Mutation Research 702 (2010) 86–91

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

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Community address: www.elsevier.com/locate/mutres

Oxidative DNA damage contributes to the toxic activity of propylparaben in mammalian cells

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ARTICLE INFO

Article history:
Received 3 March 2010
Received in revised form 23 June 2010
Accepted 16 July 2010
Available online 2 August 2010

Keywords:
Propylparaben
DNA damage
Cytotoxicity
Cell-cycle arrest
Oxidative stress

ABSTRACT

Propyl *p*-hydroxybenzoate, commonly referred to as propylparaben, is the most frequently used preservative to inhibit microbial growth and extend shelf life of a range of consumer products. The objective of this study was to provide further insight into the toxicological profile of this compound, because of the current discrepancy in the literature with regard to the safety of parabens.

The Vero cell line, derived from the kidney of the green monkey, was selected to evaluate the adverse effects of propylparaben by use of a set of mechanistically relevant endpoints for detecting cytotoxicity and genotoxic activities. Our results demonstrate that exposure to the compound for 24 h causes changes in cell-proliferation rates rather than in cell viability. A significant and dose-dependent decline in the percentage of mitotic cells was observed at the lowest concentration tested, mainly due to cell-cycle arrest at the G0/G1 phase. Immunodetection techniques revealed that induction of DNA double-strand breaks and oxidative damage underlies the cytostatic effect observed in treated Vero cells. Additional studies are in progress to extend these findings, which define a novel mode of action of propylparaben in cultured mammalian cells.

1. Introduction

Parabens are alkyl esters of *p*-hydroxybenzoic acid that are extensively used as preservatives in the food, pharmaceutical and cosmetic industries due to their broad antimicrobial spectra and low toxicity [1]. In the past few years, these compounds have been characterized as emerging pollutants with an ubiquitous presence in sewage influents and effluents from treatment plants [2,3], surface waters [4,5] and indoor dust [6].

Despite the fact that parabens are considered as relatively safe compounds with a low bioaccumulation potential [1], their detection in human fluids [7,8] and human breast tumors [9] is attracting considerable attention nowadays. Furthermore, it has been unequivocally demonstrated in various *in vivo* and *in vitro* screening tests that parabens have endocrine-disrupting activity that may represent a potential risk to human health [10]. In contrast to the detailed knowledge on interference of parabens with the endocrine system, much less is known about their potential effects on basic cellular processes.

Propyl *p*-hydroxybenzoate, commonly referred to as propylparaben (PPB), is the most frequently used preservative to inhibit microbial growth and extend the shelf life of a range of consumer products [11]. The results of a variety of *in vitro* and *in vivo* studies, reviewed extensively [1,11], have shown that PPB is practically a non-toxic compound. The European Union authorizes the use of PPB in cosmetic products with a maximum concentration of 0.4% (EU Cosmetics Directive 76/768/EEC), but the most recent regulatory reviews recommend its withdrawal in foods, because of well-documented adverse effects [12]. Consequently, PPB safety is again in question and there is an ongoing debate regarding the potential harmful effects on human health, caused by unwitting and continued exposure to this common preservative.

It is generally recognized that cell-based assays can provide reliable information about the mechanistic basis of chemical-induced effects [13,14], and play an important role in the development of integrated testing strategies for chemical hazard identification [15]. Nevertheless, it should be noted that endpoints used to determine potential toxicity must be carefully selected and evaluated because certain chemicals may give different results, depending on their specific mechanism of action [16]. The present study was conducted on Vero cells, an *in vitro* model particularly effective for the toxicological analysis of chemicals [17], by use of a set of mechanistically relevant endpoints for detecting cytotoxicity and genotoxic activity. Our results provide additional data regarding the toxic effects induced by PPB in mammalian cells, which should be considered for future *in vivo* studies.

