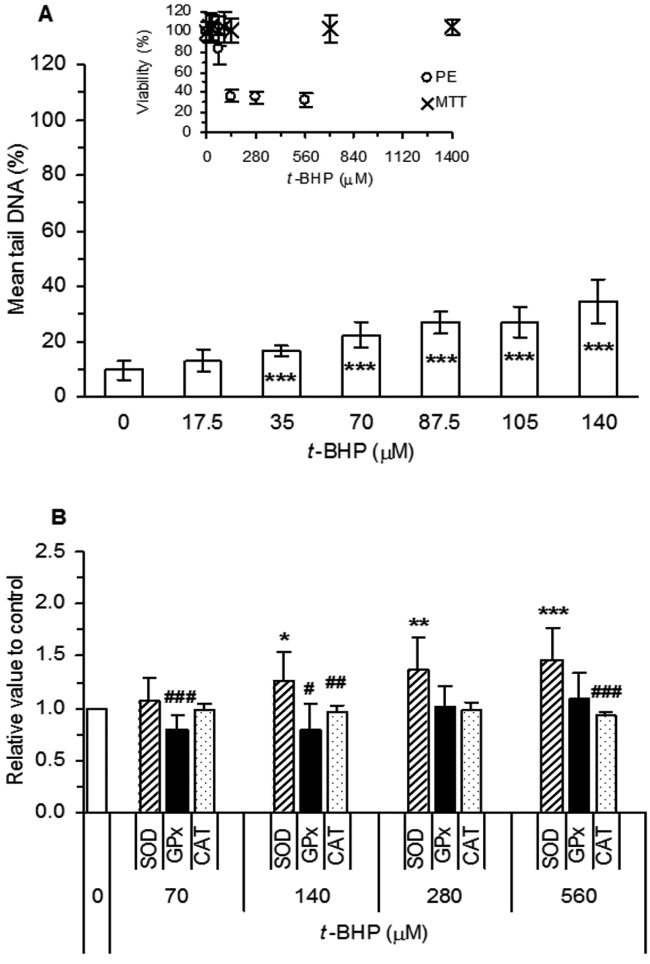

Figure 4. The influence of *t*-BHP on V79 cells. Part A – The influence of various *t*-BHP concentrations on the level of DNA strand breaks measured by the comet assay technique. The data represent means ± SD from 3 experiments. ***p<0.001 indicate significant differences compared to the control. Insert in Figure 4A shows the results of MTT test: × and PE test: ○. Part B – The activities of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in V79 cells treated with *t*-BHP. White bar represents the activity of enzymes in cells without treatment (expressed as relative value of 1). Individual enzymes are presented as stripped (SOD), black (GPx) and stippled (CAT) bars, expressed as relative values to 1. The data represent means ± SD of 3-5 independent experiments. *, $^{\#}$p<0.05; **, $^{\#\#}$p<0.01; ***, $^{\#\#\#}$p<0.001 indicate significant increases or decreases compared to the control, respectively.

