Induction of oxidative DNA damage by the marine toxin okadaic acid depends on human cell type

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Abstract:

The marine toxin okadaic acid (OA) is the main representative of diarrhoeic shellfish poisoning (DSP) toxins. Its ingestion induces nausea, vomiting, diarrhoea and abdominal ache. It has also been found to trigger cellular and molecular effects at low concentrations. Its mechanism of action has not been described yet. Results of a previous study showed that OA can induce cytotoxic and genotoxic effects, both directly and indirectly, and modulations in DNA repair processes in three different types of human cells (leukocytes, SHSY5Y neuroblastoma and HepG2 cells). These effects varied depending on the type of cell and the concentration employed (Valdiglesias et al., 2010). On that basis, the ability of OA to induce oxidative DNA damage on the same cell types was investigated in the present study. To this end, the antioxidant enzymes catalase and N-acetylcysteine, and the human DNA- glycosylase hOGG1 were used in combination with the alkaline Comet assay. The cells were treated with a range of OA concentrations (5–1000 nM) in the presence and absence of S9 fraction. The results of this study showed that OA induces oxidative DNA damage directly in leukocytes, directly and indirectly in SHSY5Y cells, while it does not induce oxidative DNA damage in HepG2 cells. Combining the outcomes of both studies, the data showed that OA induces both cytotoxicity and genotoxicity, including DNA strand breaks and oxidative DNA damage, in the cells evaluated. However, the extent of these effects are cell type dependent.

Keywords:

Okadaic acid; Oxidative damage; hOGG1; Comet assay; Catalase; *N*-Acetylcysteine

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Introduction

Okadaic Acid (OA) is a polyether fatty acid produced mainly by dinoflagellates of the Dinophysis genus. Consumption of shellfish contaminated by this marine toxin is the cause of diarrhoeic shellfish poisoning (DSP), displayed by gastrointestinal symptoms in humans and other animals (Daranas et al., 2001; Tubaro et al., 2008). At the cellular level, OA can induce both genotoxic (Traoré et al., 2001; Le Hegarat et al., 2003; Carvalho et al., 2006) and cytotoxic effects (Ghosh et al.,1992; Matias et al.,1999; Berven et al., 2001; Túnez et al., 2005). Furthermore, this marine toxin can also act both as a tumour promoter and apoptosis inductor (Gehringer, 2004). It has long been recognized that OA exerts most of these effects by binding and inhibiting serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (Bialojan and Takai, 1988); however this property cannot explain all the cellular effects induced by this compound (Xing et al., 2008).

The European regulation establishes a maximum limit of OA in food of animal origin of 160 μg/kg (Regulation EC. No 853/2004), so small quantities of this toxin may be ingested by consumers of molluscs that passed legal controls. Chronic effects of this consumption are still to be unveiled; to our knowledge there is no literature available addressing the chronic effects of OA.

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Reactive oxygen species (ROS) are produced in cells by cellular metabolism and by exogenous agents that include ionizing radiation, air pollution and a wide range of chemicals (Halliwell, 2007). They react with different biomolecules such as the DNA causing oxidative DNA damage. In turn, it poses a major threat to the genetic integrity of cells (Klungland and Bjelland, 2007). The consequences include mutagenesis of various kinds ranging from simple oxidation of bases to large deletions through single and double strand breaks (Rao, 2009).

While modified proteins and lipids can be degraded and resynthesised, DNA must be repaired before replication and cell division takes place (Klungland and Bjelland, 2007). Defence mechanisms have evolved within the body to limit the levels of ROS and the damage they induce. These defence systems include antioxidant enzymes such as catalase (CAT), and non-enzymatic antioxidants as N-ace-tylcysteine (NAC). CAT is an enzyme present in the cells of plants, animals and aerobic bacteria (Matés and Sánchez-Jiménez, 1999). It is located in a cell organelle called the peroxisome. This enzyme very efficiently promotes the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen. NAC is a well-established antioxidant and intracellular ROS scavenger, and it has also been considered to be a precursor of glutathione synthesis (Meister and Anderson, 1983; Aruoma et al., 1989).

