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Cytotoxic and genotoxic effects of environmental relevant concentrations of bisphenol A and interactions with doxorubicin

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ABSTRACT

Bisphenol A (BPA) is one of the most widely utilized endocrine disruptors to which humans are exposed, particularity through ingestion. BPA is an aneugenic compound with a putative association to tumorigenesis. Although extensively studied in estrogen responsive cells, information regarding its effects on cells from the upper gastrointestinal tract exposed to free/active forms of BPA is still scarce. Similarly, BPA interactions with other drugs have been neglected, although it has been suggested to have a potential role in doxorubicin (DOX) chemoresistance. This study is intended to assess potential cytotoxic and genotoxic effects of BPA, as well as its interactions with DOX, in Human epithelial type 2 cells (Hep-2) originated from a human laryngeal carcinoma and in a DNA damage responsive cell line, the human lung fibroblasts (MRC-5). Cell viability was analyzed through the resazurin assay. The G protein-coupled estrogen receptor 1 (GPER) expression was visualized by immunodetection. Genotoxicity, namely DNA damage and oxidative DNA damage, were assessed by comet assay and micronuclei induction, and mitotic disruption was evaluated cytologically by fluorescent microscopy with DAPI staining. Cytotoxicity analysis showed that exposure to BPA per se does not affect cellular viability. Nevertheless, the genotoxic analysis showed that BPA induced an increase of DNA damage in the Hep-2 cell line and in oxidative damage in the MRC-5 cell line. An increase of micronuclei was also observed in both cell lines following BPA exposure. BPA and DOX co-exposures suggested that BPA acts as an antagonist of DOX effects in both cell lines. The interaction with DOX appears to be cell type dependent, exhibiting a non-monotonic response curve in MRC-5 cells, a GPER expressing cell line. Our study emphasizes the need for a deeper knowledge of BPA interactions, particularly with chemotherapeutic agents, in the context of risk assessment and public health.

1. Introduction

In recent decades, the chemical industry has grown and enlarged the production of synthetic chemicals, namely Endocrine Disruptor Chemicals (EDCs), defined by the World Health Organization (WHO) (2012) as “an exogenous substance or mixture that alters function(s) of the endocrine system, and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” [1]. Awareness that there is persistent and continuous human exposure to these compounds, as well as their well-known capacity for bioaccumulation, has raised public concern regarding possible health associated effects [2]. Several studies have reported positive correlations between human exposure to these compounds and hazardous effects in fertility, fetal development and cancer (breast cancer, ovarian cancer, prostate cancer, thyroid cancer, brain cancer, among others) [3,4].

Among EDCs, one that raises particular concern is bisphenol A

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(BPA), an industrial chemical with one of the largest productions worldwide, employed in the manufacture of numerous consumer products such as polycarbonate plastics and epoxy resins. BPA is a xenoestrogen, capable of triggering distinctive estrogen-signaling pathways with potential consequences for human health (reviewed in Rubin (2011)) [5]. Its estrogenic activity has nevertheless been categorized as being weak, because its binding affinity to classical estrogen receptors (ERα and ERβ) is 10,000- and 1,000-fold lower than that of, respectively, endogenous estrogen (E2) for ERα and ERβ [6]. However, tissue specific alterations induced by BPA, as well as developmental ones, including cellular proliferation, are thought to be mediated also by non-nuclear estrogen receptors, such as the transmembrane estrogen receptor (GPER), associated with several cell-signaling pathways [7–10].

Although BPA is not classified as a carcinogen for humans (IARC, group 3), this compound has been previously characterized as an aneugenic chemical [11], and evidence has demonstrated its involvement in the development of tumors [12,13].

At concentrations equal or greater than 100 μM, BPA has reported genotoxic effects associated with decreased cellular proliferation and viability [10,14]. It has been also suggested that BPA directly interferes with the mechanisms of cell division [15], with association to aberrations in spindle morphology, congression of chromosomes malfunctions at metaphase, nondisjunction at anaphase, and abnormal microtubule organization in both cultured somatic cells and oocytes [11,16–19]. Furthermore, the expression of genes involved in mitotic processes are also affected by BPA exposure in a variety of cell lines, even when BPA is at extremely low concentrations [20–24].

BPA-induced alterations on transcriptional patterns have been correlated to the epigenetic effect of BPA, which was first demonstrated in mice after maternal exposure to BPA. This resulted in decreased DNA methylation upstream of the Agouti gene which was prevented by maternal dietary supplementation with folic acid [25]. Toxicological assessment of epigenetic changes is of particular importance, as these alternations can have permanent effects on gene expression patterns and be passed to future generations [26]. Exposure to BPA of normal-like human breast epithelial cells (MCF-10 F) was shown to alter DNA methylation patterns of several genes including those involved in apoptosis and DNA repair [27].

BPA exposure effects have been exceedingly studied in regard to carcinogenesis in hormone responsive organs [28], but information regarding potential interactions with chemotherapeutic drugs is scarce. Nevertheless, previous studies have demonstrated its counteracting effects in breast cancer cells [29,30] and HT29 cells [31] in co-exposure with Doxorubicin (DOX), one of the antineoplastic drug most used in cancer treatment all over the world [32], whose action is based on promoting the apoptosis of cancer cells by inducing DNA damage on them [33].