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doi:10.1016/j.mrgentox.2010.07.012

2. Materials and methods

2.1. Cell culture and PPB treatments

Vero cells (derived from monkey kidney) were grown at 37 °C in 75-cm^2 flasks (Falcon, Becton Dickinson, USA) under a 5% CO_2 humidified atmosphere, with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all from Lonza, Switzerland). Exponentially growing cells were seeded at a density of 80,000 cells/ml into 24-well plates for quantitative evaluation, or into 12-well plates containing a sterile glass cover-slip in each well, for microscopy studies. After removing cell culture medium and washing in phosphate-buffered saline (PBS), Vero cells were incubated for 24 h with new medium containing serial dilutions of PPB (CAS No. 94-13-3). Stock solutions of PPB (Sigma, USA) were prepared in dimethyl sulphoxide (DMSO) and maintained in the dark at room temperature. The working solutions, ranging from 50 to 500 μM (9.01–90.1 μg/ml) were freshly prepared in DMEM and sterilized by filtration through a 0.22-μm Millipore® filter. Maximum DMSO concentration in medium was 0.5% including the control groups.

2.2. Cytotoxicity assessments

Three colorimetric assays were used to evaluate the cytotoxic activity of propylparaben. Neutral red uptake (NRU) into the lysosomes of viable cells was evaluated as described by Borenfreund and Puerner [18]. In brief, after PPB exposure, culture medium was replaced with new medium containing 50 μg/ml neutral red (Merck, Germany). Following an incubation period of 3 h, the medium was removed and intracellular dye was extracted by addition of 50% aqueous ethanol containing 1% acetic acid. The MTT assay, which involves reduction of the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) by viable cells to purple formazan, was performed according to Mossmann [19]. Briefly, after PPB treatments, cells were incubated for 2 h with MTT (Sigma) in DMEM at a final concentration of 0.5 mg/ml. The medium was then replaced with DMSO in order to solubilize the formazan. Cell growth and/or cell detachment was estimated by quantifying total protein content (TPC) according to the method of Bradford [20], with Coomassie® Brilliant Blue G-250 reagent and bovine serum albumin (Sigma) as standard.

The absorbance values at appropriate wavelengths were recorded with a Spectrafluor microplate reader (Tecan, Austria).

2.3. Proliferation assays

Alterations in cell proliferation were assessed by microscopic analysis of the mitotic index and by flow cytometry. Variation in mitotic index after treatment with PPB was determined in cells cultured on cover-slips, fixed with ice-cold methanol for 6 min and stained with 5 μg/ml Hoechst 33258 (Riedel de Haen, Germany). Three thousand cells were counted per experimental point and the mitotic index was calculated as the ratio between the number of cells in mitosis and the total number of cells.

To analyze cell-cycle distribution, Vero cells treated with 500 μM PPB were collected by trypsinisation and fixed with ice-cold 70% ethanol. After gentle washing with PBS, cells were re-suspended in 1 ml of staining buffer consisting of 0.1% sodium citrate, 50 μg/ml propidium iodide and 50 μg/ml RNase A (all from Sigma) and incubated in the dark for 30 min. Nuclei were then analyzed by use of a Coulter Epics XL-MCL flow cytometer with the Expo 32 ADC software (Beckman Coulter Inc., USA).

2.4. Evaluation of genotoxic damage

To further investigate PPB toxicity in Vero cells, induction of DNA double-strand breaks (DSBs) was examined by indirect immunofluorescence against the phosphorylated form of the variant histone H2AX (γ-H2AX). Possible oxidative DNA damage was evaluated by immunocytochemical analysis of 8-hydroxydeoxyguanosine (8-OHdG). For immunofluorescence, cells were fixed in 10% formaldehyde for 10 min, washed twice in PBS, permeabilized with 0.5% Triton X-100 (Sigma) and blocked in PBS with 5% BSA at room temperature. The cover-slips were incubated with mouse monoclonal anti-γ-H2AX (Upstate, Lake Placid, NY; 1:500 dilution) for 1 h in a humidified chamber at 37 °C. Following extensive washing in PBS, samples were incubated for 45 min at 37 °C with secondary, FITC-conjugated anti-mouse IgG antibody (1:1000 dilution; Sigma). The cover-slips were subsequently rinsed in PBS, counterstained for 1 min with 5 μg/ml Hoechst 33258 and mounted in ProLong Gold anti-fading reagent (Invitrogen, UK). From at least three different experiments, a total of 1000 randomly chosen interphases were scored per slide, and divided into cells with and without DSB depending on positive label to γ-H2AX.

