EVALUATION OF OKADAIC ACID-INDUCED GENOTOXICITY IN HUMAN CELLS USING THE MICRONUCLEUS TEST AND γH2AX ANALYSIS

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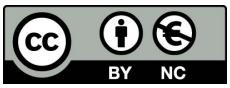
ABSTRACT Marine algal blooms have become a public health concern due to increasing frequency in the environment and severity of exposure consequences. Human intoxications produced by phycotoxins occur globally through consumption of marine fish products containing bioaccu-mulated toxins. Okadaic acid (OA) is the main representative of diarrheic shellfish poisoning (DSP) toxin. OA was found to inhibit protein phosphatases and to produce oxidative damage, as well as to disturb different cellular functions including cell cycle, gene expression, and DNA repair mechanisms. The aim of this study was to determine whether OA induced genotoxic-ity by using a micronucleus (MN) test and γH2AX analysis, and to elucidate the underlying mechanisms. Human peripheral blood leukocytes, neuroblastoma cells (SHSY5Y), and hep-atoma cells (HepG2) were treated with a range of OA concentrations in the presence and absence of S9 fraction. MN induction was observed in leukocytes at all concentrations tested, and in SHSY5Y and HepG2 cells only at the highest concentration (1000 n*M*). In contrast, γH2AX analysis was only positive for HepG2 cells. Taking together these data, in addition to the comet assay results obtained in a previous study in this issue, OA was found to exert a either a clastogenic or aneugenic effect dependent upon the cell types examined.

This is an Accepted Manuscript version of the following article, accepted for publication in *Journal of Toxicology and Environmental Health, Part A*.

Vanessa Valdiglesias, Blanca Laffon, Eduardo Pásaro & Josefina Méndez (2011) Evaluation of Okadaic Acid-Induced Genotoxicity in Human Cells Using the Micronucleus Test and γH2AX Analysis, Journal of Toxicology and Environmental Health, Part A, 74:15-16, 980-992, DOI: 10.1080/15287394.2011.582026



Harmful algal blooms (HAB) have emerged as a global concern due to the increase in environmental occurrence and severity of exposure consequences on fishing, fish, and shellfish cultures and marine ecosystems (Vale and Botana 2008; Franchini et al. 2010). Harmful algal blooms are produced by excessive and prolonged overgrowth of algae and other plant-like organisms such as dinoflagellates, diatoms, and cyanobacteria. It is well known that HAB deplete oxygen levels in the aquatic environments, produce metabolites that result in undesirable taste and odor, and, more importantly, produce natural toxins (Dionysiou 2010).

Diarrheic toxins are a group of marine toxins responsible for human poisoning associated with seafood consumption (Yasumoto et al. 1978), termed diarrheic shellfish poisoning (DSP). Diarrheic toxins are present most abundantly and frequently along European coasts (Aune and Yndestad 1993). Included within the DSP toxins are okadaic acid (OA) and at least eight related congeners (Van Dolah and Ramsdell 1992). It was reported that OA poses a threat to human health not only as a DSP toxin but also at a molecular level since OA is an inhibitor of DNA and RNA synthesis (Matias et al. 1996), is a potent and specific inhibitor of Ser/Thr protein phosphatases (Bialojan and Takai 1988), and induces crucial cellular alterations such as cell cycle disruption (Ghosh et al. 1992; Gotoh et al. 1995; Lerga et al. 1999), increase in apoptotic rate (Morimoto et al. 1997; Cabado et al. 2004; Lago et al. 2005), cytoskeleton modulation (Vale and Botana 2008), and changes in

expression levels of different genes (Chin et al. 2000; Ao et al. 2008).

