Assessment of okadaic acid effects on cytotoxicity, DNA damage and DNA repair in human cells

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Abstract:

Okadaic acid (OA) is a phycotoxin produced by several types of dinoflagellates causing diarrheic shell-fish poisoning (DSP) in humans. Symptoms induced by DSP toxins are mainly gastrointestinal, but the intoxication does not appear to be fatal. Despite this, this toxin presents a potential threat to human health even at concentrations too low to induce acute toxicity, since previous animal studies have shown that OA has very potent tumour promoting activity. However, its concrete action mechanism has not been described yet and the results reported with regard to OA cytotoxicity and genotoxicity are often contradictory. In the present study, the genotoxic and cytotoxic effects of OA on three different types of human cells (peripheral blood leukocytes, HepG2 hepatoma cells, and SHSY5Y neuroblastoma cells) were evaluated. Cells were treated with a range of OA concentrations in the presence and absence of S9 fraction, and MTT test and Comet assay were performed in order to evaluate cytotoxicity and geno-toxicity, respectively. The possible effects of OA on DNA repair were also studied by means of the DNA repair competence assay, using bleomycin as DNA damage inductor. Treatment with OA in absence of S9 fraction induced not statistically significant decrease in cell viability and significant increase in DNA damage in all cell types at the highest concentrations investigated. However, only SHSY5Y cells showed OA induced genotoxic and cytotoxic effects in presence of S9 fraction. Furthermore, we found that OA can induce modulations in DNA repair processes when exposure was performed prior to BLM treatment, in co-exposure, or during the subsequent DNA repair process.

Keywords:

Okadaic acid; Cytotoxicity; Genotoxicity; DNA repair; Human cells

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1. Introduction

Okadaic acid (OA) is a marine toxin produced by several dinoflagellate species. It is responsible for frequent food poisonings associated with shellfish consumption [1]. This toxin was firstly isolated from the black sponge *Halichondria okadaii* [2] and is frequently found in several types of molluscs usual in the human diet as those from *Mytilus* or *Ostrea* genus.

The ingestion of OA contaminated shellfish results in a syndrome known as diarrhoeic shellfish poisoning (DSP) which is characterized by severe gastrointestinal symptoms including nausea, vomit, diarrhoea, and abdominal ache [3]. These symptoms begin within 4 h and continue for about 3 days, but they do not appear to be fatal [4]. DSP toxins are not considered as neurotoxins [5]. This is despite some studies in the last years, which described a possible neuronal apoptosis-inductor role for OA [6,7].

The current European regulation on the level of DSP toxins in shellfish for human consumption exclusively focuses on reduction of the gastrointestinal symptoms. Following the Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 [8], laying down specific hygiene rules for food of animal origin, the maximum limit of OA is 160 μg/kg. This means that small quantities of OA may be present in molluscs that have passed legal controls before its marketing, and therefore chronic exposure to this toxin may exist in regular consumers.

In addition to DSP, it has been reported that OA poses a threat to human health even at concentrations within the nanomolar range [9]. OA and its derivates (dinophysistoxins DTX1 to DTX5) are specific inhibitors of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) [10,11]. These inhibitions in turn affect intracellular processes such as metabolism, contractility, gene transcription, and the maintenance of cytoskeletal structure [12]; in fact inhibition of

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OA-sensitive phosphatases was suggested to be likely responsible for many, if not all, of the observed cellular responses to OA [13]. Among these responses, OA has been reported to induce micronuclei formation [14,15], DNA strand breaks [16], 8-OH-deoxyguanine adducts [17], minisatellite mutations [18], mitotic spindle alterations [19,20], and cytotoxicity in mammalian cultured cells [1,21]. Besides, this toxin has very potent tumour promoting activity in two-stage carcinogenesis experiments involving mouse skin [22] and mucosa of the rat glandular stomach [23]. However, the results obtained with regard to OA cytotoxicity and genotoxicity are often contradictory [1,24–26].

The aim of this work was to evaluate the genotoxic and cytotoxic effects of OA on three different types of human cells: peripheral blood leukocytes, hepatoma cells (HepG2), and neuroblastoma cells (SHSY5Y). Cells were treated with a range of OA concentrations from 5 to 1000 nM, in the presence and absence of S9 fraction, with the purpose of determining if this toxin acts directly or requires metabolic activation. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test was employed to evaluate cell viability and the alkaline Comet assay was used to determine the induction of primary DNA damage (DNA single and double strand breaks and alkali-labile sites). Furthermore, the DNA repair competence assay was performed in order to evaluate OA effects on DNA repair after exposure to bleomycin (BLM).