Immunocytochemical detection of 8-OHdG was carried out essentially as described by Yarborough et al. [21] with minor modifications. Cells were fixed with cold acetone for 10 min and treated with 100 μg/ml RNase A (Sigma) for 1 h at 37 °C. Following DNA denaturation for 5 min at 4 °C (70 mM NaOH, 0.14 mM NaCl, dissolved in 40% ethanol) samples were treated with 0.1% Triton X-100 for 5 min at 4 °C. After blocking endogenous peroxidase for 10 min with 3% H_2O_2 at 4 °C and non-specific antibody binding with 5% BSA in PBS for 10 min, the slides were incubated overnight at 4 °C with 5 μg/ml anti 8-OHdG antibody (JaICA, Japan) and subsequently with biotin-conjugated secondary antibody at 37 °C for 10 min and streptavidin-peroxidase for another 10 min (both from Vector Laboratories). Finally, cells were treated with diaminobenzidine for 3 min to localize peroxidase and analyzed by means of light microscopy after mounting cover-glasses with Eukitt® (Fluka, Germany). The signal intensity of the 8-OHdG immunostaining of at least 100 randomly selected cells per concentration was measured by use of the Image J 1.41 software (National Institutes of Health, USA). The formula used for quantification was as follows: 8-OHdG index = $\Sigma[(X - \text{threshold}) \times \text{area}\ (\mu^2)]$/total cell number, where X is the staining density indicated by a number between 0 and 256 on a grey-scale.

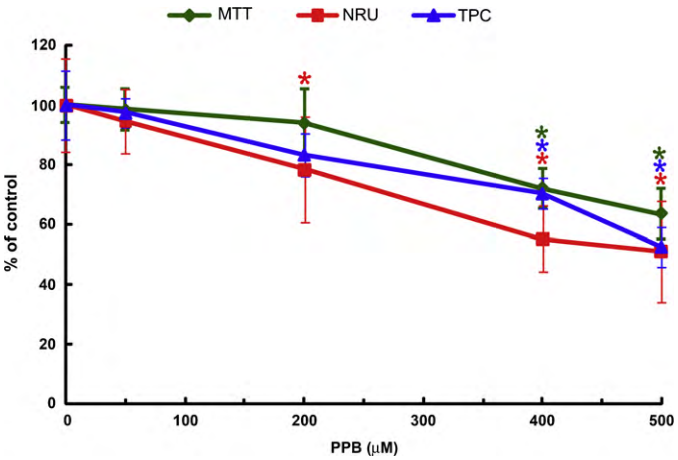

Fig. 1. Dose–response curves obtained in the three cytotoxicity assays after treatment of Vero cells with increasing PPB concentrations for 24 h. Data are expressed as percentage of the values found in the respective control cultures. Asterisks indicate statistically different values in treated cells compared with control cells.

2.5. Microscopy

Microscopic observations were carried out by means of an Olympus BX-61 epi-fluorescence microscope (Tokyo, Japan) equipped with an HBO 100-W mercury lamp and ultraviolet (UV, 365 nm), blue (450–490 nm) and green (546 nm) excitation filters. The images were acquired with a CCD camera Olympus DP-70 and processed with the software Olympus DP controller 1.1.1.65, and Adobe Photoshop 8.0 (Adobe Systems Inc.). All comparative images (treated *vs* untreated samples) were obtained under identical microscope and camera settings.