Oakadaic acid is a well-established genotoxic agent since it was reported to induce micronuclei (MN) in CHO cells (Le Hégarat et al. 2006) and DNA strand breaks in Caco2 cells (Traoré et al. 2001) as well as in leukocytes, SHSY5Y cells, and HepG2 cells (Valdiglesias et al. 2010). In the same cell lines OA produced oxidative DNA damage (Creppy et al. 2002, Valdiglesias et al. 2011a), DNA adduct formation in BHK21 C13 fibrob-lasts and HESV keratinocytes (Fessard et al. 1996), and minisatellite mutations in NIH 3T3 cells (Nakagama et al. 1997). However, results reported are contradictory. Some authors suggested that OA was aneugenic (Le Hégarat et al. 2004; Carvalho et al. 2006), while others proposed a clastogenic effect for this toxin (Traoré et al. 2001). There are also studies reporting that OA needs to be metabolically activated to exert its mutagenic action (Nakagama et al. 1997; Le Hégarat et al. 2003; 2004), contrasting with other investigations indicating a direct effect (Rogers et al. 1994). Furthermore, a role for OA as a tumor promoter in both in vitro and in vivo systems was also proposed (Suganuma et al. 1988; Sakai and Fujiki 1991; Fujjiki et al. 1992), and two human epidemiological studies, based on information from questionnaires, reported a significant association between diarrheic intoxication and different types of cancer, mainly of gastrointestinal etiology, in both men and women (Cordier et al. 2000; López-Rodas et al. 2006). Despite data collected regarding OA-induced genotoxic effects, a precise mechanism of action has not yet been described. The results reported with regard to OA-mediated cytotoxicity and genotoxicity are often contradictory and differ from one cell type to another. Based upon this uncertainty, further studies were conducted on the mechanisms underlying OA-induced genotoxicity, especially in different human cell types.

The aim of this study was to determine OA-induced genotoxic effects using the MN test and analysis of histone H2AX phosphorylation at serine 139 (γH2AX), a marker of double-strand break repair initiation, in three different types of human cells—peripheral blood leukocytes, neuroblastoma cells (SHSY5Y), and hepatoma cells (HepG2)—in order to elucidate the underlying mechanism(s) of action. Peripheral blood leukocytes were selected since there are some in vivo studies reporting a rapid absorption of OA and distribution throughout the organism (Matias and Creppy 1996; Ito et al. 2002). The neuroblastoma SHSY5Y cells were chosen on the basis of previous studies describing potential neurotoxic effects attributed to OA (Nuydens et al. 1998; He et al. 2001). Finally, the hepatoma HepG2 cells were selected since OA was suggested to accumulate in liver due to similarity to some known hepatotoxins (Aune and Yndestad 1993). Cells were treated with a range of OA concentrations (5–1000 n*M*) in the presence and absence of S9 fraction. Flow cytometric methodologies were used for MN and γH2AX determination.

METHODS

Chemicals

OA (CAS number 78111-17-8), mitomycin C (MMC) (CAS number 50-07-7), benzo[a]pyrene (BaP) (CAS number 50-32-8), camptothecin (Campt) (CAS number 7689-03-4), RNase A, and propidium iodide (PI) were purchased from Sigma-Aldrich Co. (Madrid, Spain). OA was dissolved in dimethyl sulfoxide (DMSO), and MMC, BaP, and Campt were dissolved in sterile distilled water. The metabolic activation fraction used was S9 from male Sprague-Dawley rats, from Sigma-Aldrich Co. The freshly prepared S9 mix consisted of 10% S9, 3.3% 1 *M* KCl, 3.2% 0.25 *M* $MgCl_2.6H_2O$, 2.5% 0.2 *M* glucose 6-phosphate, 10% 0.04 *M* NADP, 21% distilled H_2O, and 50% phosphate buffer (pH 7.4).

Cell Culture

Human peripheral blood was collected using heparinized vials from three healthy nonsmoker male donors aged 23–30 yr. The University of A Coruña Research Ethics Committee approved the investigations.

Written consent was obtained from each donor prior to joining the study. Mononuclear cells (lymphocytes and monocytes) were isolated on a Ficoll-Hypaque density gradient as described earlier (Laffon et al. 2002) and cultured in supplemented RPMI 1640 medium containing 15% heat-inactivated fetal bovine serum (FBS), 1% phytohemagglutinin (PHA), 1% L-glutamine (200 m*M*), and 1% penicillin (5000 U/ml)/streptomycin (5000 μg/ml), all from Invitrogen (Barcelona, Spain), for 24 h prior to the OA treatments. SHSY5Y cells (human neuroblastoma cell line) and HepG2 cells (human hepatocellular carcinoma cell line) were obtained from the European Collection of Cell Cultures. SHSY5Y cells were grown in nutrient mixture EMEM/F12 (1:1) medium with 1% nonessential amino acids and 1% antibiotic and antimycotic solution, and supplemented with 10% heat-inactivated fetal bovine serum, all from Invitrogen. HepG2 cells were cultured in DMEM medium with 1% antibiotic and antimycotic solution and supplemented with 10% FBS, all from Invitrogen. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

For the assays using SHSY5Y and HepG2 cells, 96-well plates (flat bottom) were prepared with cells obtained from a 90 to 100% confluence flask. Cell suspension (200 μl) was added to each well and cells were cultured at 37°C for 24 h prior to the experiments. Cell densities were approximately in the range of 6–8 $\times$ 10^5 cells/well at the beginning of culturing. For OA treatments, all cells were incubated at 37°C in the presence of OA (5, 10, 20, 50, 100, or 1000 n*M*) or control solutions at 1% of final volume. The range of OA concentrations was selected on the basis of the few previous studies reporting cytotoxic and genotoxic effects produced by OA in other different cell types (Traoré et al. 2001; Le Hégarat et al. 2003; 2004).