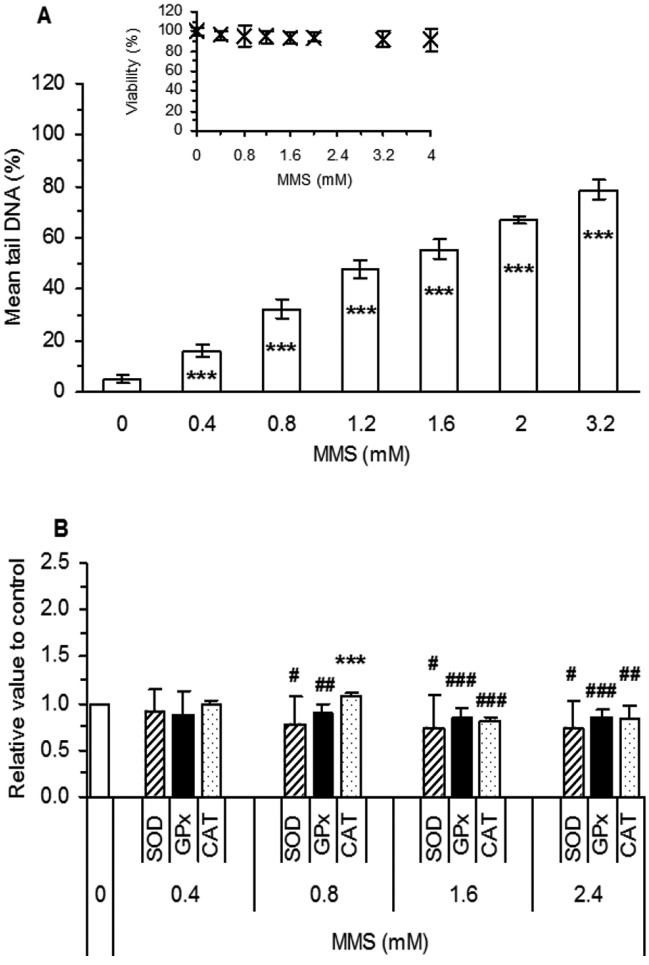

Figure 5. The influence of MMS on HepG2 cells. Part A – The influence of various MMS concentrations on the level of DNA strand breaks measured by the comet assay technique. The data represent means ± SD from 3 experiments. ***p<0.001 indicate significant differences compared to the control. Insert in Figure 5A shows the results of MTT test. Part B – The activities of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in HepG2 cells treated with MMS. White bar represents the activity of enzymes in cells without treatment (expressed as relative value of 1). Individual enzymes are presented as stripped (SOD), black (GPx) and stippled (CAT) bars, expressed as relative values to 1. The data represent means ± SD of 3-5 independent experiments. $^{\#}$p<0.05; $^{\#\#}$p<0.01; ***, $^{\#\#\#}$p<0.001 indicate significant increases or decreases compared to the control, respectively.

MMS induces significant increase of the enzyme SOD in V79 cells. Figures 5B and 6B represent activities of SOD, GPx and CAT expressed as multiples of controls. MMS-treated HepG2 cells showed, at all tested concentrations, reduced level of SOD as well as GPx. Only the activity of CAT was increased at concentration 0.8 mM. On the other hand, V79 cells manifested significantly increased level of SOD in the concentration range 0.4-2.4 mM. Activities of GPx and CAT were reduced.

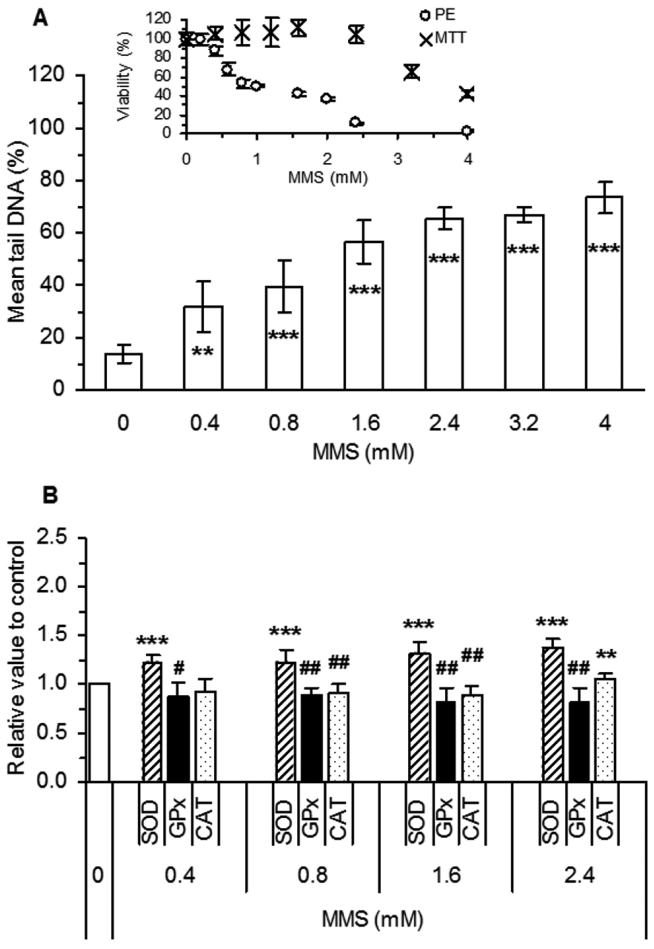

Figure 6. The influence of MMS on V79 cells. Part A – The influence of various MMS concentrations on the level of DNA strand breaks measured by the comet assay technique. The data represent means ± SD from 3 experiments. $^{**}p<0.01$; $^{***}p<0.001$ indicate significant differences compared to the control. Insert in Figure 6A shows the results of MTT test: × and PE test: ○. Part B – The activities of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in V79 cells treated with MMS. White bar represents the activity of enzymes in cells without treatment (expressed as relative value of 1). Individual enzymes are presented as stripped (SOD), black (GPx) and stippled (CAT) bars, expressed as relative values to 1. The data represent means ± SD of 3-5 independent experiments. $^{\#}p<0.05$; **, $^{\#\#}p<0.01$; $^{***}p<0.001$ indicate significant increases or decreases compared to the control, respectively.