Ingestion of contaminated food is responsible for more than 90% of overall human exposure to BPA at all age groups [34], therefore tissues from the upper gastrointestinal tract, such as laryngeal cells, are particularly exposed to non-conjugated BPA (active form). In the present study, we evaluate BPA effects in Human epithelial type 2 (Hep-2) cells, originated from a human laryngeal carcinoma. We use concentrations of BPA commonly found in human biological samples, as reviewed in Ribeiro et al. and Vandenberg et al. [35–37], which are also considered safe in terms of human exposure. Since we also intend to evaluate BPA and DOX genotoxic effects, alone and in co-exposure, we have also assessed their isolated and joint effects in MRC-5 cells, a DNA repair-proficient cell line.

2. Materials and methods

2.1. Cell cultures and reagents

Hep-2 and MRC-5 cell lines were kindly offered by Centro Hospitalar Lisboa Ocidental; Hospital Egas Moniz, Microbiology and Molecular Biology Laboratory, and cultivated in 75cm2 flasks with RPMI media containing GlutaMAX™ I, 25 mM HEPES (Invitrogen), supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Cell cultures were maintained in a 5% (v/v) CO2 humidified atmosphere at 37 °C. Subculture cells were allowed to stabilize for 24 h in standard growth medium before treatments.

2.2. Drugs and treatments

Bisphenol A (Sigma) was freshly diluted in ethanol and added to the culture media in concentrations of 4.4 μM (1 μg/ml), selected based on the reference value of 50 μg/kg bodyweight/day, assuming an average bodyweight of 70 kg and a total water intake of 3 l per day. The U.S. Environmental Protection Reference Dose for Chronic Oral BPA Exposure (RfD) is 50 μg/kg body weight/day [38], which is concordant with BPA Tolerable Daily Intake (TDI) set by the European Food Safety Authority up until January 2015 and more recently reduced to 4 μg/kg body weight/day [39]. We have also assessed dosages of 4.4 nM (1 ng/ml) and 0.44 nM (0.1 ng/ml), which correspond to the range of detected levels of BPA in human biological samples due to environmental exposure. DOX (AppliChem) was dissolved in water and added to the culture medium until it reached a final concentration of 4 μM (2.5 μg/ml), corresponding to free DOX concentration in blood in clinical cancer chemotherapy [40]. For the combined BPA/DOX exposures, cells were pre-exposed to BPA for 24 h followed by additional 24 h of simultaneous exposure to BPA and DOX. Correspondingly, for single drug exposures, cells were incubated with BPA for 48 h after the 24 h stabilization period, whereas for DOX standard medium was substituted by medium with DO × 48 h after subculture and maintained. Controls were used for all experiments, using cells grown in standard culture medium or in medium supplemented with ethanol 170 μM (vehicle concentration for BPA).

2.3. Cytotoxicity assay

Cell viability was evaluated by CellTiter-Blue assay (Promega), which is a fluorometric method that assesses cell metabolic capacity. For this, cells were plated on 96-well dishes at a density of 3.2 × 104 cells/well. After treatments, CellTiter-Blue Solution Reagent was added to each well according to the manufacturer's instructions, cells incubated for 4 h and viability was determined by measuring fluorescent emission at 590 nm using a Synergy HT Bio-Tek plate-reader. Experiments were repeated three times with at least three replicates per treatment.

2.4. Immunofluorescence

Cells were plated on glass coverslips. After treatments cells were fixed in acetone PA for 10 min at room temperature. For the immunodetection procedure, cells were incubated with the primary antibody anti-GPR30 (ab39742, Abcam) diluted 1:200 in 1% (w/v) BSA/PBS and incubation was carried out at 37 °C for 1h30 h. After washing with PBS, conjugated anti-rabbit-FICT IgG (1:200, Abcam), a secondary antibody was added at 1% (w/v) BSA/PBS and incubated for 60 min at 37 °C. Cells were then washed three times with PBS, and DAPI stained, coverslips were mounted on glass slides with antifade AF1 (Citifluor). Immunofluorescence was recorded using an epifluorescence microscope Zeiss Axioskop2 equipped with a Zeiss AxioCam MRc5 digital camera. Images were captured using the appropriate excitation and emission filters and merged with Adobe Photoshop 7.0 (Adobe Systems) software. Two replicates per treatment were analysed for two independent experiments.

2.5. Comet assay

Cells from different treatments were cryopreserved in a freezing mix (90% v/v of FBS and 10% v/v DMSO) and stored at −80 °C for comet assay analysis. Analysis of DNA damage and oxidative damage, a modification of the comet assay (originally described by Singh et al. 1988) [41], was performed to assess the DNA oxidation in these cells as described in Collins et al. [42].