All treatments in leukocytes and SHSY5Y were performed in the presence and absence of metabolic activation. The protocol described by Pérez-Machado et al. (2004) was followed for the treatment with S9 fraction. HepG2 cells were not cultured in the presence of S9 fraction due to their demonstrated ability to activate compounds without the addition of exogenous enzymes (Knasmüller et al. 2004). DMSO was used as a negative control in all cases; Campt (10 μ*M*) or MMC (3 μ*M* for leukocytes and 1.5 μ*M* for HepG2 and SHSY5Y cells) was used as a positive control in experiments without metabolic activation, and BaP (200 μ*M* for leukocytes and 40 μ*M* for HepG2 and SHSY5Y cells) was used as a positive control in experiments with metabolic activation. The positive controls and their concentrations were selected for each assay on the basis of previous studies (Le Hégarat et al. 2004; Redon et al. 2006; Colognato et al. 2007).

MN Evaluation by Flow Cytometry

Cells were incubated with OA for 24 h, cultured for another 24 h in fresh medium, and subsequently a suspension of nuclei and MN was prepared according to the procedure described by Nüsse et al. (1994), with some modifications (Roman et al. 1998). Cells were centrifuged and the cell pellet was resuspended in solution I (584 mg/L sodium chloride, 1 mg/L sodium citrate, 50 μg/ml PI, 50 μg/ml RNase A, and 0.3 ml/L Nonidet P-40), to yield a final concentration of 10^6 cells/ml. Samples were maintained in the dark at room temperature for 15 min. Then an equal amount of solution II (1.5% citric acid and 0.25 *M* sucrose) was added and cells were incubated for 30 min in the dark at room temperature. Finally, the suspension was filtered through a 50-μm nylon mesh, and flow cytometry measurements were carried out. This two-step procedure destroyed the cell membrane and cytoplasm such that the suspension contained relatively pure nuclei and MN. This suspension was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Madrid, Spain). For each sample, at a minimum, 50,000 events were evaluated from the PI signal detected in the FL2 channel. Data analysis was carried out with Cell Quest Pro software (Becton Dickinson), following the instructions described by Avlasevich et al. (2006).

Cytokinesis-Block MN Test

Cultures were established in duplicate by adding 0.5 ml of whole blood to 4.5 ml of culture medium (RPMI 1640 containing 15% heat-inactivated FBS, 1% phytohemagglutinin, 1% L-glutamine [200 m*M*], and 1% penicillin [5000 U/ml]/streptomycin [5000 μg/ml], all from Invitrogen). Cells were incubated at 37°C in the dark. OA was added at 24 h and maintained until the end of the incubation time (64 h). Cytochalasin-B (6 μg/ml) was added at 44 h to prevent cytokinesis. Cells were collected by centrifugation, treated with a mild hypotonic solution (0.075 *M* KCl at 4°C), and then centrifuged immediately and fixed in Carnoy (3:1 methanol–acetic acid). Air-dried slides were prepared and stained with 5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in antifade solution.

Microscope analyses were performed using a Leica DM-RXA fluorescence microscope, equipped with a 100-W mercury lamp and a 100× magnification objective. In total, 2000 binucleated cells with well-preserved cytoplasm, half from each duplicate culture, were scored "blindly" by the same reader to determine the number of MN, binucleated cells with MN (BNMN), nuclear buds (BUD), and nucleoplasmic bridges (NPB). Furthermore, 1000 cells were examined to determine the cytokinesis-blocked proliferation index (CPBI) and the number of apoptotic and necrotic cells. The criteria described by Fenech (2007) were followed for scoring (MN, BNMN, BUD, NPB, and cells undergoing apoptosis and necrosis).