2. Materials and methods

2.1. Chemicals

OA (95%, CAS No. 78111-17-8), benzo(a)pyrene (B(a)P) (96%, CAS No. 50-32-8), and BLM (1.2–1.7 units/mg, CAS No. 9041-93-4) were purchased from Sigma–Aldrich Co. (Madrid, Spain). OA was dissolved in dimethylsulfoxide (DMSO) and BLM and B(a)P were both dissolved in sterile distilled water. The metabolic activation fraction used was S9 from male Sprague-Dawley rats from Sigma–Aldrich Co. (Madrid, Spain). The freshly prepared S9 mix consists of 10% S9, 3.3% 1 M KCl, 3.2% 0.25 M $MgCl_2 \cdot 6H2O$, 2.5% 0.2 M glucose-6-phosphate, 10% 0.04 M NADP, 21% distilled water and 50% phosphate buffer (pH 7.4).

2.2. Cell cultures and OA treatments

Human peripheral blood was collected using heparinized vials from three healthy non-smoker male donors aged 23–30. 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 and frozen as previously described [27]. Cells were quickly thawed at 37 °C when commencing the experiment. Then, they were cultured in supplemented RPMI 1640 medium containing 15% heat-inactivated foetal bovine serum, 1% phytohaemagglutinin (PHA), 1% L-glutamine (200 mM), and 1% penicillin (5000 U/mL)/streptomycin (5000 μg/mL). HepG2 cells (human hepatocellular carcinoma cell line) were obtained from the European Collection of Cell Cultures and cultured in DMEM medium with 1% antibiotic and antimycotic solution and supplemented with 10% foetal bovine serum. SHSY5Y cells (human neuroblastoma cell line) were obtained from the same Collection and grown in nutrient mixture EMEM/F12 (1:1) medium with 1% non-essential aminoacids, 1% antibiotic and antimycotic solution and supplemented with 10% heat-inactivated foetal bovine serum. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

With regard to the preparation for OA treatments, leukocytes were thawed and cultured at 37 °C for 24 h; SHSY5Y and HepG2 cells (6×10^5 cell/well) were seeded in 96-well plates and allowed to adhere for 24 h at 37 °C. Then, leukocytes (10^6 cells/mL) were incubated at 37 °C for 3 h in the presence of OA or the controls at 1% of final volume. HepG2 and SHSY5Y cells were exposed to OA or the controls at 1% of final volume also for 3 h. The range of OA concentrations were selected on the basis of the few previous studies reporting cytotoxic and genotoxic effects of OA in other different cell types [14,16,26]. All the treatments for cytotoxicity and DNA damage evaluation were performed in the absence or presence of S9 fraction for leukocytes and SHSY5Y cells. Treatment with S9 fraction was performed as described by Pérez-Machado et al. [28]. HepG2 cells were not cultured in the presence of S9 fraction in any case due to their demonstrated ability to activate compounds without the addition of exogenous enzymes [29]. DMSO was used as negative control, and B(a)P (50 μg/mL for leukocytes and 5 μg/mL for HepG2 and SHSY5Y cells) and BLM (15 μg/mL for leukocytes and 1 μg/mL for HepG2 and SHSY5Y cells) were used as positive controls in the experiments with or without S9 fraction, respectively.

2.3. MTT assay

Cytotoxicity was evaluated by the MTT assay, according to the method of Mosmann [30] with minor modifications [31,32]. The cells were exposed to OA (5, 10, 20, 50, 100, and 1000 nM) or the controls, in the presence or absence of S9 fraction, for 3 h at 37 °C. After OA treatment, medium was discarded and 100 μL of new medium with 10 μL MTT dye (5 mg/mL solution in PBS) was added to each well and re-incubated for another 4 h at 37 °C. After this time, the medium was removed and 200 μL of DMSO was added to each well and mixed thoroughly to dissolve the released purple formazan dye. After 10 min, absorbance was measured at 510 nm using a microplate absorbance reader (MRXII, DYNEX Technologies). The percentage of cell viability was used as a cytotoxicity parameter.