Oxidative damage is produced when the antioxidant defence system is overwhelmed. 8-oxo-7,8-dihydroguanine (8-oxoGua) is the main result of oxidative DNA damage (Angerer et al., 2007). These lesions are often used as suitable biomarkers of oxidative stress (Kuo et al., 2007) since its presence in cells of animals and humans may lead to point mutations (Loft et al., 2008).

On the basis of our previous study in which OA effects on DNA damage and repair were described (Valdiglesias et al., 2010), in the present work we aimed to evaluate oxidative DNA damage induction by OA in human periph-eral blood leukocytes, human SHSY5Y cells and human HepG2 cells. The alkaline comet assay was adjusted in order to study different mechanisms of oxidative DNA damage. Incubation of OA-treated cells with the human enzyme hOGG1, a DNA-glycosylase responsible for the excision of 8-oxoGua, allowed the specific detection of these oxidized bases, both in the presence and absence of S9. This was in order to determine if OA acts directly or needs metabolic activation. Co-incubation with the anti-oxidant enzyme CAT or the ROS scavenger NAC was employed to find out the mechanism through which OA generates ROS, if any.

The comet assay has been selected in the present study because of its higher accuracy in determining the levels of 8-oxoGua compared to other methods (Gedik and Collins, 2005). Furthermore, its versatility allows it to be used in co-treatments as is the case for the antioxidants.

Material and methods

Chemicals

OA (CAS No. 78111-17-8), benzo(a)pyrene (B(a)P) (CAS No. 50-32-8), bleomycin (BLM) (CAS No. 9041-93-4) and H_2O_2 (CAS No. 7722-84-1) were purchased from Sigma–Aldrich Co. (Madrid, Spain). NAC (CAS No. 616-91-1) and CAT were purchased from (Sigma–Aldrich, Poole, UK); hOGG1 was purchased from New England Biolabs (Herts, UK). 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 consisted of 10% S9, 3.3% 1 M KCl, 3.2% 0.25 M $MgCl_2{\cdot}6H_2O$, 2.5% 0.2 M glucose-6-phosphate, 10% 0.04 M NADP, 21% distilled water and 50% phosphate buffer (pH 7.4).

Cell culture and OA treatment

Heparinised vials were used to collect human peripheral blood from three healthy non-smoker male donors aged 23–30, who signed an informed consent form. The University of A Coruña Research Ethics Committee approved the investigations.Leukocytes were isolated and frozen as previously described (Laffon et al., 2010). At the beginning of the experiments, cells were quickly thawed at 37 °C 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)/strepto-mycin (5000 µg/ml) (all from Invitrogen, Spain).

HepG2 cells (human hepatocellular carcinoma cell line) and SHSY5Y cells (human neuroblastoma cell line) were both obtained from the European Collection of Cell Cultures. HepG2 cells were cultured in DMEM medium with 1% antibiotic and antimycotic solution and supple-mented with 10% foetal bovine serum (all from Invitrogen, Spain). SHSY5Y cells were grown in nutrient mixture EMEM/F12 (1:1) medium with 1% non essential amino-acids, 1% antibiotic and antimycotic solution and supple-mented with 10% heat-inactivated foetal bovine serum (all from Invitrogen, Spain). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

For the OA treatments, leukocytes were thawed and cultured at 37 °C for 24 h; SHSY5Y and HepG2 cells were seeded in 96-well plates (6 × 10 cells/well) and allowed to adhere for 24 h at 37 °C. Then, leukocytes (10^6 cells/ml), HepG2 cells and SHSY5Y cells were incubated at 37 °C for 3 h in the presence of OA or the controls at 1% of final volume. In the treatments for oxidative DNA damage evaluation by means of hOGG1 enzyme, six OA doses were employed (5, 10, 20, 50, 100 and 1000 nM), and they were performed in the absence and presence of S9 fraction for leukocytes and SHSY5Y cells. Treatment with S9 fraction was performed as described by Pérez-Machado et al.(2004). 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 (Knasmüller et al., 2004). Two OA doses (100 and 1000 nM) were assayed in the CAT and NAC experiments. DMSO was used as negative control. 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 0.1 µg/ml for HepG2 and SHSY5Y cells) were used as positive controls

in the hOGG1 experiments with and without S9 fraction, respectively, and H_2O_2 (25 μM) was used as the positive control in CAT and NAC experiments.