γH2AX Analysis

The γH2AX analysis was performed following the protocol described by Tanaka et al. (2009). Cells (5×10^5/ml) were incubated for 24 h at 37°C and then incubated with OA (5–1000 n*M*) or controls at 1% for 3 h. The cell suspension was then centrifuged for 5 min at 210 × g before the supernatant was aspi-rated. The remaining cell pellet was fixed in 1%*p*-formaldehyde for 15 min at 4°C. The suspension was centrifuged again and cells were postfixed in 1 ml of cold 70% ethanol (–20°C) and stored at 4°C overnight. An aliquot of fixed cells (250 μl) was centrifuged (5 min, 955 × g), washed in phosphate-buffered solution (PBS), and incubated with 100 μl anti-human γH2AX-Alexa Fluor 488-conjugated antibody (Becton Dickinson) (1:20 dilution in 1% bovine serum albumin [BSA] in PBS) for 15 min at room temperature. Subsequently, cells were centrifuged for 5 min at 955 × g and resuspended in PBS containing 0.1 mg/ml RNase A and 50 μg/ml PI (Becton Dickinson) for 30 min at room temperature. At minimum, 10,000 events were acquired with a FACSCalibur Flow cytometer, obtaining data from FL1 (Alexa Fluor 488) and FL2 (PI) detectors. Data were analyzed using Cell Quest Pro software (Becton Dickinson).

Statistical Analysis

Three independent experiments were performed for each experimental condition tested. Experimental data were expressed as mean ± standard error. Distribution of the response variables departed significantly from normality (Kolmogorov–Smirnov goodness-of-fit test) and therefore nonparametric tests were considered adequate for the statistical analysis of these data. Differences between groups were tested with the Kruskal–Wallis test and Mann–Whitney *U*-test. The associations between two variables were analyzed by Pearson's correlation. A p value of $<.05$ was considered significant. Statistical analysis was performed using the SPSS for Windows statistical package (version 16.0).

RESULTS

In this study, OA-induced genotoxic effects were investigated in three types of human cells—leukocytes, SHSY5Y cells and HepG2 cells—by means of the MN test (microscopy and flow cytometry evaluation) and γH2AX analysis. Results obtained from the flow cytometry evaluation of MN frequency are shown in Figure 1. No significant differences with regard to control were observed in leukocytes treated with OA either without or with S9 fraction, except for a numerical increase at the 20 n*M*

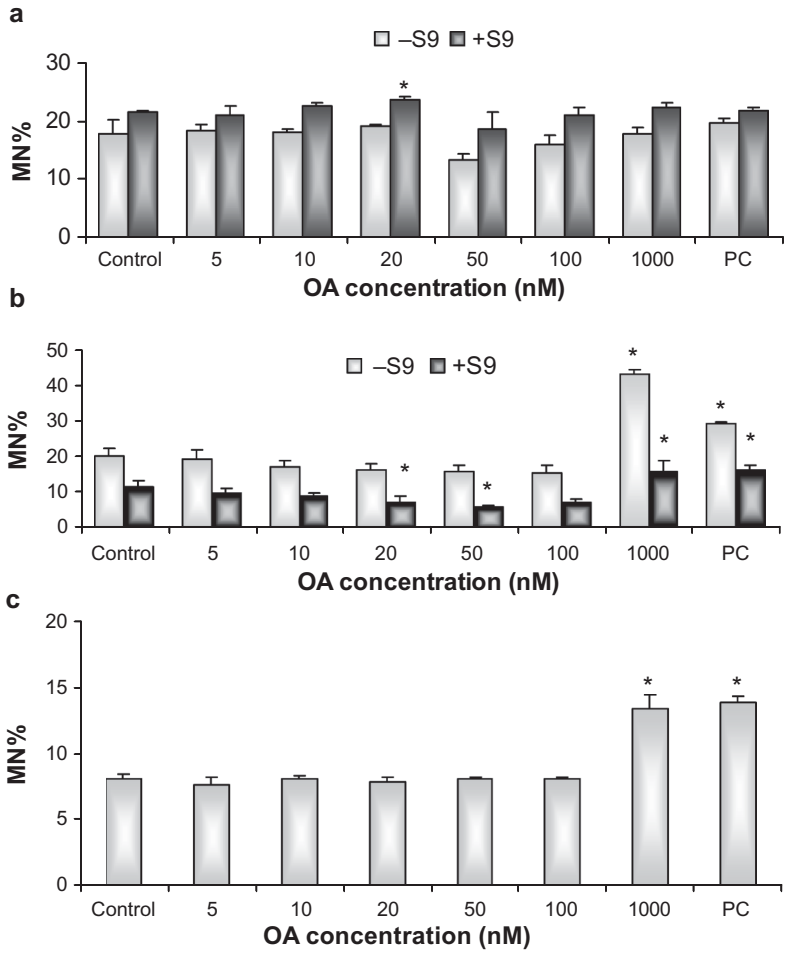


FIGURE 1. MN frequency determined by flow cytometry in human cells treated with OA: leukocytes (a), SHSY5Y cells (b), and HepG2 cells (c). PC: positive control. Asterisk indicates significant difference from control, $p < .05$.