2.4. Comet assay

Cells were incubated with OA (5, 10, 20, 50, 100, and 1000 nM) or the controls, in the presence or absence of S9 fraction, for 3 h at 37 °C. After treatment time, the alkaline Comet assay was performed following the general protocol proposed by Singh et al. [33] with minor changes for leukocytes [34] and for the cell lines [35]. Image capture and analysis were performed using the Comet IV Software (Perceptive Instruments) for leukocytes (University of A Coruña laboratory), and using the Kinetic Imaging Komet 4.0 for both cell lines (University of Bradford laboratory). In all cases 50 cells were scored from each replicate slide (i.e. 100 cells in total) and percentage of DNA in the Comet tail (%TDNA) was used as a DNA damage parameter.

2.5. DNA repair competence assay

In order to evaluate OA effects on DNA repair this experiment comprised three consecutive phases: pretreatment (phase A), treatment of cells with BLM for DNA damage induction (phase B), and DNA repair (phase C). Cells were treated with OA (50 and 100 nM) in each separate phase. Phase A: cells were incubated for 3 h with or without OA at 37 °C. Phase B: cells were then treated with BLM (20 μg/mL for leukocytes and 8 μg/mL for HepG2 and SHSY5Y cells) for 30 min at 37 °C in the presence or absence of OA. Phase C: Subsequently, cells were washed, resuspended in fresh medium and incubated with or without OA for 15 min at 37 °C to allow DNA repair. The Comet assay was performed as described above after phase B (half of cells from each different condition tested, data labelled as before repair) and phase C (the other half, data labelled as after repair). Comet IV Software (Perceptive Instruments) was used for image capture and analysis.

The cells were treated with OA (50 and 100 nM) or DMSO for 15 min and the Comet assay was performed immediately after. This was to confirm that a 15 min incubation with OA (phase C) does not induce significant DNA damage, and therefore the possible effects observed in this phase do not arise from OA genotoxicity but from OA effects on DNA repair.

2.6. Statistical analysis

Three independent experiments were performed for each experimental condition tested. Experimental data were expressed as mean ± standard error. Distribution of the response variable departed significantly from normality (Kolmogorov–Smirnov goodness of fit test) and therefore non-parametric tests were considered adequate for the statistical analysis of these data. The differences between groups were tested with Kruskal–Wallis test and Mann–Whitney *U*-test. Dose–response relationships for OA were determined by Pearson's correlation. A *P*-value of <0.05 was considered significant. Statistical analysis was performed using the SPSS for Windows statistical package (version 16.0).

3. Results

3.1. Effects of OA on cytotoxicity

Results obtained for the MTT assay in the three types of human cells evaluated, in the absence and presence of S9 fraction, are shown in Fig. 1. OA diminished the viability of leukocytes to 90% at 1000 nM in the absence of S9 fraction, but no cytotoxic effects were observed in the presence of S9 fraction (Fig. 1a). However, a reduction in viability was found in SHSY5Y cells both with and without S9 fraction. In the absence of metabolic activation this reduction was to 89% at 100 nM and 71% at 1000 nM, and in the presence it was to 86% at 100 nM and 77% at 1000 nM (Fig. 1b). Besides, OA also diminished the percentage of viability in HepG2 cells to 91% at 50 nM, 71% at 100 nM, and 66% at 1000 nM (Fig. 1c). No statistically significant differences were observed. Furthermore, the possible existence of dose–response relationships were investigated for the three types of cells in the presence or absence of S9 fraction, but no statistically significant dose–responses relationships were found.