Discussion

Oxidatively modified DNA is, despite extensive DNA repair, abundant in many human tissues. ROS are formed in living cells continuously, as a consequence of metabolic and other biochemical reactions, as well as external factors [16]. To protect cells against dangerous effects of free radicals organisms have evolved several enzymatic as well as non-enzymatic defense mechanisms that are capable of removing, neutralizing, or scavenging ROS, RNS, and their intermediates. Oxidative stress may be caused by increased exposure to oxidants or decreased protection against them. Both of these problems may even occur simultaneously. The most important antioxidant enzymes represent superoxide dismutases [22], occurring in four forms.

One of these, located in the cytosol and inter-membrane space of mitochondria of eukaryotic cells, contains copper and zinc (Cu/Zn-SOD). This SOD is entirely unrelated, except for its activity, to the other three. There are 2 kinds of SODs that contain manganese (Mn-SOD). The fourth type of SOD contains iron (Fe-SOD) and has been found in periplasmic cells of *E. coli*. Superoxide dismutases are believed to be present in all oxygen-metabolizing cells and in extracellular fluids but they are lacking in most obligate anaerobes. SOD enzymes catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide ($O_2 + O_2 + 2H = O_2 + H_2O_2$). H_2O_2 resulting from this reaction is converted to H_2O in the peroxisomes by the antioxidant enzyme catalase (CAT), and in the cytoplasm by the enzyme glutathione peroxidase (GPx). Catalases use either an iron or manganese cofactor; their only substrate is hydrogen peroxide. The glutathione peroxidase (GPx) is an enzyme, containing four selenium-cofactors, which catalyses the breakdown of hydrogen peroxide and organic hydroperoxides. The glutathione system (glutathione, glutathione reductase, glutathione peroxidase and glutathione-S-transferase) occurs at a particularly high level in liver. Non-enzymatic antioxidants represent small molecular weight compounds, which directly scavenge free radicals and related reactants: α-tocopherol (vitamin E), β-carothene (vitamin A), ascorbate (vitamin C), glutathione, estrogens, creatine (a nitrogenous compound), xanthophylls (yellow pigments related to carothene), flavonoids (aromatic oxygen heterocyclic compounds that are highly distributed in higher plants) and many other compounds.

Molecular basis of changes in enzyme activity may consist in transcriptional and/or post-transcriptional regulation of gene expression. Sequence analysis of the mouse GPx and CAT genes had revealed putative binding motifs for NF-κB and AP-1, transcriptional regulators that are activated in response to oxidative stress in various tissues [23]. Two different characteristic regulatory elements, the antioxidant responsive element (ARE) and xenobiotic responsive element (XRE) have been located in the promoter region of the Cu/Zn-SOD in human liver cells [24]. Wakabayashi et al. [25] and other authors e.g. Bercht et al. [26] stated that induction of various

cytoprotective proteins and glutathione levels is in eukaryotic cells up-regulated by the transcription factor NRF2, which is activated by both oxidants and alkylation agents via the sensor protein KEAP1. DNA repair genes have not been identified among the various targets of NRF2. Kou et al. [27] suggested that both induction of antioxidative enzymes and induction of phase II detoxifying enzymes are mediated by a *cis*-acting element ARE. In the expression of ARE mediated genes the transcription factor NRF2 (NF-E2-related factor 2) plays a key role. Activation of this pathway by natural compounds contained in fruits and vegetables protects cells from oxidative stress-induced cell death. On the other hand, elevated levels of ROS may also lead to the activation of tumor-inducing transcription factors and their corresponding tumor-inducing genes. Together with increased level of DNA damage this may, in some cases, create a selection pressure for the malignant phenotype seen in cancer [28]. Li et al. [29] found that one of the primary antioxidant enzymes manganese superoxide dismutase (Mn-SOD) has the ability to reverse malignant phenotype. However, a variety of human tumor cells is low or absent in Mn-SOD expression. These authors suggested that tumor suppression by over-expressing Mn-SOD is related to a modulation of activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB). These cause a down-regulation of genes responsible for tumor malignant phenotype. This fact is in a good conformity with an older discovery of Oberley and Buettner [30], who found only diminished or no level of Mn-SOD in all tumors studied. These findings imply that the antioxidant enzymes can be induced by the transcription factors to defend cells from oxidative stress.