concentration in the presence of metabolic activation (Figure 1a). In SHSY5Y cells treated in the absence of metabolic activation, an elevated MN frequency was observed at 1000 n*M* OA. Moreover, a significant concentration-response relationship was obtained ($r = 0.428$, $P < 0.01$). Similarly, OA induced a rise in MN rate at 1000 n*M* in the presence of S9 fraction; however, the MN frequency was reduced at 20 and 50 n*M* (Figure 1b). In HepG2 cells, the MN frequency was increased in a concentration-dependent manner after OA treatment ($r = 0.539$, $P < 0.01$), reaching statistical significance at 1000 n*M* (Figure 1c).

The MN test following a standard protocol cytokinesis block with microscopic evaluation was carried out in leukocytes. The genotoxicity parameters determined (MN, BNMN, BUD, and NPB) are shown in Table 1. Increases in MN and BNMN frequencies were obtained at all concentrations tested, both in the absence (Table 1, top half) and presence (Table 1, bottom half) of S9 fraction. In addition, BUD rates were higher in the OA-exposed cells compared to control, but statistical significance was only reached at 5, 50, and 100 n*M* OA with metabolic activation, and there was a significant concentration-response relationship observed in this case ($r = 0.389$, $P < 0.05$). Further, all OA concentrations tested showed increased NPB frequencies, being statistically significant at 10 and 50 n*M* in the absence of S9 fraction and at 5 and 50 n*M* in the presence of

TABLE 1. Cytokinesis-Blocked MN Test in OA-Treated Leukocytes

Dose	CBPI	MN (‰)	BNMN (‰)	BUD (‰)	NPB (‰)	Apoptosis (‰)	Necrosis (‰)
−S9 Control	1.6 ± 0.01	12.33 ± 2.24	11.67 ± 2.06	2.67 ± 0.56	1.67 ± 0.76	11.67 ± 2.75	2.33 ± 0.75
5 n*M*	1.58 ± 0.02	25.5 ± 2.23*	22.83 ± 1.83*	4.17 ± 1.17	7.67 ± 4.34	15.33 ± 1.31	3.33 ± 0.56
10 n*M*	1.66 ± 0.02	29.67 ± 4.4*	26.17 ± 3.44*	3.33 ± 0.62	6.17 ± 1.92*	20.67 ± 2.04	2.67 ± 0.49
20 n*M*	1.62 ± 0.02	25.33 ± 3.58*	23.5 ± 3.64	3.67 ± 0.76	5.5 ± 2.29	16.67 ± 1.48	3.00 ± 0.22
50 n*M*	1.67 ± 0.02	22. ± 2.27*	20.17 ± 1.89*	3.67 ± 0.62	9.67 ± 4.62*	14. ± 1.84	3.00 ± 0.34
100 n*M*	1.62 ± 0.01	27.33 ± 3.43*	25. ± 3.28*	4. ± 1.13	11.17 ± 4.85	27. ± 3.62*	3.67 ± 0.65
MMC	1.54 ± 0.04	64.5 ± 10.11*	57.83 ± 8.04*	4.17 ± 0.54	1.67 ± 0.84	13.33 ± 1.63	4.00 ± 0.86
+S9 Control	1.61 ± 0.01	8.67 ± 0.88	8.50 ± 0.85	1.5 ± 0.67	2.67 ± 0.84	8 ± 0.68	1. ± 0.5
5 n*M*	1.58 ± 0.01	23.5 ± 2.92*	21.67 ± 2.38*	6.50 ± 0.5*	6.83 ± 1.49*	8. ± 2.31*	3. ± 1.5
10 n*M*	1.6 ± 0.02	20.83 ± 2.94*	18.5 ± 2.26*	3.50 ± 0.67	4.5 ± 1.45	14. ± 1.39*	4. ± 2.
20 n*M*	1.61 ± 0.02	24.67 ± 2.89*	22.83 ± 2.73*	4.83 ± 1.22	6.5 ± 2.49	16. ± 1.77*	2. ± 1.
50 n*M*	1.64 ± 0.03	20.33 ± 2.49*	17.83 ± 2.21*	4.5 ± 0.92*	7.67 ± 1.82*	17. ± 2.83*	4. ± 2.
100 n*M*	1.61 ± 0.03	20.33 ± 2.84*	9.33 ± 2.60*	5.67 ± 0.67*	6.5 ± 1.8	13. ± 1.18*	3.33 ± 1.67
BaP	1.58 ± 0.01	29.67 ± 2.91*	6. ± 2.57*	5.67 ± 0.62*	2.33 ± 0.92	9.67 ± 1.35	1.33 ± 0.67