Comet assay and antioxidants treatments

After treatment time, the alkaline comet assay was performed following the protocol proposed by Singh et al. (1988) with minor changes for leukocytes (Laffon et al., 2002) and for the cell lines (Cemeli et al., 2006). Image capture and analysis were performed using the Comet IV Software (Perceptive Instruments) for hOGG1 treatment in leukocytes (University of A Coruña laboratory), and the Kinetic Imaging Komet 4.0 for everything else (University of Bradford laboratory). In all cases fifty cells were scored from each replicate slide (i.e. 100 cells in total) and the percentage of DNA in the comet tail (%TDNA) was used as a DNA damage parameter.

The possible protective effect of the antioxidants CAT and NAC was evaluated by simultaneous exposure of cells to OA and CAT (100 U/ml or 500 U/ml) or NAC (0.5 mM or 1 mM), after which the comet assay was performed as described in the previous paragraph.

Modified comet assay for hOGG1

A modified comet assay incorporating incubation with hOGG1 was carried out as described by Smith et al. (2006). Briefly, after the lysis period the slides were washed 3 times for 5 min each with a buffer consisting of 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA), 0.1 M KCl and 40 mM Hepes pH 8. Then, 50 ml of hOGG1 prepared in buffer (0.0016 U/ml) were added to each slide and incubated at 37 ºC for 10 min. The controls followed the same protocol but by using 50 ml of buffer without enzyme. The following unwinding, electrophoresis and staining steps were as for the standard comet assay.

Statistical analysis

At least three independent experiments were per-formed for each experimental condition tested. Experi-mental data are expressed as mean ± standard error. Differences between groups were tested with Kruskal–Wallis test and Mann–Whitney U-test. The dose-response relationship for OA was determined by Pearson's correla-tion. A P-value of <0.05 was considered significant. Statis-tical analysis was carried out using the SPSS for Windows statistical package (version 16.0).

Results

This work investigated the possible induction of oxida-tive DNA damage by the marine toxin OA in human leukocytes, SHSY5Y cells and HEPG2 cells. We used three modified versions of the comet assay. In the first one we used incubation with the repair enzyme hOGG1 to specif-ically recognize oxidized guanines (8-oxo-dG). The other two versions consisted of simultaneous treatments OA and CAT or NAC, to determine the protection against OA induced oxidative DNA damage offered by these antioxi-dants. Our previous study determined the generation of DNA damage at the concentrations of 100 and 1000 nM (Valdiglesias et al., 2010). For this reason, these concen-trations were selected for the antioxidant experiments. S9 fraction treatment was not used in the antioxidant exper-iments since our previous results showed that in its absence DNA damage was induced. Furthermore, there were lower levels of DNA damage in SHSY5Y cells in the presence of metabolic fraction (Valdiglesias et al., 2010).

The incubation of human leukocytes with OA (5–1000 nM) in the absence of S9 fraction resulted in increases in DNA oxidative damage at all the concentrations evaluated with hOGG1 and it was statistically significant for 10, 100 and 1000 nM (Fig. 1a). A significant dose-response rela-tionship was also found in these cells after hOGG1 incuba-tion (r ¼ 0.457, P < 0.01). However, no differences were observed for any OA concentration in leukocytes treated in the presence of S9 fraction after hOGG1 incubation (Fig. 1b). When CAT was employed simultaneously with OA in human leukocytes, a statistically significant reduction in the levels of DNA was found for both concentrations 100 and 1000 nM (Fig. 2a). No decrease of %TDNA was obtained by NAC co-treatment; on the contrary, a statistically significant increase in this parameter was observed in control cells and in cells exposed to OA 100 nM (Fig. 2b).