Applegate et al. [31] reported that antioxidant enzymes are only slightly inducible by the oxidants in mammalian cells cultured *in vitro*. In contrast, Christova et al. [32] assumed that exposure to different cytotoxic agents often leads to changes in antioxidant enzymes expression. It follows that the cell survival critically depends on the ability of cells to respond to oxidative stress by induction of different antioxidant enzymes. Similarly Rodriguez et al. [33] and some other authors hypothesized that moderate levels of toxic reactants induce rise in antioxidant enzymes activities, while high levels of reactants reduce enzymatic activities. This can be a result of molecular machinery damage required for their induction.

In our study we tried to verify these different views utilizing two cell lines cultured *in vitro* treated with three types of chemical compounds: H_2O_2, *t*-BHP and MMS. In all cases we studied survival, induction of single strand DNA breaks and the activity of antioxidant enzymes SOD, GPx and CAT. H_2O_2 induces lesions similar to those resulting from ionizing radiation. It rapidly crosses cell membranes through water channels (aquaporins), reaches the nucleus and in the presence of the metal ions it is converted to free hydroxyl radicals ($OH^{\cdot}$). OH radicals attack DNA backbone and modify purines and pyrimidines to their hydroxyl derivatives. It is supposed that exogenous H_2O_2 could be at least partially converted inside the cells to $H_2O + O_2$ by reductive processes catalyzed by the enzymes CAT and GPx similarly to endogenously emerging H_2O_2. PE test used in our experiments showed significant reduction of colonies in H_2O_2-treated V79 cells (similar results of PE test were received by Bose (Girigoswami) et al. [34]). MTT assay did not show the cytotoxic effect of H_2O_2 either in HepG2 or in V79 cells. We cannot explain exactly the reasons why MTT test did not reflect reduced viability of cells treated with H_2O_2 but it could not be excluded that a longer time for the restitution of oxidoreductase enzymes was needed. As it was mentioned in part Material and methods, NAD(P)H-dependent cellular oxidoreductase enzymes reflect the number of viable cells as they are capable of reducing the tetrazolium dye (MTT) to its insolubile formazan, which can be measured colorimetrically. Our assumption is supported by finding of Sarkar and Sil (35) who found out that *t*-BHP-induced loss of cell viability (measured by MTT test) was time-dependent up to 180 min of post-*t*-BHP treatment.

We observed that H_2O_2 induced the most significant increase of the enzyme CAT in HepG2 cells and the enzyme GPx in V79 cells. The level of SOD was increased in HepG2 cells only at concentration 0.1 mM while in V79 cells it was not increased at all. Dittmar et al. [3], who investigated the role of oxidative DNA damage in human aging, revealed that in blood of older people (that contains a higher amount of oxidative DNA lesions) the SOD/GPx ratio is lowered. This means that the activity of GPx is elevated and the amount of DNA lesions is inversely related to SOD activity. HepG2 cells exposed to higher doses of H_2O_2 (0.2-0.4 mM) and V79 cells exposed to a scale of H_2O_2 concentrations (0.2-1.6 mM) acted according to this model.