2.6. Statistical analysis

Statistical analysis was performed by use of SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) and Microsoft® Excel 2007. The results were analyzed by Student's *t*-test for comparing paired samples and analysis of variance (ANOVA) with Bonferroni as *post hoc* test for multiple samples. Differences were considered statistically significant at $p \leq 0.05$. Each data point represents the arithmetic mean ± standard deviation of at least three independent experiments. EC50 (50% effective concentration) values were obtained via non-linear regression. Partial correlation coefficients (r) were calculated to assess the association between the various measured parameters.

3. Results

3.1. Cytotoxic effects of PPB in Vero cells

The three biochemical assays performed in mammalian cells indicated that a 24-h exposure to PPB induces a dose-dependent cytotoxic effect (Fig. 1). It was not possible to obtain EC50 values, since in all the cases they were greater than the highest concentration tested. A significant correlation between TPC results and both MTT ($r = 0.84$, $p \leq 0.0001$) and NRU data ($r = 0.77$, $p \leq 0.0001$) was obtained, suggesting that PPB causes primarily a reduction of cell number in treated cultures.

To confirm the possible interference of the compound with proliferative activity of Vero cells, the mitotic index was estimated after a 24-h exposure to increasing concentrations of PPB. As shown in

Mitotic index (% of control)				
Control	PPB 50 μM	PPB 200 μM	PPB 400 μM	PPB 500 μM
100.0 ± 11.8	74.4 ± 15.4*	42.5 ± 0.1*	30.7 ± 18.0*	24.0 ± 6.9*

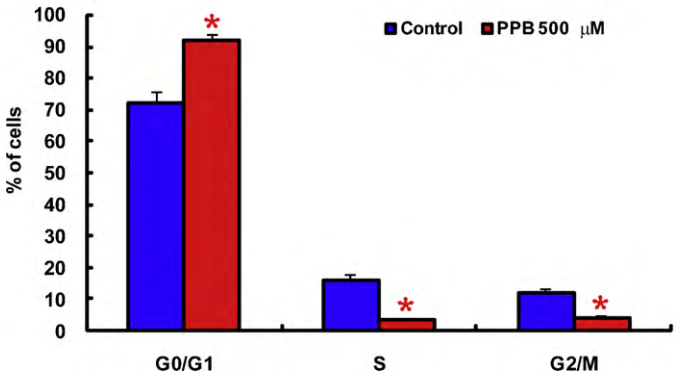

Fig. 2. Effect of PPB on Vero cell proliferation after a 24-h exposure period. The top panel shows mitotic index values expressed as percentage of that found in respective untreated cultures. The bottom panel shows cell-cycle profiles of cells treated with 500 μM PPB. Data represent mean ± SD from at least three independent determinations and asterisks indicate statistically different values in treated cells compared with control cells.

Fig. 2, a significant and dose-dependent decline in percentage of mitotic cells was observed, from the lowest concentration tested, reaching values approximately 4 times smaller than that of the control at 500 μM. In this case, flow-cytometric analysis of DNA content was performed, revealing a significant G0/G1 cell-cycle arrest, with a concomitant decrease in the proportion of cells in the S and G2/M phases.

3.2. DNA damage caused by PPB in Vero cells

Exposure of Vero cells to PPB for 24 h led to the appearance of nuclear γ-H2AX foci, an early indicator of the presence of DNA double-strand breaks. However, as shown in Fig. 3, 500 μM was the only concentration that significantly increased by two-fold the percentage of γ-H2AX-positive cells, when compared with untreated control values.

Interestingly, in the same experimental conditions, Vero cells were also positive for 8-OHdG staining. Control cultures exhibited a faint background labelling that was gradually increased in treated cells, to reach the maximum intensity at 500 μM PPB (Fig. 4). Calculation of the 8-OHdG index indicated a significant induction of oxidative DNA damage from the lowest concentration tested (Fig. 5).