Note. Asterisk indicates significant difference from control (Mann-Whitney *U*-test), $p < .05$. CBPI: cytokinesis-blocked proliferation index; MN: micronucleus; BNMN: binucleated cells with micronuclei; BUD: nuclear buds; NPB: nucleoplasmic bridges; MMC: mitomycin C (3 μ*M*); BaP: benzo[a]pyrene (200 μ*M*).

S9. A positive concentration-response relationship was obtained in both cases (without S9: $r = 0.360$, $P < 0.05$; with S9 $r = 0.367$, $P < 0.05$).

The results obtained for the cytotoxicity parameters evaluated microscopically in the MN test (CBPI, and apoptosis and necrosis rates) are shown in Table 1. No marked differences were observed in the CBPI in OA-exposed cells either without (Table 1, top half) or with (Table 1, bottom half) metabolic activation. Regarding the percent necrosis, a significant concentration-response relationship was obtained in OA-treated cells in the presence of S9 fraction ($r = 0.339$, $P < 0.05$), although statistical significance was not reached at any concentration tested. Furthermore, exposure to OA induced a rise in apoptotic frequency, both without and with S9 fraction, with a significant concentration-response relationship in the former case ($r = 0.430$, $P < 0.01$).

γH2AX levels were determined by flow cytometry in all the cell types tested after treatment with OA (Figure 2). In the absence of S9 fraction, decreases in the γH2AX percentage were observed in leukocytes at 5, 10, 20, and 1000 n*M* OA, with an inverse significant concentration-response relationship ($r = -0.362$, $P < 0.05$) (Figure 2a). However, no marked differences were obtained in these cells when treated in the presence of S9 fraction. No marked effect of OA exposure was found in SHSY5Y cells, except for a quantitative increase in H2AX phosphorylation at 20 n*M* in the absence of metabolic activation (Figure 2b). In contrast, HepG2 cells showed general and concentration-dependent elevation in γH2AX levels at all concentrations tested ($r = 0.412$, $P < 0.01$) (Figure 2c).

DISCUSSION

Two different procedures were followed for evaluating the induction of MN following OA exposure: flow cytometry analysis and cytokinesis-block standard protocol with microscopy scoring. First, MN frequency was investigated in the three cell types by means of flow cytometry. The main advantages of this technique are (1) the large number of cells that can be analyzed in short time intervals, (2) apparent objective criteria for identifying MN in suspension, and (3) a high degree of automation during data acquisition and analysis (Nüsse and Marx 1997).

Data obtained from flow cytometry evaluation of MN rates showed increases at 1000 n*M* OA in SHSY5Y cells, in both the absence and presence of S9 fraction, and in HepG2 cells. These results are in agreement with the previous studies of Le Hégarat et al. (2003; 2004), who reported OA-induced elevation in MN frequency in CHO-K1 cells. In addition, they combined MN test with fluorescence in situ hybridization (FISH) demonstrated that OA-induced MN is a consequence of aneugenic effects. Le Hegarat et al. (2006) also observed that OA produced MN formation and concentration- and time-dependent rises in mitotic arrest and multinucleated cells in Caco-2 cells. However, despite these previous investigations showing positive results in MN evaluation after exposure to similar OA concentrations, no marked effects were observed in the present study for OA-treated leukocytes, either without or with metabolic activation. Furthermore Viaggi et al. (1995) reported that in some cases it is difficult to discriminate between MN and nonspecific background from debris present in the suspension of nuclei and MN using DNA content measurements alone, especially in the region of low fluorescence intensities.