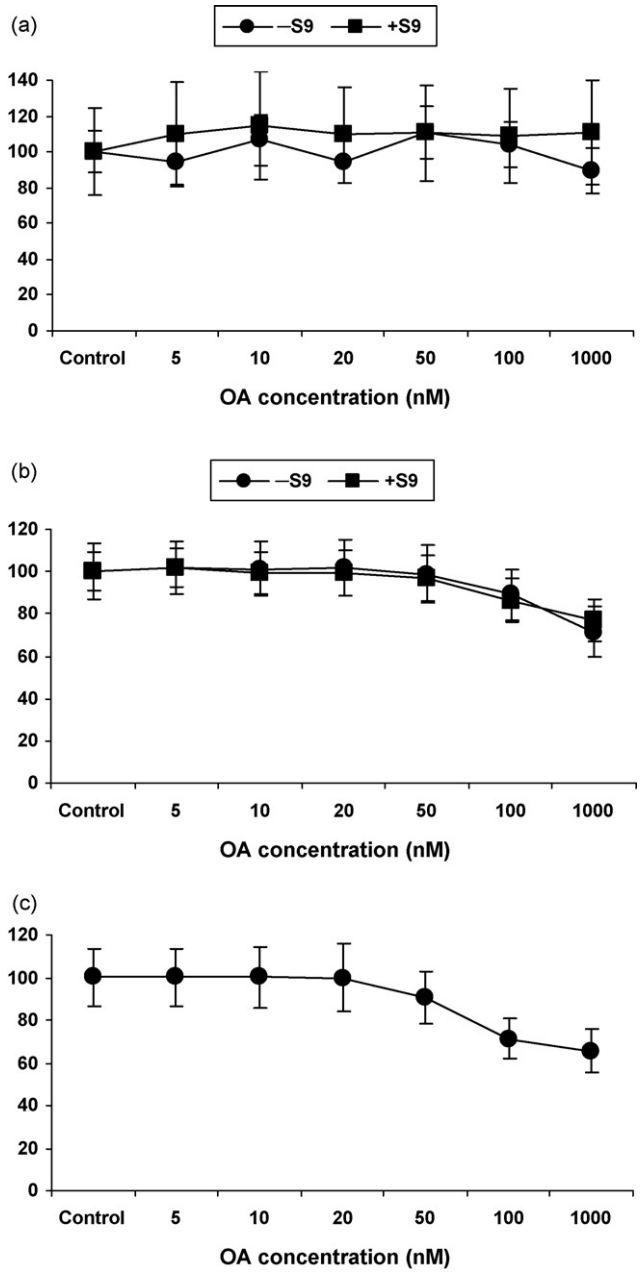

Fig. 1. Cytotoxicity (% viability) in leukocytes (a), SHSY5Y cells (b), and HepG2 cells (c) treated with OA in the presence and absence of S9 fraction.

3.2. Effects of OA on DNA damage

Data on the OA effects on cell genotoxicity, in the absence and presence of S9 fraction, obtained by means of the Comet assay for all the cells studied are summarized in Fig. 2. The incubation of human leukocytes with OA (5–1000 nM) in the absence of S9 fraction resulted in increases in DNA damage at the highest concentrations investigated, significant only for 100 nM (Fig. 2a). Statistically significant values of %TDNA were also observed in SHSY5Y cells, in the presence (10, 50, 100, and 1000 nM) and absence (50, 100, and 1000 nM) of S9 fraction (Fig. 2b). HepG2 cells treated with OA concentrations (50–1000 nM) yielded a statistically significant increase (Fig. 2c). Nevertheless, no DNA damage induction was detected in leukocytes incubated with any OA concentration in presence of S9 fraction (Fig. 2a).