Briefly, cells were thawed at 37 °C and centrifuged to eliminate freezing mix; 30 μL of cell suspension was diluted in 140 μL of 1% low melting-point agarose (Pronadisa), and 70μL of this mix was transferred as a drop onto the slide pre-coated with 1% standard agarose (SeaKem®), totalizing 2 drops per slide, each was then covered with a coverslip to set the gels. Cells treated with 100 mM H_2O_2 were used as a control. Slides were then placed in a lysis solution (2.5 M NaCl, 0.1 M Na2EDTA, 10 mM Tris and 1% Triton® X-100, pH 10), for 1 h at 4 °C. For the detection of oxidative damage, slides were incubated with the FPG (kindly donated by Prof. Andrew Collins) for 30 min at 37 °C. After this treatment, all slides were submerged into an electrophoresis solution (10 M NaOH and 0.5 M EDTA) for 40 min at 4 °C and subsequently electrophoresis was conducted in the same solution under 20 V for 20 min at 4 °C. After washed and air dried, the gels were stained with DAPI. The scoring of 100 comets per slide was performed with Comet Assay IV Perceptive Instruments® software. The visualization of slides was performed by a single observer [43].

2.6. Cytology analysis

Cells were cultivated on shell vials (129 AX; PVL). After treatments, cells where fixed with methanol for 20 min at room temperature, DAPI stained and mounted on glass slides with mounting medium for evaluation of micronuclei formation. The slides were evaluated for micronuclei, mitosis and mitosis abnormalities in a Zeiss Axiovert 40 CFL microscope with immersion oil and 1000× amplification by a single observer.

2.7. Statistical analysis

Comet assay results were statistically analyzed by ANOVA and the Games-Howell post hoc test. Variables evaluated by cytology analysis were statistically analyzed by Logistic Regression. Student´s *t*-test was used for statistical comparison of pairs of means.

3. Results

3.1. Environmentally relevant BPA concentrations per se do not affect cell proliferation or viability but interact with DOX in the MRC-5 repair-proficient cell line with a non-monotonic curve like effect

Potential cytotoxic effects of BPA and DOX exposure alone and in combination were evaluated by analyzing cell viability in Hep-2 and MRC-5 cells. We have tested three environmental relevant BPA concentrations 0.44 nM, 4.4 nM and 4.4 μM and one commonly used therapeutic dosage of DOX, namely 4 μM. The CellTiter-Blue assay was utilized to measure viability after 48 h exposure to vehicle and to BPA, 24 h exposure to DOX and 24 h of co-exposure to DOX and BPA concentrations. Our results show no significant differences in cellular viability following exposition to different concentrations of BPA in both cell lines (Fig. 1). DOX exposure however induced a significant decrease in Hep-2 viability, whereas in MRC-5 cells no significant effects in viability were observed. Also, in Hep-2 cells, BPA co-exposure with DOX did not affect the DOX effect, whereas in MRC-5 cells we observed an increase in cellular viability after co-exposure to both the lower and higher BPA dosages suggesting a U shaped response curve. Additionally, the lower and middle BPA concentrations tested induced significant divergent effects in relation to DOX alone as demonstrated in Fig. 1B.

3.2. MRC-5 cells express GPER

Immunocytofluorescence analysis of MRC-5 cells, confirm the presence of GPER restricted to the cytoplasm, in a characteristic endoplasmic reticulum association (Fig. 2), as previously observed by Otto and coworkers in MDA-MB231 and HEC50 cells [44] and in our previous work in HT29 cells [24].

3.3. Genotoxic effects of BPA and DOX, when alone and in co-exposure

Comet assay was used to evaluate two dependent variables: global DNA damage, and oxidative damage – a particular type of DNA damage caused by the oxidation of nucleotides.

These two dependent variables are represented by percentage of DNA in the tail, whose length is proportional to percentage of damage. The statistical analysis of comet assay results compares the mean percentage of damage due to different treatments by ANOVA. The Games-Howell test was used for post-hoc comparisons of all the treatments with the control group for both dependent variables. The results, for the Hep-2 and MRC-5 cell lines, are presented in Fig. 3A and B, respectively.

In Hep-2 cells, the highest values of DNA damage were observed in exposure to DOX alone and in co-exposures of DOX and BPA. As for the effect of BPA exposure, we have found divergent results, since BPA 0.44 nM induced a significant increase in DNA damage, whereas BPA 4.4 nM induced a decrease. Regarding oxidative damage, treatments with vehicle, BPA 4.4 nM and BPA 0.44 nM, induced a significant decrease in this type of damage. Comparing the DOX treatment alone with co-exposure treatments, it was found that mean DNA damage was significantly different between the treatment with DOX and the treatment with DOX + BPA 4.4 μM and DOX + BPA 4.4 nM ($p < 0.001$ and $p = 0.036$, respectively). In regard to oxidative damage, there was also statistically significant differences between the means following DOX treatment and DOX + BPA 4.4 nM treatment ($p = 0.021$).

In MRC-5 cells, a significant increase in DNA damage was found only after exposure to DOX alone and a decrease was found after DOX + BPA 4.4 μM co-exposure.

Oxidative damage results also indicated increased values after BPA 4.4 μM, BPA 4.4 nM and DOX + BPA 0.44 nM treatments. Comparing the DOX treatment with the co-exposure treatments, we found that mean DNA damage was significantly different between DOX treatment and DOX + BPA 4.4 μM and DOX + BPA 0.44 nM treatments ($p < 0.001$ for both). The mean oxidative damage was also significantly different when the DOX treatment was compared with the DOX + BPA 4.4 μM treatment ($p = 0.006$).