When SHSY5Y cells were treated with OA (5–1000 nM) in the absence of S9 fraction, increases in oxidative DNA damage as detected by hOGG1 incubation were found in the lowest concentrations and they were statistically significant for 10, 20 and 50 nM. No oxidative damage was obs-erved at the highest OA concentrations (Fig. 3a).

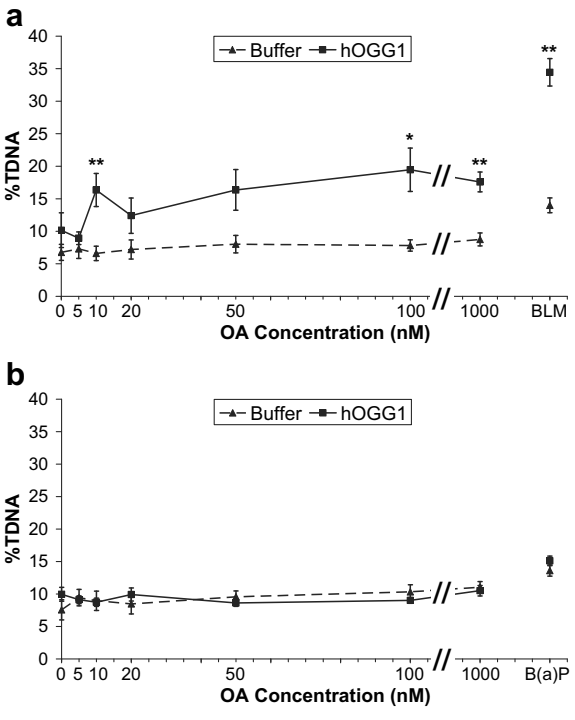

Fig. 1. Results of hOGG1-modified comet assay in leukocytes treated with OA in the absence (a) and presence (b) of S9 fraction. BLM: bleomycin; B(a)P: benzo(a)pyrene. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference with regard to the corresponding buffer.

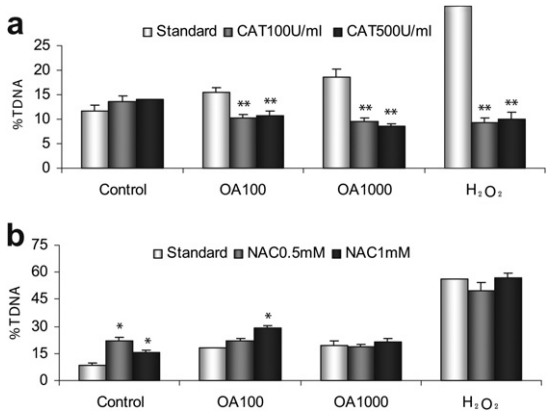

Fig. 2. Results of standard and modified comet assay with catalase (CAT) (a) or *N*-acetylcysteine (NAC) (b) in human leukocytes treated with OA. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference with regard to the corresponding control without enzyme.

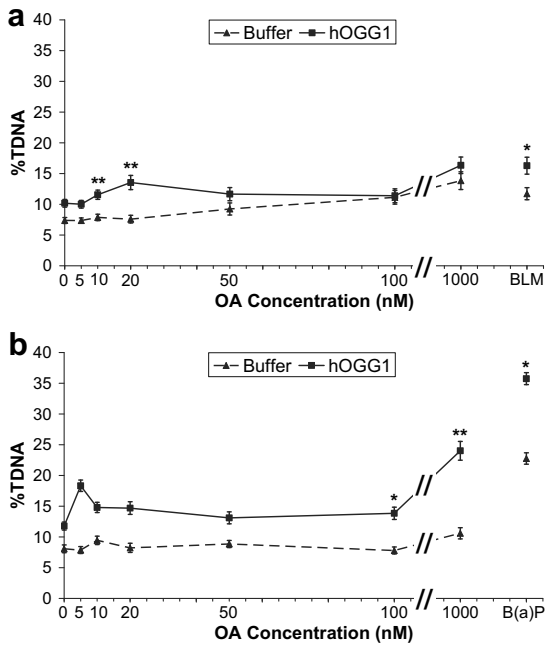

Fig. 3. Results of hOGG1-modified comet assay in SHSY5Y cells treated with OA in the absence (a) and presence (b) of S9 fraction. BLM: bleomycin; B(a)P: benzo(a)pyrene. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference with regard to the corresponding buffer.