4. Discussion

Because of their widespread use in cosmetics and body-care products, the potential toxicity of parabens has been extensively studied both *in vivo* and *in vitro* to assess a variety of adverse effects relevant to human health. Despite this, controversial results have been published and consequently further information is required to meet current regulatory standards. The aim of this study was to

γ-H2AX-positive cells (%)				
Control	PPB 50 μM	PPB 200 μM	PPB 400 μM	PPB 500 μM
4.96 ± 1.60	5.00 ± 0.28	5.10 ± 0.14	5.05 ± 0.19	7.97 ± 0.04*

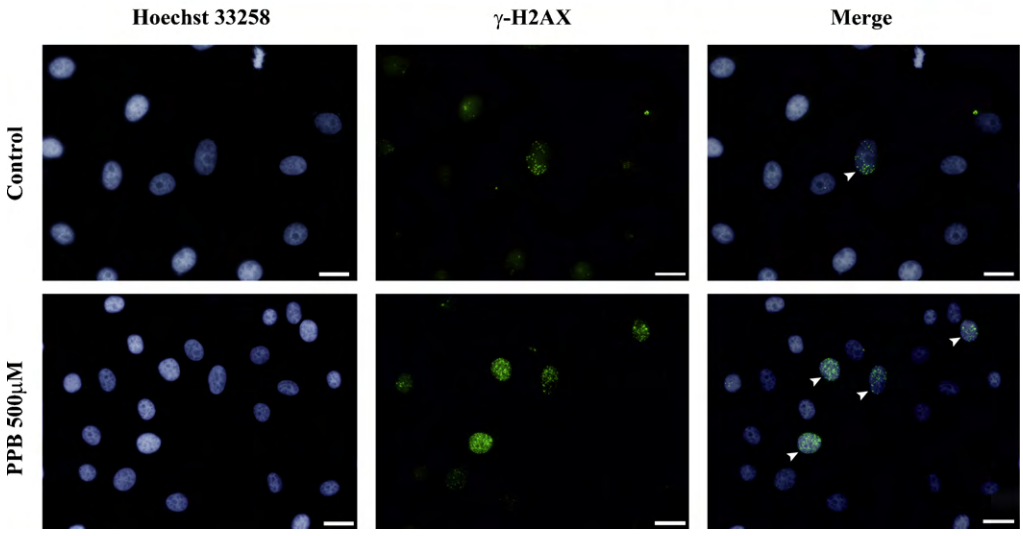

Fig. 3. Formation of double-strand breaks in Vero cells following PPB treatments for 24 h. Top panel: γ-H2AX-positive cells expressed as percentage of that found in respective untreated cultures. Bottom panel: γ-H2AX immunofluorescence after a 24-h exposure to 500 μM PPB. Arrowheads indicate γ-H2AX-positive nuclei. Bar: 20 μm.

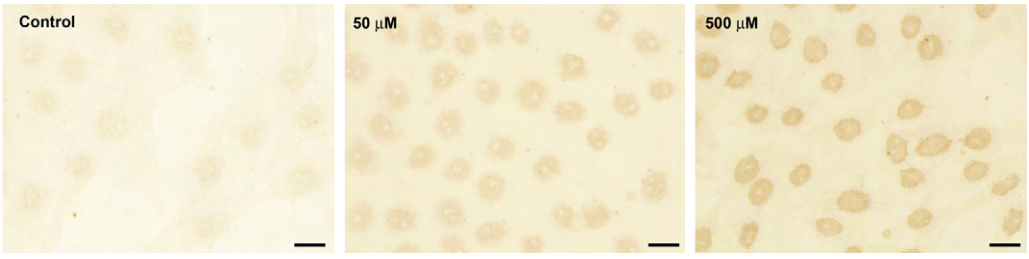

Fig. 4. Representative immunocytochemical staining for 8-OHdG in Vero cells treated with PPB for 24 h. Bar: 20 μm.