3.4. BPA reveals aneugenic potential in a dose dependent manner

3.4.1. Micronuclei induction

The MN was used to evaluate the aneugenic potential of exposure to BPA, when alone and in co-exposure with DOX. Results of the MN Assay are percentages of the MN observed for each treatment and were analyzed by Logistic Regression, using the control group as the reference. The comparisons between control and treatments for Hep-2 and MRC-5 cell lines are presented in Fig. 4A and B, respectively.

In Hep-2 cells we have found a significant increase in the percentage of MN after the BPA 4.4 μM (2.35%) and 4.4 nM (2.5%) treatments, and a significant decrease induced by exposure to DOX alone (0.65%) in relation to the control (1.35%).

Regarding the MRC-5 cells, 4.4 μM BPA (3.5%) and 4.4 nM BPA (3.25%) treatments prompted a significant increase of the percentage of MN ($p = 0.008$ and $p = 0.025$, respectively) whereas DOX (1.25%) exposure resulted in a significant decrease ($p = 0.038$) as compared to the control (2.1%).

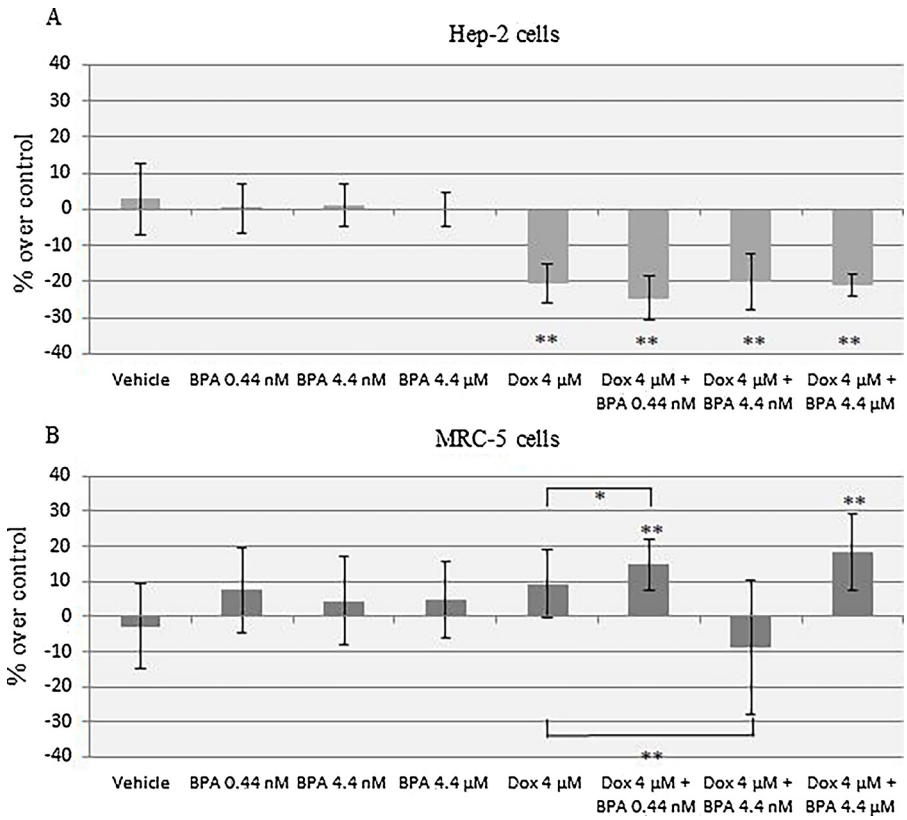

Fig. 1. Evaluation of BPA and DOX exposure effects when alone and in co-exposure on the cellular viability of Hep-2 (**A**) and MRC-5 (**B**) cell lines. Variation in cell viability after 48 h exposure to vehicle and BPA at 4.4 µM, 4.4 nM and 0.44 nM concentrations, 24 h exposure to DOX 4 µM and 24 h of co-exposure to DOX and BPA concentrations. Results are presented as percentage of variation in relation to equivalent cells maintained in standard medium (control – which is graphically represented as zero). Experiments were repeated with at least three biological replicates, three times per experiment. Student's *t*-test (** $p < 0.01$ and * $p < 0.03$) in relation to control and to DOX alone.

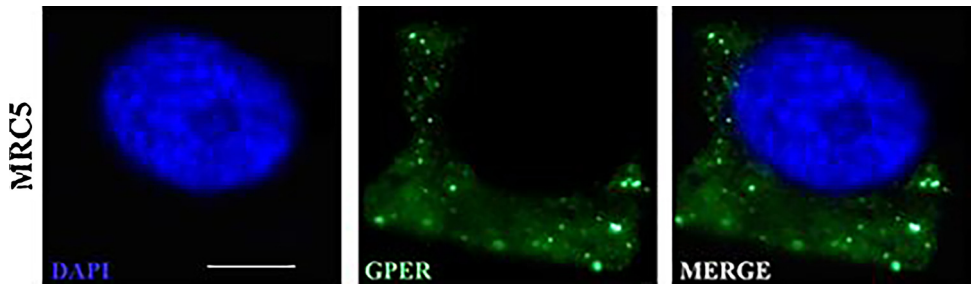

Fig. 2. GPER is expressed in MRC-5 cells. Immunocytofluorescence images of representative interphase MRC-5 cells showing GPER distribution signals (center), corresponding DAPI-staining of DNA (left) and merged images (right), bar = 5 µm.