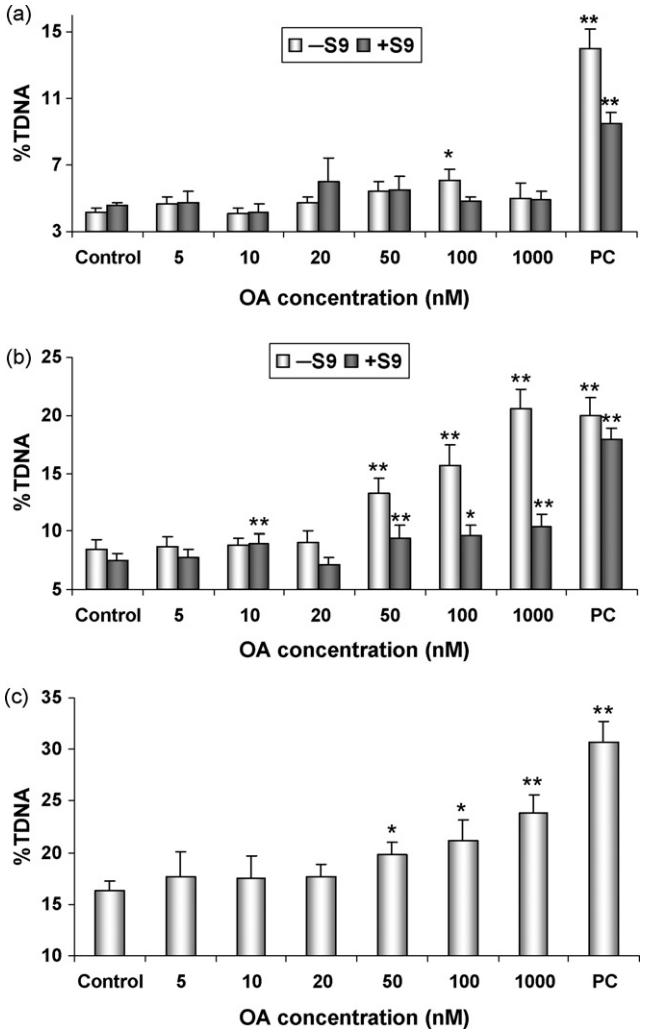

Fig. 2. Results of Comet assay in human leukocytes (a), SHSY5Y cells (b), and HepG2 cells (c) treated with OA in the presence or absence of S9 fraction. PC, positive control: BLM for experiments in absence of S9 fraction; B(a)P for experiments in the presence of S9 fraction. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference with regard to the corresponding control.

Furthermore, a statistically significant dose–response relationship was obtained for cells treated with OA in the absence of S9 fraction ($r = 0.359$, $P < 0.05$ for leukocytes, $r = 0.782$, $P < 0.01$ for SHSY5Y cells, and $r = 0.494$, $P < 0.01$ for HepG2 cells), and for SHSY5 cells exposed to OA in the presence of S9 fraction ($r = 0.559$, $P < 0.01$), but not for leukocytes with metabolic activation.

3.3. Effects of OA on DNA repair

Fig. 3 shows the results obtained in the DNA repair competence assay for the three types of cells. Data on treatment with OA for 15 min are included in Fig. 3 in order to prove no additional damage in any of the cell types. On the basis of the results obtained in the assessment of the effects of OA on DNA damage, only two OA concentrations (50 and 100 nM) were selected for the evaluation of the possible effects of this compound on the repair of BLM-induced DNA damage, since they produced genotoxicity in the three cell types evaluated.

When DNA damage was induced by BLM and no OA treatment was carried out, it was statistically significantly repaired in all cells after the 15 min incubation. In leukocytes, DNA damage decreased significantly after the repair period when treated with OA during phase C, but not during phases A and B (Fig. 3a). Data obtained