Among the array of chemical compounds inducing oxidative stress *in vitro*, *t*-BHP is an important agent used in experiments with cell cultures. *T*-BHP decomposition to alkoxyl and peroxyl radicals, in which metal ions assist, induces creation of ROS including H_2O_2 in cells. This leads to lipids peroxidation, damage to DNA, cell toxicity, depletion of glutathione and to the altered intracellular calcium homeostasis and apoptosis [36]. We proved previously that *t*-BHP promotes formation of oxidative-labile sites and single strand DNA breaks in HepG2 cells [37]. MTT assay did not show any toxic effects of *t*-BHP either in HepG2 or in V79 cells. The same negative results of MTT assay were obtained by Lapshina et al. [38] in Chinese hamster B14 cells treated with *t*-BHP (100-1000 μM) during 60 min. On the contrary, Alia et al. [39] proved, with help of LDH method, cytotoxicity of *t*-BHP (200 and 500 μM) after 3 h treatment of HepG2 cells. Similarly other results described in the literature are varied and depend greatly on the experimental conditions and on the method used for cytotoxicity testing of *t*-BHP. It is evident that not all methods are suitable. We observed that *t*-BHP induces the most significant increase of the enzyme CAT in HepG2 cells and the enzyme SOD in V79 cells. The activities of GPx were in V79 cells reduced mainly at lower concentrations. Selective inhibition of GPx (without effect on glutathione reductase activity) was described in *t*-BHP-treated V79 cells also by Ochi

and Miyaura [10]. These authors observed *t*-BHP-induced depletion of glutathione (GSH, a radical scavenger) causing increased cytotoxicity in V79 cells. They concluded that the cellular antioxidant system cannot efficiently play a protective role against exogenous hydroperoxides. We have shown that unlike V79 cells, HepG2 cells treated with *t*-BHP (70-700 μM) did not manifest any reduction of GPx. Differences between the effects of *t*-BHP on metabolically active HepG2 cells and metabolically inactive V79 cells are explicable, as *t*-BHP is metabolized to free radicals by cytochrome P450 in HepG2 cells and it is also converted by the glutathione system leading to glutathione depletion. In V79 cells *t*-BHP is converted only by the glutathione system. Reduction of GPx in *t*-BHP-treated V79 cells is therefore obvious. Significant increase of ROS and GPx levels in *t*-BHP-treated HepG2 cells was also described by Goya et al. [40], who studied a protective effect of the olive oil phenol hydroxytyrosol on *t*-BHP-influenced HepG2 cells. We assume that *t*-BHP in HepG2 cells stimulates (at a certain concentration range) the activity of all antioxidant enzymes studied and this stimulation is in a good correlation with the degree of DNA damage.

MMS reacts with DNA and other nucleophils by the so-called SN_2 mechanism (a bimolecular reaction). The extent of cellular methylation by MMS is 81-85% of N^7-guanine and 0.8% of O^6-guanine of the total DNA methylation. MMS is able to directly break DNA strands, but it does not manifest any strong mutagenic or carcinogenic activity. Pohanka [41] suggested that there exist numerous examples of the alkylation-induced oxidative cell injury. Liu et al. [42] reported that the alkylating agents provide the depletion of the enzyme glutathione-S-transferase and the generation of ROS in mammalian cells. This may play a role not only in the MMS-induced genotoxicity but also in activation of cellular antioxidant defense. The increased activity of SOD and reduced activities of GPx and CAT, which we observed in MMS-treated V79 cells, were identical in *t*-BHP-treated V79 cells, characterized by depletion of glutathione. The inhibition of the glutathione redox cycle (glutathione reductase and glutathione peroxidase) was proved also in renal proximal tubular cells damaged by alkylation nefrotoxicant DCVC [43], in Swiss female mice fed with pellet diet containing bifunctional alkylating agent sulfur mustard [15] and in humans damaged by chemical warfare weapons containing sulfur mustard [41]. Also various other authors consider that cytotoxicity and genotoxicity of alkylation agents rest in depletion of reduced glutathione (GSH) and subsequent lipid peroxidation and free radical generation.

Based on all the results obtained we can conclude that regarding induction of antioxidant enzymes, HepG2 and V79 cells do not respond equally to chemical influence of H_2O_2, *t*-BHP and MMS. We found that stimulation of CAT in HepG2 cells (by H_2O_2 and *t*-BHP), SOD in V79 cells (by *t*-BHP and MMS) and GPx in V79 cells (by H_2O_2) could result from transcriptional or post-transcriptional regulation of the expression of the genes CAT, SOD and GPx. One of the objectives of our work was to verify the hypothesis that moderate levels of reactants induce a rise of the enzymatic activities and high levels of reactants reduce this induction. Only findings obtained in *t*-BHP-treated HepG2 cells correlated with this hypothesis. Moreover, our results showed that detection of the activities of SOD, GPx and CAT in cells treated with chemical agents couldnot be considered an appropriate indicator of genotoxicity.

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