3.4.2. Mitotic anomalies

The results of mitotic aberrations are expressed by the mitotic index, which is the number of cells undergoing cellular division divided by the total number of cells in the study population. The results were analysed by Logistic Regression, using the control group as reference, in Hep-2 and MRC-5 cell lines, and are presented in Fig. 5A and B, respectively.

BPA 4.4 µM exposure in Hep-2 cells resulted in the highest mitotic index, although this was not found significantly different from the control. No significant differences were also observed between the control and the other treatments in relation to mitotic index.

In MRC-5 cells all BPA concentrations induced a significant increase in mitotic index when compared with the control. BPA 4.4 nM (2.05%) has the highest mitotic index, being statistically higher than the control (0.35%). BPA 0.44 nM (1.35%) and BPA 4.4 µM (1.05%) also presented a statistically significant increase of the mitotic index.

Although BPA 4.4 µM showed the higher abnormal mitotic index, no significant differences between treatments and control were observed.

4. Discussion

The continuous and widespread human exposure to BPA raises questions regarding its potential effects to human health and interference with some drugs used for human benefit. This research intended to address possible genotoxic effects of low concentrations of BPA upon human cells which are known to be in direct contact with BPA in its active form, due to ingestion of contaminated food and water. Also, it was evaluated its interference with DOX, a very common antineoplastic used with therapeutic purposes. Evidence that BPA alone and in co-exposure with DOX, have effects according with cell type and may be dose-dependent, originating non-monotonic response curves, is provided by the results.

Although in recent years BPA has been intensively studied in estrogen responsive cancer tissues and cells, information regarding potential interactions with chemotherapeutic drugs is still scarce. It is currently acknowledged that chemicals can interact and these interactions can be classified as i) additive – when the effect of the two chemicals together equals the sum of the effect of them separately; ii) synergetic – the effect of the two substances together is greater than the sum of their separate effect; and iii) antagonistic – the effect of the compounds together is less than their effects in separate [45,46].

Previous studies have demonstrated that BPA induces counteracting effects in co-exposure with commonly used therapeutic dosages of DOX,

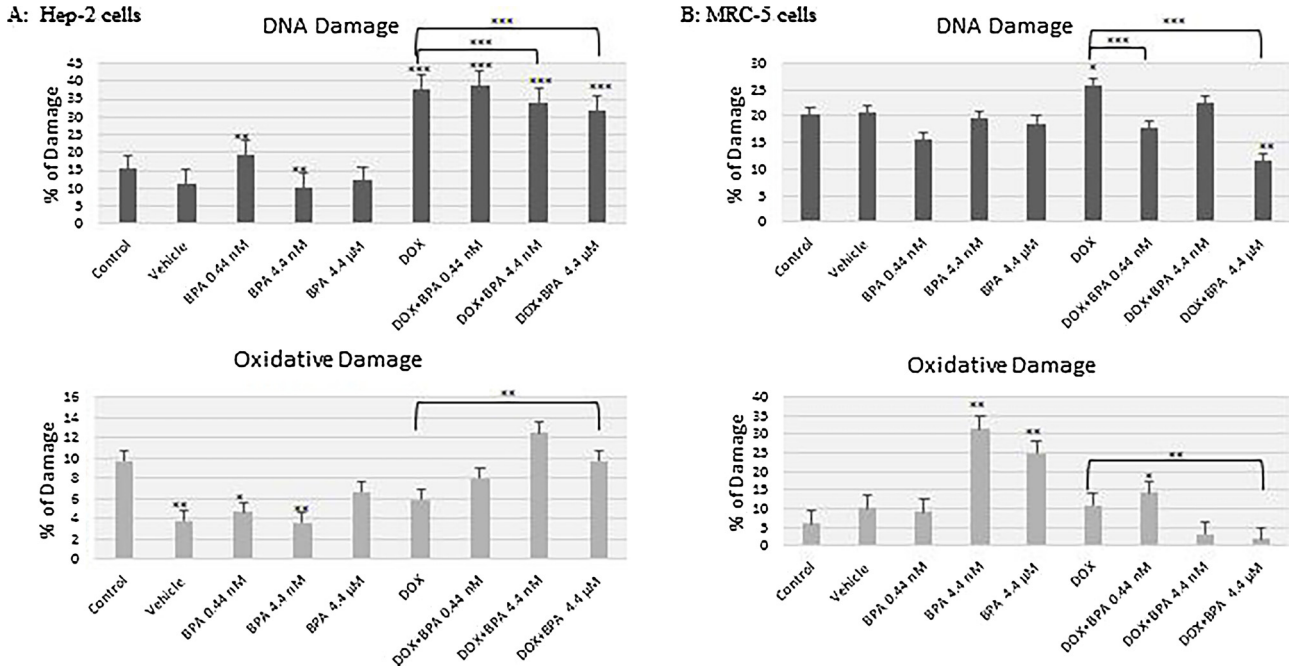

Fig. 3. Effects on DNA damage and oxidative damage of exposure to BPA and DOX, exposure effects de per se when alone and in co-exposure, on DNA damage and oxidative damage in the A) Hep-2 cell line and B) MRC-5 cells. The total number of cells scored was n = 300 *per* treatment and control for Hep-2 cell line and n = 100 *per* treatment and control for MRC5 cells. Bars show (mean + /- SE). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for ANOVA post hoc Games-Howell test for paired comparisons between treatments and control.