Statistically significant dose-response relationships were also observed in these cells after buffer (r ¼ 0.708, P < 0.01) and hOGG1 incubation (r ¼ 0.436, P < 0.01). In the presence of S9 fraction, all treatments showed increased DNA damage after incubation with hOGG1 and they were statistically significant for 100 and 1000 nM (Fig. 3b). No decrease in the levels of DNA damage was observed when SHSY5Y cells were exposed to either CAT or NAC during the OA treatment (Fig. 4a and b).

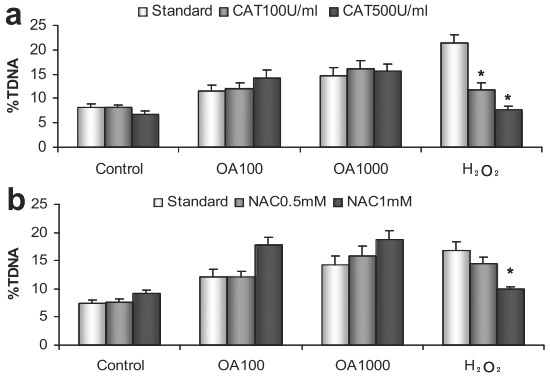

Fig. 4. Results of standard and modified comet assay with catalase (CAT) (a) or *N*-acetylcysteine (NAC) (b) in SHSY5Y cells treated with OA. $^{*}P < 0.05$, significant difference with regard to the corresponding control without enzyme.

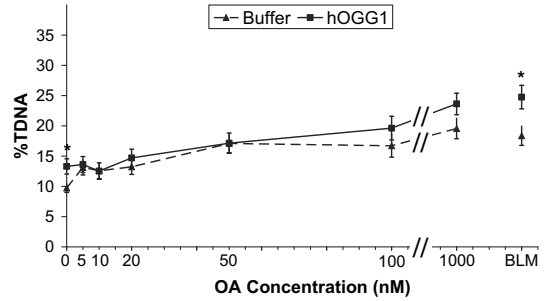

Fig. 5. Results of hOGG1-modified comet assay in HepG2 cells treated with OA. BLM: bleomycin. $^{*}P < 0.05$, significant difference with regard to the corresponding buffer.

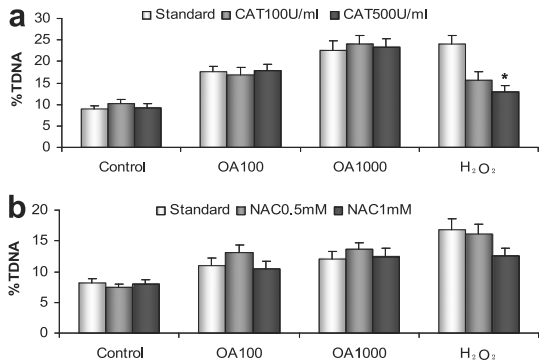

Fig. 6. Results of standard and modified comet assay with catalase (CAT) (a) or *N*-acetylcysteine (NAC) (b) in HepG2 cells treated with OA. $^{*}P < 0.05$, significant difference with regard to the corresponding control without enzyme.

No difference in DNA damage levels after incubation with and without hOGG1 was observed in HepG2 cells exposed to any OA concentration (Fig. 5). Likewise to leukocytes and SHSY5Y cells, statistically significant dose-response relationships were generated in the absence of hOGG1 (r ¼ 0.445, P < 0.01) and in the presence (r ¼ 0.484, P < 0.01). No %TDNA decrease was found when CAT or NAC were employed in co-treatment with OA (Fig. 6a and b).