The cytokinesis-block MN test was therefore performed in leukocytes. Results obtained for the cytotoxicity parameters showed that OA did not produce cell cycle delay (determined by CBPI index), but induced apoptosis both in the absence and presence of S9 fraction. In previous studies, OA was found to induce apoptosis in different kind of cells, in agreement with our results (Davis et al. 1996; Morimoto et al. 1997; Cabado et al. 2004; Lago et al. 2005, Valdiglesias et al. 2011b). However, our data for CPBI index differ from other studies in which OA was found to induce mitotic arrest or premature

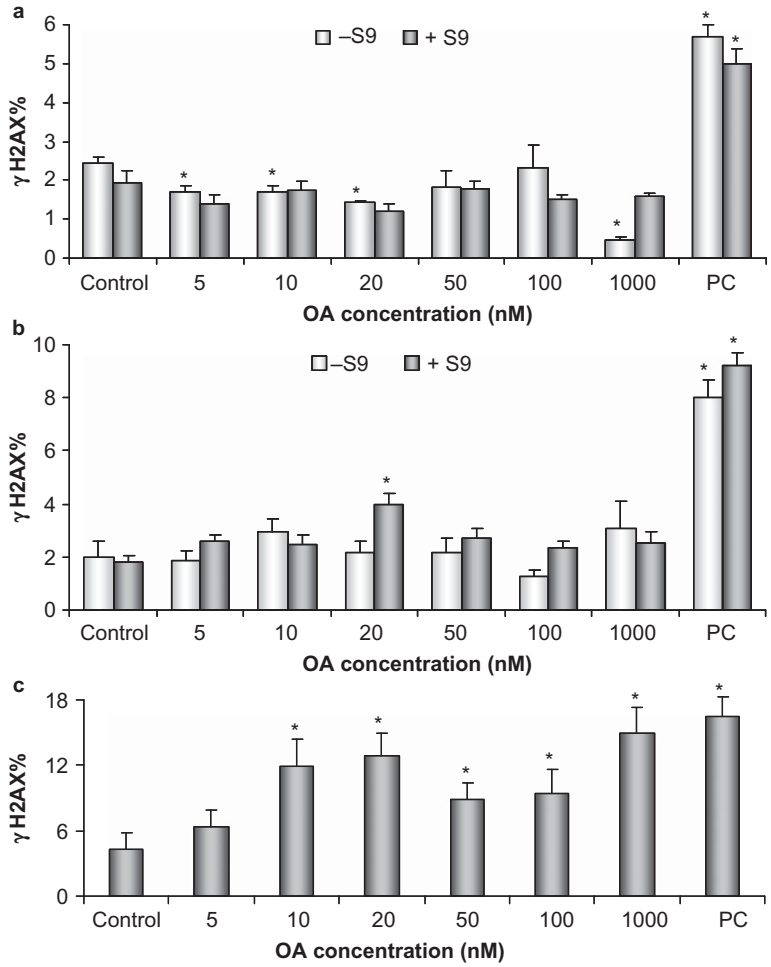


FIGURE 2. γH2AX analysis in human cells treated with OA: leukocytes (a), SHSY5Y cells (b), and HepG2 cells (c). PC: positive control. Asterisk indicates significant difference from control, $p < .05$.

chromosome condensation (Ghosh et al. 1992; Gotoh et al. 1995; Nuydens et al. 1998; Lerga et al. 1999).

Regarding genotoxicity, increases in the MN, BNMN, and NPB frequencies were observed with and without metabolic activation, and in BUD frequency only in the presence of S9 mix. In some cases increases did not reach statistical significance, as in the case of NPB, but this might be considered important from the biological point of view. Moreover, significant linear concentration-response relationships were not obtained in the frequency of MN. Taking into account that the highest OA concentration evaluated in this test was 100 n*M*, it may be possible that the effect of OA on MN induction did not follow a linear behavior in the concentration range tested. The role of OA as a genotoxic agent was also previously described in other studies with different cells systems (Traoré et al. 2001; Le Hégarat et al. 2003; 2006; Souid-Mensi et al. 2008; Valdiglesias et al. 2010), but there is some contradiction regarding the type of OA-induced DNA damage. Some authors suggested that OA was aneugenic (Le Hégarat et al. 2004; Carvalho et al. 2006), while others proposed a clastogenic effect (Traoré et al. 2001). It was also propsed that OA effects vary depending on the cell type and experimental conditions (Souid-Mensi et al. 2008; Valdiglesias et al. 2010).