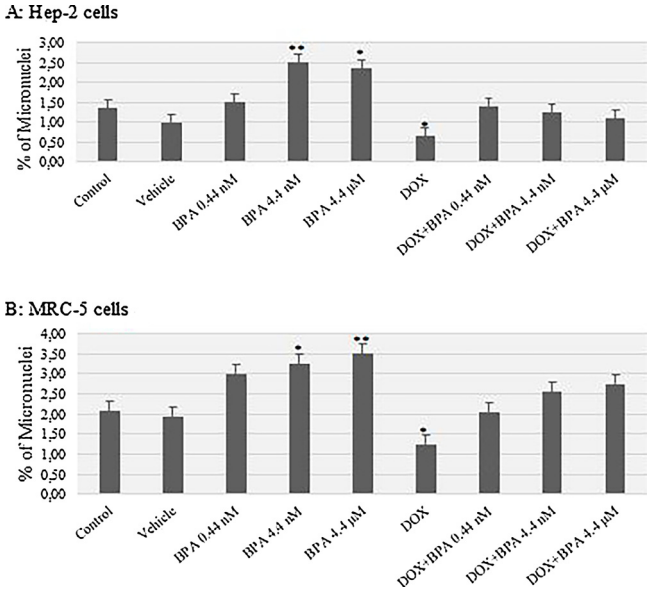

Fig. 4. Evaluation of BPA and DOX exposure effects when alone per se and in co-exposure on the percentage of Micronuclei in A) Hep-2 cell line and B) MRC-5 cells. The total number of cells scored was n = 1000 *per* treatment and control. Bars show (mean + /- SE). * $p < 0.05$ and ** $p < 0.01$ from Logistic Regression conducted for comparison between treatments and control.

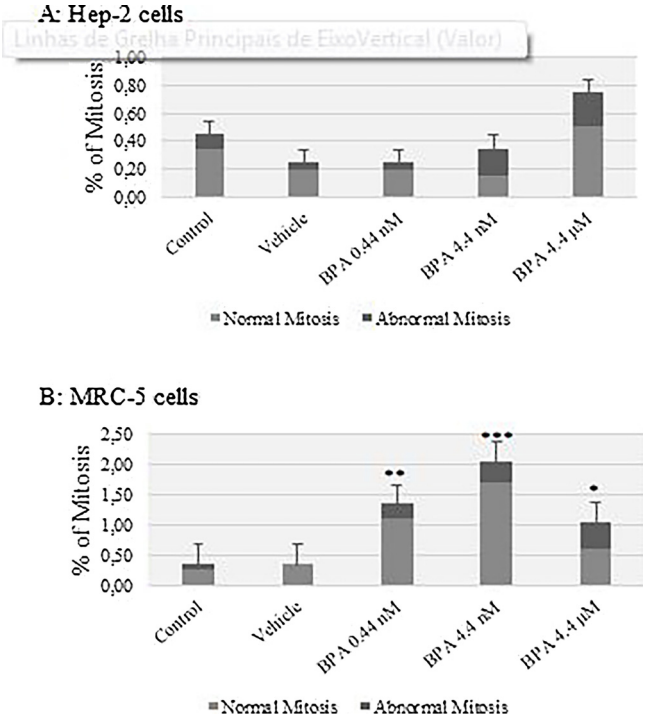

Fig. 5. Evaluation of BPA and DOX exposure effects alone per se and in co-exposure on the Mitotic Index in A) Hep-2 cell line and B) MRC-5 cells. The total number of cells scored was n = 1000 *per* treatment and control. Bars show (mean + /- SE). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ from Logistic Regression for comparison between treatments and control.

a drug which induces cell cycle arrest and apoptosis [47], associated with increased levels of anti-apoptotic proteins [29,30]. Additionally, our previous studies performed in the HT29 cell line, revealed that BPA can alter the effect of DOX on transcript levels of crucial genes involved in cancer biology and interacts with DOX in a dose dependent manner although having no effects on cell viability [31].

Here we also did not observe any effect in both cell lines after BPA exposure. However, in Hep-2 cells, DOX per se induced a severe decrease in cellular viability which is in agreement with the expected effects of this drug in therapeutic doses [47]. In MRC-5 cells, the absence of DOX effects in cellular viability is also in agreement with previous studies [48]. On the other hand, whereas in Hep-2 BPA co-exposure with DOX did not alter the DOX effect, just as previously

observed in HT29 cell lines [31], BPA in MRC-5 cells induced an increase of cellular viability in a non-monotonic manner, since the effects were observed in the lower and higher doses of BPA tested.