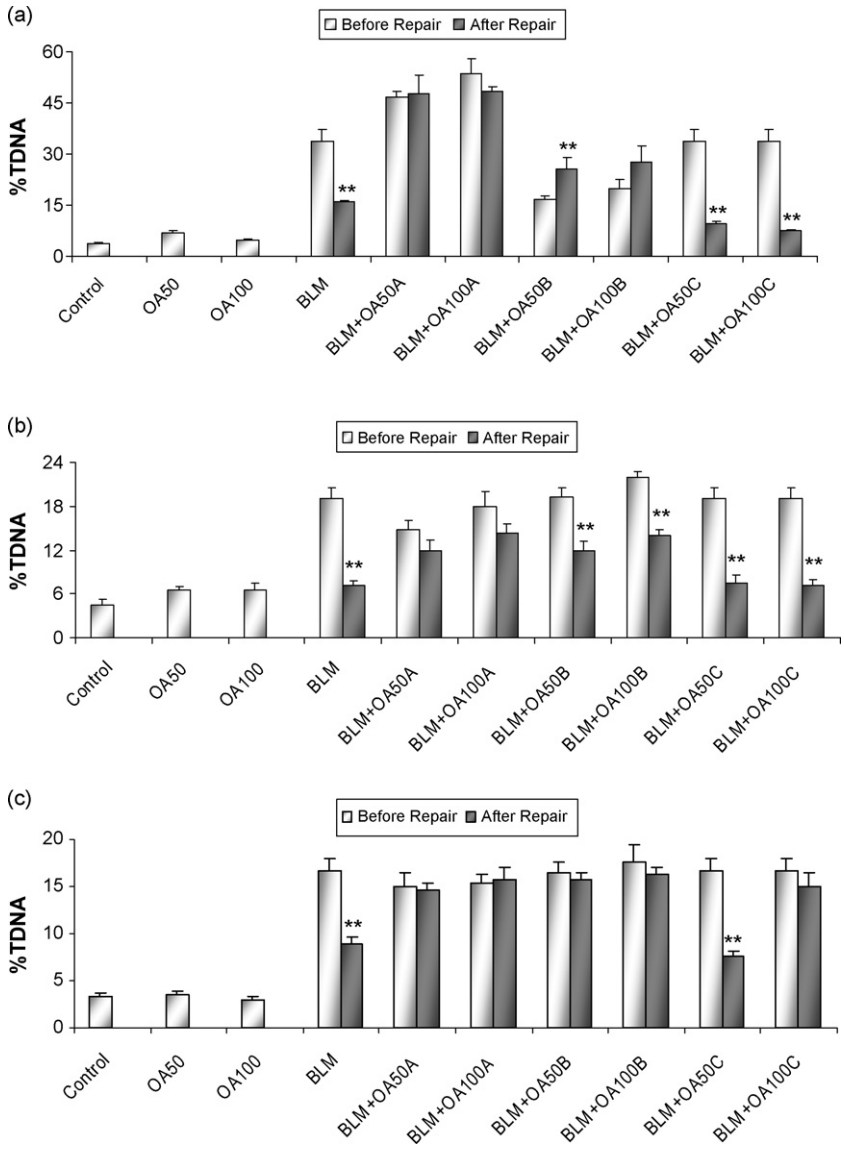

Fig. 3. Effect of OA treatment during phases A, B or C on DNA repair in human leukocytes (a), SHSY5Y cells (b), and HepG2 cells (c) challenged with BLM. $^{**}P < 0.01$, significant difference with regard to the same treatment before repair.

from SHSY5Y cells resulted in a statistically significant decrease in the DNA damage after the repair period in the case of treatment with OA in phases B and C (Fig. 3b). No decrease in BLM-induced DNA damage was observed during the repair period in HepG2 cells, except for OA exposure to 50 nM in phase C (Fig. 3c).

4. Discussion

The marine polyether OA is a phycotoxin produced by several types of dinoflagellates, mainly of the genus *Dynophysis* and *Prorocentrum*, which has a dramatic impact upon human health, economy, and science [36]. The major reason is that OA is accumulated by molluscs and several fishes by eating the phytoplankton, and they are consumed by humans causing DSP. This toxin has been reported to pose a hazard to human health at concentrations too low to induce acute toxicity [16]. It has long been recognized that OA is a tumour promoter that exerts its cellular effects by binding and inhibiting type 1 and 2A Ser/Thr protein phosphatases [10,23]. OA is also known to induce growth inhibition or apoptosis in many cell types like intestinal cells, neuronal cells, leukemic cells (reviewed in Refs. [37,38]), and OA effects cannot always be explained by phosphatases inhibition. Little is known about the molecular mechanisms and the components involved in the cellular responses induced by this toxin [39].

In this study the OA effects on genotoxicity, cytotoxicity, and DNA repair were evaluated in three different types of human cells: peripheral blood leukocytes, neuronal cells (the SHSY5Y cell line), and hepatic cells (the HepG2 cell line). Peripheral leukocytes were chosen on the basis of the scarce *in vivo* studies in mammals that report a rapid absorption and distribution of OA all over the organism [4,40]. The SHSY5Y cell line was selected to evaluate the OA neurotoxic effects on the basis of previous studies in which a possible neuronal apoptosis-inductor role of OA was described [6,41]. Finally, the HepG2 cell line was selected because of the great similarity of OA with some known hepatotoxins, suggesting that it could be accumulated in the liver and induce toxicity to this organ [3].

OA induced low levels of cytotoxicity at 100 and 1000 nM in leukocytes, SHSY5Y cells, and HepG2 cells after 3 h of culture in the absence of S9 fraction. Previous studies described the cytotoxic effects of OA on different mammalian cells including Caco-2, C6 glioma, DOK, V79, and NIH3T3 cells [1,11,16,21,42]. Among the three different cell types evaluated, HepG2 was the most sensitive

to OA. This is in agreement with the findings of Souid-Mensi et al. [1] who determined OA induced cytotoxicity in DOK, Caco-2, HepG 2, and C6 cell lines among which HepG2 was the most sensitive.