Data obtained in the evaluation of MN frequency by flow cytometry and by the standard

cytokinesis-block test showed that the microscopic procedure is more sensitive for detecting OA-induced genotoxicity. Nevertheless, the rapid speed and low sample size required for flow cytometric measurement make this technique a reliable tool for a preliminary genotoxicity evaluation.

In the present study, the levels of histone H2AX phosphorylation at serine 139 (γH2AX) in leukocytes, SHSY5Y, and HepG2 cells were determined in order to characterize the type of DNA damage induced by OA in these cells. H2AX phosphorylation is well established as a marker of DNA damage as it accumulates in chromatin domains near the sites of DNA double-strand breaks (DSB) (Andrievski and Wilkins 2009). Following the generation of DSB, a rapid kinase-based signaling pathway is activated that coordinates DNA repair with the induction cell-cycle checkpoints (Zhou and Elledge 2000; Rouse and Jackson 2002). γH2AX has two functions in this repair process: a role in promoting changes in the structural configuration of chromatin, and a role in chromatin tethering of repair factors (Fernández-Capetillo et al. 2004). γH2AX appears within minutes of induction of DSB, increasing with time up to 20–30 min and subsequently dephosphorylating over the next few hours (Rogakou et al. 1998; Pilch et al. 2003; Antonelli et al. 2005).

Results obtained from the γH2AX analysis showed that the three cell lines investigated respond differently to OA exposure; HepG2 were the most sensitive cells. In leukocytes treated in the presence of metabolic activation no marked changes in γH2AX levels were found, whereas in the absence of S9 these cells showed a decrease at several OA concentrations. Since these last results seem to lack biological plausibility, they may be a result of relatively few experimental data included in each comparison group, and probably by raising the number of replicas these differences might be blurred.

SHSY5Y cells showed no marked effects both in the absence and presence of S9 microsomal fraction. Finally, hepatic cells were most sensitive to OA treatment, with increased levels of γH2AX at all the concentrations tested. The differences between cell lines reflect their different sensitivity to the genotoxic effects produced by OA, including direct damage induced by OA on DNA, but also different effects produced by this toxin on DNA repair processes (Valdiglesias et al. 2010). OA induction of DNA strand breaks, determined by means of the comet assay, was previously described by Traoré et al. (2001) in Caco2 cells, and by Valdiglesias et al. (2010) in the same cell types used in the present study. Comet assay and γH2AX assessment methodologies have a similar basis (detection of DNA strand breaks, directly in the case of comet assay or as consequences in the case of γH2AX analysis), such that results are expected to be analogous when used to evaluate genotoxicity induced by a particular agent. However, while H2AX phosphorylation only occurs in the presence of DSB, the alkaline version of the comet assay detects not only DSB but also other kinds of DNA damage, such as single-strand breaks (SSB), abasic lesions, and incomplete excision repair sites; thus, comparable outcomes are not always necessarily obtained. Thus, present negative results from γH2AX assessment in leukocytes and neuronal cells, contrasting with previously reported positive results from the comet assay, suggest that OA-induced DNA damage may originate not from DSB but from other different DNA lesions.

In conclusion, our results showed that OA induced MN formation, both directly and indirectly, in all cell types tested, but DSB only occurred in HepG2 cells. Taken together, these results and our previous data from the comet assay in the same cell types (Valdiglesias et al. 2010) indicated that in leukocytes OA induced minimal DNA damage (only in the absence of S9 fraction), no significant effects in the γH2AX analysis, and increased MN frequency. This suggests that this toxin acts predominantly as an aneugenic agent in these specific cells. In contrast, in SHSY5Y cells OA induced clearly positive results in the comet assay but negative results in the γH2AX analysis, pointing to

clastogenic damage different from DSB (single-strand breaks [SSB], abasic sites, incomplete excision repair sites). A reason might be that these lesions were efficiently repaired at low OA concentrations, becoming fixed as MN only when OA concentration was high (1000 n*M*). Similarly, OA was found to produce clastogenic DNA damage in HepG2 cells, including DSB in this case as demonstrated by the positive results obtained in γH2AX analysis, which only resulted in MN formation at the highest OA concentration. Nevertheless, most genotoxic agents induce both structural and numerical chromosomal damage, that is, both clastogenic and aneugenic alterations (Schuler et al. 1997), although one type of alteration usually occurs more frequently. At present in the case of OA no genotoxicity mechanism can be discarded in any cell type.

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This work was funded by a grant from the Xunta de Galicia (INCITE08PXIB106155PR).
V. Valdiglesias was supported by a fellowship from the University of A Coruña.

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