Studies suggested that BPA promotion of cell proliferation is associated with the transmembrane estrogen receptor (GPER) that holds much higher affinity for BPA than the nuclear ER implicated in low dose responses [9,49]. Immuno-cytofluorescence shows that GPER was restricted to the cytoplasm in MRC-5 cells (Fig. 2), with identical distribution observed in HT29 cells, characteristic of its association with the endoplasmic reticulum [24]. To our knowledge, this is the first time that the expression of GPER is demonstrated in this cell line. Although GPER may have a role in mediating the responses to BPA exposure in some cell types, its expression does not result in induced cell proliferation in all GPER-positive cells [24].

Considering the previous data regarding BPA aneugenic potential [11,24] and as a player in carcinogenesis [12,13], DNA damage was also assessed by comet assay. This is a widely used tool in biomonitoring which evaluates the genotoxic effects of exposure to specific substances (like antineoplastic drugs or chemicals) *in vivo* and *in vitro* studies. In this study, the introduction of FPG enzyme, which converts the nucleotides oxidized into strand breaks [50,51], also allowed to assess DNA oxidative damage

Comet assay results regarding the exposure of Hep-2 cells to BPA, revealed that the highest DNA damage took place with the lower BPA concentration (0.44 nM), which is in agreement with other studies, demonstrating DNA damage as a consequence of exposure to BPA [52–54]. Iso et al. (2006) showed that the capacity of BPA to cause damage in DNA is higher in a cell line expressing ERs, but BPA also causes significant damage in ER-negative cell lines [53]. Apparently, the binding to ERα mimics estrogenic activity leading to the activation of the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK), whereas the binding to ERß prevents this to signal its downstream targets, acting as an antagonist [55].

Significant decrease in mean DNA damage of cells exposed to BPA 4.4 nM was observed, although without being significantly lower than those exposed to BPA 4.4 μM. This result may be explained by the non-monotonic response to BPA, namely hormone concentration and receptor occupancy interaction [7], reviewed in foregoing studies [56–59].

The comparison between cell viability and comet assay results reports that even at low non cytotoxic BPA concentrations, can lead to genotoxic effects, being in agreement with Pfeifer et al. [60]. The concentration of BPA is so low that it does not reach toxic level, however high enough to bind to the receptor and interfere with cell DNA [7]. Audebert et al. showed that higher concentrations (1–100 μM) of BPA presented cytotoxic effects but not genotoxic ones. This supports the assumption that BPA machinery is similar to hormones, so a high concentration may lead to cytotoxic acute reaction.

These findings are in concordance with other studies that have described BPA genotoxic effects and associate BPA to epigenetic effects and reproductive dysfunction [61–65]. These genotoxic effects not only have an impact on the exposed individual but also on their offspring [7,66]. As reviewed by Roy et al., prenatal exposure to BPA can lead to a precocious puberty in girls and genital abnormalities in boys [66]. Still regarding the epigenetic effects of BPA, a study on placental cells revealed that BPA induced alterations in expression levels of microRNAs, which can lead to negative effects for offspring development [62].

In the MRC-5 cell line there was no significant differences between the means of DNA damage in response to BPA treatments and the control. Pfeifer et al. showed that low-doses of BPA up-regulates c-Myc, which induces DNA damage in ERα-negative cells [60]. Dong et al. also described the BPA binding to the trans-membrane estrogen receptor (GPR30) leading to an activation of Erk1/2 in breast cancer cells [67]. This suggests that BPA is not entirely dependent on the ER, which can help explain the absence of significant damage in the ER-negative MRC-5 cell line [68,69].

Regarding the oxidative damage presented by the MRC-5 cells after exposure, there are statistically significant differences between BPA 4.4 μM, 4.4 nM, and the controlin agreement with other studies describing BPA as capable of promoting oxidative damage [70,71]. In Hep-2 cell line, oxidative damage was significantly lower in vehicle, BPA 4.4 nM and 0.44 nM in comparison with control. These differences were not expected since BPA is known to cause oxidative damage. Since MRC-5 is an ER-negative cell line and HEp-2 is an ER-positive one, a possible explanation is that these differences are a consequence of different mechanisms of action of this chemical agent – ER-dependent and independent, as reported by Pfeifer et al. [53,60,68,69,72,73].

In Hep-2 cell line, DNA damage mean for all DOX treatments was significantly higher than in controls ($p < 0.001$), possibly being explained by DOX mechanism of action based on DNA damage and subsequent cell cycle arrest [32,74]. These findings are in agreement with other studies of exposition to antineoplastic drugs, showing DNA damage caused by DOX [75–78].

In the MRC-5 cell line, exposure to DOX also led to a significant increase in DNA damage when compared to control. However, the same did not happen in co-exposures to DOX and BPA, as the DOX + BPA 4.4 μM presented a significantly lower damage than the control.

Comparing the exposure of Hep-2 cells to DOX alone with co-exposure to DOX and BPA, the observed DNA damage of DOX co-exposure with BPA 4.4 μM and 4.4 nM were significantly lower than after DOX alone. Similar decrease was found for the MRC-5 cells, where the DNA damage following exposure to DOX, was significantly different from the co-exposures to DOX + BPA 4.4 μM and BPA 0.44 nM ($p < 0.0001$ for both).