Discussion

Okadaic acid (OA), the most widely distributed and most common marine toxin in Europe, is accumulated in the digestive tracts of shellfish causing DSP in consumers (Ito et al., 2002; Creppy et al., 2002). The primary cellular targets of OA are several classes of protein serine/threonine phosphatases that play central roles in the regulation of many essential cellular processes, including metabolism, growth, division, and death (Daranas et al., 2007). OA has been shown to induce these cellular effects even at low concentrations; however results reported with regard to its molecular effects, especially those related to genotoxicity, are often contradictory. Moreover, although it is well-known that OA can inhibit specifically the serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (Bialojan and Takai, 1988), the cellular and molecular effects of this toxin cannot always be explained by this inhibition, and the existence of other targets different from phosphatases cannot be excluded (Xing et al., 2008).

In order to shed light on effects of OA in the human organism, genotoxic and cytotoxic endpoints at different OA concentrations (5–1000 nM) on the same three types of human cells were evaluated in a previous study by our group (Valdiglesias et al., 2010). In such a study, we found that OA can induce DNA damage, viability decrease, and several DNA repair modulations, both in the presence and absence of S9 fraction. However, the effects observed were lower in the presence of S9 fraction. On particular occa-sions, the cells were able to repair the DNA damage in a short period of time (15 min) and this might indicate that the damage was produced through oxidative stress (Fracasso et al., 2006). On the basis of this hypothesis, in the present study the possible induction of oxidative DNA damage by OA was evaluated by means of the comet assay in human peripheral leukocytes, human neuronal cells (SHSY5Y cell line) and human hepatic cells (HepG2 cell line). The comet assay was adjusted depending on the investigation.

Amongst the cellular defence mechanisms against oxidative stress, antioxidant enzymes, e.g. catalase, and ROS scavengers, e.g. N-acetylcysteine, play a central role in maintaining the cellular redox balance that is essential for cell survival (Valko et al., 2006). Oxidative DNA damage will occur when ROS have sufficiently high frequency to exceed the antioxidant ability of the cell and the capacity of the cell for DNA repair (Ferrero-Gutiérrez et al., 2008; Loft et al., 2008). In this regard, the comet assay has been established as the most popular assay in antioxidant intervention trials since it can detect DNA strand breaks in its standard alkaline version or oxidized purines (including the most represen-tative DNA oxidation lesion 8-oxoGua) using an enzyme-modified version (Gedik and Collins, 2005; Loft et al., 2008). In the present study, we used hOGG1 to reveal 8-oxoGua, because it is more specific that formamidopyrimidine DNA-glycosylase that also recognizes alkylation damage (Smith et al., 2006). Co-incubation of OA with CAT and NAC allowed the determination of whether if the OA induced oxidative damage and how this was triggered.

An increased %TDNA was found in all the concentrations evaluated in the absence of S9 fraction in leukocytes when OA induced oxidative DNA damage was determined in leukocytes using incubation with the hOGG1 enzyme. Likewise, the antioxidant enzyme CAT effectively prevented free radical formation and reduced toxicity significantly. This confirms that OA-induced DNA damage in leukocytes may be, at least partially, the result of a redox imbalance between the reactive oxygen production and the antioxi-dant protective systems. However, no oxidative damage was observed in the presence of S9 fraction. The current outcomes corroborate those of our previous work (Valdiglesias et al., 2010) in which no DNA damage was found when leukocytes were exposed to OA in the presence of S9 fraction but a significant dose-response relationship was obtained when leukocytes were treated in the absence of S9 fraction. Hence, it seems that, in leukocytes, OA directly induces oxidative DNA damage which could lead to DNA strand breaks if antioxidant defences are over-whelmed. Further, the products of OA biotransformation generated by incubation with S9 fraction are ineffective in producing oxidized DNA damage. No antioxidant effect was found in either the positive control (H_2O_2)orany OA concentration when NAC was employed. The selection of the concentrations to be employed with the antioxidants is critical since they may exert different responses. For instance, in relation to NAC there are different views regarding the concentrations displaying therapeutic prop-erties (Liu et al., 2007). In some cases, it can reduce oxidative damage induced by other compounds (Arbillaga et al., 2007; Bhaskar et al., 2008), it can also have no effect (Leehey et al., 2005) or even cause oxidative damage (Okiawa et al., 1999). Thus, it is possible that a wider range of concentrations might have provided antioxidant responses as those described previously (Zhang et al., 1999; Morley et al., 2003).