The treatment of human cells with OA (5–1000 nM) was carried out to assess the potential genotoxicity of this marine toxin. This resulted in increases in DNA damage in the absence of S9 fraction at 50, 100, and 1000 nM of OA in SHSYSY cells and HepG2 cells, and at 100 nM in leukocytes. Furthermore, a significant dose–response relationship was obtained in the three cell types investigated. Previous investigations reported genotoxic effects induced at particular concentrations of OA in a number of cell lines [16,17,42,43]. By contrast, no genotoxicity was found after OA treatment in other cell systems [24,26]. In fact, Souid-Mensi et al. [1] concluded that OA genotoxicity is chiefly cell type- and concentration-dependent. Nevertheless, these authors reported that HepG2 cells were highly sensitive towards OA at concentrations 10–50 nM, meanwhile the present results showed significant DNA damage only at concentrations over 50 nM. A possible explanation lies on the different methodologies used to analyse the genotoxic effects (Comet assay and 3D assay), that detect different types of damage in the DNA, and also on the different time of exposure to OA (3 h vs. 12 h).

In the presence of S9, the SHSY5Y cell line showed similar cytotoxic and lower genotoxic effects than those determined in absence of S9 fraction. These results suggest that OA acts as both direct and indirect cytotoxic and genotoxic agent, although the products generated by its metabolic transformation are less effective in genotoxicity induction than the primary toxin. Several studies agreed with the fact that OA exerts a direct effect [24,42], but also the need for OA metabolic activation was found in some other studies [14,18,26]. This could strengthen the concept that OA acts differently depending on the type of cell and study conditions. The literature provides examples of genotoxic compounds responding in a different manner *in vitro* depending on the experimental design and conditions [44–47]. In this regard, there is a review addressing this matter and the attention that the interpretation of the results should receive [48].

The influence of OA on repair of BLM-induced DNA damage was assessed by incubating the cells with OA during phases A, B, and C (pretreatment, BLM treatment and repair, respectively). No S9 fraction treatment was used in this part of the study due to the absence of an effect in leukocytes and the lower effect induced in SHSY5Y cells. DNA damage was evaluated before and after a 15 min repair period in BLM-free medium. BLM behaves as a radiomimetic agent capable of inducing a wide spectrum of mutagenic lesions in mammalian cells including DNA base damage, abasic sites, and alkali-labile sites [49–52] which result in DNA single and double strand breaks. BLM is classified as a direct clastogenic agent and acts in an S-independent fashion [53]. Challenging concentrations of BLM have been extensively used in DNA repair competence studies [54,55].

Comparison between the results obtained in the different treatments before and after the repair period showed that OA disrupts the DNA repair processes to a different extent in the three cell types. HepG2 was the most sensitive to the effects of OA on DNA repair since the repair of BLM-induced DNA damage was completely inhibited in the 3 phases. A short treatment of 15 min with OA at 100 nM (phase C) inhibited the DNA repair processes except for 50 nM OA. In leukocytes, repair was inhibited when OA was applied before BLM or in co-treatment (phases A and B). The SHSY5Y cells were the most insensitive. DNA repair alterations were only observed when cells were treated with OA before BLM treatment (phase A). This suggest that in HepG2 cells OA may, somehow, affect the DNA repair systems during the 3 h pretreatment, enhance the strand breaks production by BLM during the 30 min damage induction, and alter DNA repair processes when they are already acting on damaged DNA. However, in leukocytes the two first processes occur whereas in SHSY5Y cells only the first one. The fact that leukocytes and SHSY5Y cells repair DNA damage in a short period of time (15 min) might be indicative of damage being induced through oxidative stress, at least to some extent. It has been extensively reported the rapid DNA repair after exposure to genotoxicants inducing reactive oxygen species [56,57]. A small number of previous studies were performed in order to determine the possible DNA repair alterations induced by OA. The phosphatases inhibition caused by OA has been suggested to be able to slightly inhibit DNA repair in a dose-dependent manner [58] and to affect non-homologous end-joining double strand break repair [59]. On the contrary, results from another study showed that OA did not interact with the DNA repair process involved in *in vitro* unscheduled DNA synthesis in rat hepatocytes [26].

In conclusion, our results showed that OA acts mainly as a direct cytotoxic and genotoxic agent for human cells. However, its effects vary depending on the type of cell and the concentration employed. We also observed that OA exerts slight indirect effects on neuronal cells, but not on leukocytes. We concluded that the discordant data reported in the literature on the cytotoxic/genotoxic OA effects can be due to the differential experimental conditions. Furthermore, our data indicate that OA can induce modulations in DNA repair processes. Further work on this matter may be necessary to shed light on the cellular responses to OA exposure.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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