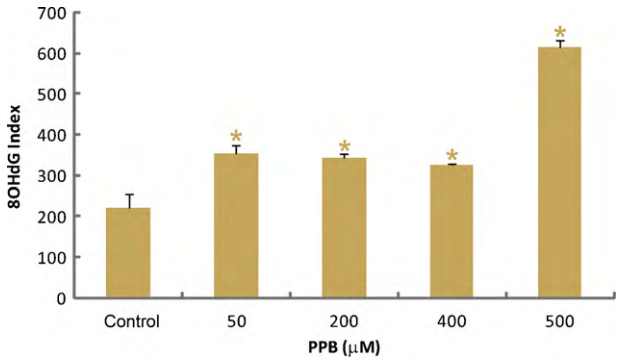

Fig. 5. 8-OHdG index obtained in Vero cells following a 24-h exposure to increasing concentrations of PPB. Asterisks indicate statistically different values in treated cells compared with control cells.

evaluate the cytotoxicity and genotoxicity of PPB in short-term *in vitro* experiments that represent the starting point in tiered testing strategies for chemical risk assessment.

Since a multitude of cellular events are involved in toxicity, a comprehensive *in vitro* study requires multi-parametric quantitative assays in order to mitigate the significant shortcomings of single-parameter measures. In a first set of experiments, we determined the cytotoxic effects induced by PPB on Vero cells by three established *in vitro* assays. The results of MTT and NRU methods, used to estimate metabolic activity and membrane integrity respectively, revealed a gradual decrease in cell viability upon treatment with PPB for 24 h. However, a significant positive correlation ($p \leq 0.0001$) was observed over the entire concentration range tested between both viability assays and total protein content that reflects the cell number in monolayer cultures. The similarity of the above results suggests that, under our experimental conditions, the compound did impair cell growth but did not appear to specifically interfere with critical cell structures such as mitochondria and membranes. These findings seem to be in contrast with previous research conducted in mitochondria and hepatocytes isolated from rat liver, indicating that cytoxicity caused by PPB is mediated primarily through mitochondrial damage [22,23]. However, it is worth mentioning that concentrations used by other authors to elucidate the mechanisms of action of parabens were about four times higher than those used in the present study. On the other hand, it has been reported that PPB readily interacts with model membrane systems leading to important changes in biophysical properties [24,25], although this effect has not been observed in complex cellular models. Therefore, the discrepancy between the results of the above-mentioned studies and our data are most likely related to the marked differences in the experimental approaches used to examine the toxicological profile of the compound.

To further investigate the mechanism underlying cell-growth inhibition induced by PPB, the mitotic index and cell-cycle profiles were analyzed in treated Vero cells. We observed that PPB reduced the mitotic activity, even at concentrations that did not change the cell density significantly (less than 400 μM). The decrease in the number of mitotic cells was dose-dependent, reaching near-zero values at 500 μM when about 52% of cell confluence was estimated using the protein assay. Flow-cytometric analysis of DNA content revealed a G0/G1 phase-arrest of the cell cycle, which confirms the cytostatic activity of PPB in Vero cells. Proliferating cells can halt the cell cycle, at virtually any transition point, by activating a number of signal-transduction pathways called checkpoints that ensure accurate duplication and segregation of the genetic material [26]. The possible causes of G1 checkpoint arrest are diverse and include exposure to ionizing or UV radiation, genotoxic chemicals and oxidative stress [27]. Irrespective of the stimulus, transient arrest at the G1/S boundary provides additional time to repair DNA damage but, if errors in this process occur, cells trigger programmed death responses or enter a senescence state [28–31]. Our results reveal that cell-cycle arrest was concomitant with the appearance of discrete γ-H2AX nuclear foci in treated cells. There is compelling evidence that histone H2AX becomes rapidly phosphorylated on Ser-139 (γ-H2AX), by ATM (ataxia-telangiectasia mutated) kinase, in response to DNA double-strand breaks (DSBs) [32–34].