The use of two antioxidants was aimed to discern the mechanism of action by which OA triggers oxidative stress. CAT breaks down H_2O_2 into O_2 and H_2O, this way it abol-ishes the major oxidative stress mediator, not harmful per se, but it gives rise to all the reactive species. Instead, NAC promotes the activity of GSH. This may also explain why NAC and CAT respond differently to OA.

In SHSY5Y cells, an increased oxidative damage was found in the lowest OA concentrations (from 5 to 50 nM) when evaluated by means of hOGG1-modified comet assay in the absence of S9 fraction. Results of a previous study already suggested that repeated exposure to low doses of OA may induce some chronic effects in humans via oxida-tive processes (Matias et al., 1999). Nevertheless, no increase in the hOGG1 detected oxidative damage was observed at 100 and 1000 nM OA. This agrees with the results of the CAT and NAC experiments in which these OA concentrations employed and no protective effects of any of the two antioxidants were found. On the other hand, when cells were treated in the presence of the S9 fraction, increases in %TDNA were found after hOGG1 incubation at all the concentrations evaluated, being statistically signifi-cant at the highest OA levels (100 and 1000 nM). The results of our previous work showed that OA could induce DNA strand breaks (alkaline comet assay) in these cells both in the presence and in the absence of S9, although the effect was more pronounced in the absence. This suggests that the products of metabolization were less effective in inducing DNA strand breaks than the toxin per se. Perhaps at higher OA concentrations, there is sufficient biotrans-formation to generate DNA reactive metabolites.

In contrast to the other cell types studied, no oxidative damage at any OA concentration was detected in the metabolically competent HepG2 cells by the hOGG1-modified comet assay. Consequently co-treatment with CAT or NAC did not exert any protection on cells exposed to OA. This might confirm that OA does not produce oxidative DNA damage in these cells. Strand break production (standard comet assay) by OA in HepG2 cells following a positive dose-response relationship was previously observed (Valdiglesias et al., 2010). This raises the question of whether such DNA damage could be induced by indirect mechanisms like endonucleases.

Previous studies showed that OA can induce oxidative damage in fibroblasts and HeLa cells (Schmidt et al., 1996), caco2 cells (Creppy et al., 2002), N1E-115 cells (Túnez et al., 2006), CCRF-CEM T-leukemia cells (Boudreau et al., 2007) and cultured astrocytes (Ferrero-Gutiérrez et al., 2008). Moreover, it has been observed in other studies that the cytotoxicity induced by this compound can be reduced by the administration of different antioxidants (Gimeno et al., 2004; Túnez et al., 2005; Levinthal and Defranco, 2005). In addition, OA was also found to induce apoptosis in a ROS-independent way in Jurkat T-leukemia cells (Boudreau et al., 2007) and to block apoptosis and ROS generation induced by 1-methyl-4-phenylpyridinium ion in SHSY5Y cells (Ahn et al., 2009).

In conclusion, data from this study showed that OA induces oxidative DNA damage directly in leukocytes, directly and indirectly in SHSY5Y cells and it does not induce oxidative DNA damage in HepG2 cells. Taken together, our previous and present results confirm the genotoxic potential of the marine toxin OA, since it induces DNA damage both in the form of strand breaks and oxidative damage. However, the mechanism leading to the DNA damage is highly dependent on the cell type. This may be related to the antioxidant status of each cell type and capability of metabolizing OA amongst other reasons. Finally, the present study selects cell types for which potential effects after OA ingestion may occur, being rele-vant targets in the organism. This investigation has been performed with the Comet assay which currently is the most accurate technique for the measurement of 8-oxoGua.

Ethical statement

Authors declare that the work described in this manuscript has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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