This study shows that low doses of BPA antagonize the induction of DNA damage by DOX in a therapeutic dosage, being in line with other studies which reported antagonist effects of BPA regarding antineoplastic drugs [29,31]. Nevertheless, it does not seem to influence the mitotic index.

When we confront the DNA damage of MRC-5 cells, following co-exposure to DOX and BPA, with the cell viability assay, it turns out that the co-exposure treatments with less damage are also those which presented a significant increase in cell viability. Saffi et al. (2010) showed that the nucleotide excision repair (NER) mechanism is involved in the repair of DNA damage caused by DOX, in this study the MRC-5 cells are defined as NER proficient [48]. This is a possible explanation to the increase in cell viability following exposure to DOX and co-exposures. Although it is noticeable that the DOX + BPA 4.4 nM presented a decrease in viability, this may be due to the antagonist mechanism of DOX by BPA that is still unknown but has previously been reported [31].

Oxidative damage cause by DOX was not significantly different from control for either cell lines. The oxidative potential of DOX has been previously described [79,80], although Wong et al. showed that the resistance of a cancer cell line to the apoptotic effects of DOX was related to the downregulation of MT-ND3 [81]. This raises the possibility that our cell lines also present a mechanism to avoid the oxidative stress cause by DOX. Nevertheless, the MRC-5 cells presented a significant increase in oxidative damage caused by DOX co-exposure to BPA 0.44 nM. This might be an effect of DOX, which oxidative effects have been described [79], not yet antagonized by BPA, since no significant differences compared to the oxidative damage cause by DOX (10.87 ± 19.56) at this BPA concentration were found.

We have also observed significant differences between oxidative damage following exposure to DOX and in co-exposure with BPA 4.4 nM in Hep-2 cells, and that oxidative damage was significantly lower in DOX + BPA 4.4 μM ($p = 0.006$). This illustrates the interference of BPA with DOX effects [31], and how the effects of BPA differ depending on cell line, as reported by Ribeiro-Varandas et al. [24] and Aghajanpour-Mir et al. [82].

The statistical analysis of the mitotic index in Hep-2 cells revealed

no significant differences between treatments and control. In this cell line, the exposure to BPA 4.4 μM exhibited the highest percentage of mitosis. It was also visible that cells exposed to BPA 4.4 μM had the highest percentage of abnormal mitoses, however without significant differences from the controls. This result might perhaps explain why this concentration also exhibited the highest percentage of MN.

The mitotic index of the MRC-5 cell line was influenced by the treatments. Following BPA treatments, the cells presented an increased mitotic index when compared to control, the highest mitotic index being for those exposed to BPA 4.4 nM, $p < 0.0001$. These findings are in agreement with other study which describes an increased mitotic index in response to BPA [83,84]. These studies also described an increase of abnormal mitosis in consequence of exposure to BPA. In our results such increase was visible in both cell lines but was not statistically significant [83,84]. Aghajanpour-Mir et al. showed that BPA causes chromosomal aberrations and their highest values were also in intermediate concentrations of BPA [82].

Both Hep-2 and MRC-5 cell lines did not present mitosis following DOX exposures and co-exposures, suggesting that BPA at these concentrations does not interfere with the cell cycle arrest promoted by DOX [31,85]. The MN assay allows an assessment of damaged DNA not repaired by the cell, since it is necessary that a cell undergoes at least one division to originate MN.

The MN percentage in the Hep-2 cell line is significantly higher in exposure to BPA 4.4 μM and BPA 4.4 nM than in the control ($p = 0.02$ and $p = 0.009$, respectively). The analysis of MN percentages in MRC-5 cells revealed an increase of MN formation in the same exposures observed for the Hep-2 cells (BPA 4.4 μM and BPA 4.4 nM), which also presented values significantly different than the control ($p = 0.008$ and $p = 0.025$, respectively). This is in agreement with other researchers that describe BPA as a MN inducer [11,24,84,86,87].

In both cell lines, the formation of MN following DOX presented a significant decrease when compared to the control, $p < 0.01$.

Interestingly, the DOX + BPA 4.4 μM and DOX + BPA 4.4 nM treatments for the MRC-5 cell line and the DOX + BPA 0.44 nM for the Hep-2 cell line, led to an increase of MN percentages when compared to the control, although the differences were not statistically significant.

It was also visible in both cell lines that all co-exposures had a highest percentage of MN thancontrols. A main conclusion from these results is that BPA antagonizes the DOX effects. This BPA interference upon DOX had already been established by Delgado et al. (2015), who described as BPA alters the transcript levels of *AURKA, p21, CLU, c-fos* and *bcl-xl*, all genes related to cell cycle progression, mitotic regulation and apoptosis control [31].

5. Conclusions

This study provides evidence that the effect of BPA is dependent on cell type and is non-monotonic. Furthermore, we show that low doses of BPA endorse genotoxic effects and interferes with DOX effects at therapeutic concentrations, a relevant point for cancer patients undergoing this treatment. More importantly, other than genotoxicity, is this new evidence for the antagonist effect on DOX – one of the most used antineoplastic drugs in cancer treatment.

Conflicts of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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