It is well established that a causal link exists between the generation of DSBs and the induction of mutations and chromosomal damage, as well as the triggering of apoptotic cell death [29,35]. In this regard, mutagenicity testing of PPB in conventional bacterial and yeast assays primarily gave negative results [11], whereas chromosome aberrations and sister chromatid exchange have been recently reported in CHO-K1 cells exposed to PPB at concentrations of 500 μM and above [36]. However, despite these observations little is known about the mechanisms responsible for the genotoxic effect of this paraben preservative on mammalian cells. In an attempt to understand the generation of DSBs under our experimental conditions, the possible involvement of oxidative stress was investigated, since the presence of γ-H2AX could indicate oxidative DNA lesions during progression through the cell cycle [37,38]. An important finding to emerge from the present study is that increased levels of 8-OHdG were detected in Vero cells treated with PPB, even at the lowest concentrations used. 8-OHdG is one of the major oxidative adducts, formed by a variety of chemicals with different mechanisms of action [39], which is widely recognized as a sensitive marker of oxidative stress [40]. Previous studies have suggested that different paraben derivatives may potentiate oxidative damage in the skin [41], enhance ROS production in UVB-exposed HaCaT keratinocytes [42] and cause lipid peroxidation in kidney and liver of Swiss albino mice, both *in vitro* [43,44] and *in vivo* [45]. However, to the best of our knowledge, this report reveals for the first time the ability of propylparaben to cause oxidative stress coupled with DNA damage, in a mammalian cell system. Oxidatively induced DNA lesions have been demonstrated to possess a potential role in the initiation, promotion, and progression stages of carcinogenesis [46,47]. This is of particular concern in the case of parabens, since considerable debate exists regarding the possible involvement of these compounds in tumorigenesis, through oestrogenic and genotoxic activities [10].

The extent of DNA damage caused by oxidants is the consequence of a balance between lesion induction from radical processes and repair capacity. The results presented in this study reveal that, while the γ-H2AX fluorescence signal was only present in Vero cells exposed to 500 μM PPB, significant oxidative DNA damage, expressed as 8-OHdG index, was detected over the entire range tested. It is generally recognized, although some controversy exists, that γ-H2AX nuclear foci gradually disappear as DNA-repair progresses [48,49]. Consequently, it could be reasonably assumed that DSBs caused by PPB through induction of oxidative DNA damage, were efficiently repaired in Vero cells treated with concentrations in the range of 50–400 μM. On the other hand, the persistence of the fluorescent γ-H2AX signal in cells exposed to 500 μM PPB, would probably be a sign of incomplete repair of DSBs. Alternatively, it has been shown that γ-H2AX may remain elevated after DSB rejoining, until total removal of γ-H2AX molecules from the chromatin was achieved, or as a result of defective DNA-repair processes [50,51].

Our data, although simplified with respect to the *in vivo* situation, indicate the need for further research on the genotoxic effects of parabens in mammalian cells. An important question that remains to be resolved concerns the identity and source of the radicals responsible for oxidative DNA damage. It is generally recognized that 8-OHdG is mainly produced by hydroxyl radicals, singlet oxygen or direct photodynamic action [52,53], although

the possible participation of reactive intermediates of parabens cannot be ruled out. In particular, phenoxy radicals may play a role here, which result from peroxidase-dependent activation of phenolic compounds that may react directly with dG to yield C8-dG O-adducts [54]. Likewise, in view of the fact that humans are simultaneously exposed to a large number of environmental pollutants, the possible synergistic or antagonistic effects between PPB and other chemical compounds in the body must be considered in future toxicological studies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Education and Science (CTM2005-02135, CTM2008-00311). The authors thank Sylvia Gutierrez Erlandsson from the Cytometry and confocal microscopy service at the Centro Nacional de Biotecnología for technical assistance. A. Peropadre is a recipient of a contract from Community of Madrid (Contrato de Personal Investigador de Apoyo).

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