Environmental Toxicology and Pharmacology 69 (2019) 1–8

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

# Low doses of Bisphenol A have pro-inflammatory and pro-oxidant effects, stimulate lipid peroxidation and increase the cardiotoxicity of Doxorubicin in cardiomyoblasts

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ARTICLE INFO

*Keywords:*
Inflammation
Cardiotoxicity
Bisphenol A
Peroxidation
Doxorubicin

ABSTRACT

Endocrine disrupters are strictly associated to cancer and several cardiovascular risk factors. Bisphenol A (BPA) is an endocrine disrupter commonly used in the manufacturing of plastics based on polycarbonate, polyvinyl chloride and resins. Our study aims to investigate whether BPA may cause pro-oxidative and pro-inflammatory effects on cardiomyoblasts, thus exacerbating the Doxorubicin (DOXO)-induced cardiotoxicity phenomena. We tested the metabolic effects of BPA at low doses analyzing its affections on the intracellular calcium uptake, oxidative stress, lipid peroxidation and production of nitric oxide and interleukins. Co-incubation of BPA and DOXO significantly reduced the cardiomyoblast viability, compared to only DOXO exposure cells. The mechanisms underlying these effects are based on the stimulation of the intracellular calcium accumulation and lipid peroxidation. Notably, BPA increase the production of pro-inflammatory interleukins involved in cardiovascular diseases as well as in DOXO-Induced cardiotoxicity phenomena. This study provides a rationale for translational studies in the field of cardio-oncology.

## 1. Introduction

Endocrine disrupters are chemicals like pesticides, plastic additives, cosmetics products and organic pollutants able to modify the synthesis, secretion and biological activity of endogenous hormones (Nadal et al., 2017). Humans are environmental exposed, daily, to several endocrine disruptors at very low doses (Wang et al., 2013). Main sources of endocrine disrupters are perfumes (releasing Phthalates) (Strassle et al., 2018), insecticides (like Endosulphan) (Preud'homme et al., 2015), sunscreens (as example Parabens, Nonylphenol) (Gassel et al., 2013) drugs (such as 17α-Ethynylestradiol) (Saaristo et al., 2019), fungicides, epoxy resins, flame retardants (containing Polychlorinated and Polybrominated biphenyls) (Chokwe et al., 2019), recycled paper, polycarbonate bottles, plastics and food packaging (releasing Bisphenol A) (Chen et al., 2017), lubricants (that contain Nonylphenol). Our recent review described that exposure to endocrine disruptors is strictly relate to several cardiovascular risk factors like obesity, metabolic syndrome, type 2 diabetes as well as prostate cancer (Quagliariello et al., 2017a; 2017b).

Bisphenol A (BPA), an additive for polycarbonate plastics (like plastic bottles, same food containers, thermal papers, register receipts), is the most common endocrine disruptor clearly related to increased relative risk of cancer incidence (Shafei et al., 2018) and cardiovascular diseases (Murata and Kang, 2018; Seachrist et al., 2016).

Mice exposed to BPA at environmental concentration (between 0.01- 0.1 μM) have a significant increases of diabetes, insulin resistance, precocious puberty, reduced sperm count, obesity, prostate and breast cancer (Trasande, 2017). BPA increase cellular migration in prostate and breast cancer cells thereby stimulating the epithelial mesenchymal transition (Huang et al., 2018). Levels of urinary BPA are increased in patients with prostate cancer (Tse et al., 2017) and with cardiovascular diseases (Melzer et al., 2010), compared to healthy patients. These associations are strictly related to no estrogen-like effects of BPA but based on its pro-inflammatory and pro-oxidant effects (Rezg et al., 2014; Han et al., 2016). Notably, BPA accumulates in human body fat and changes the release of leptin and adiponectin from adypocytes thereby affecting metabolic changes in the body like induction of insulin resistance and production of interleukin 1-β (Menale et al., 2015).

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https://doi.org/10.1016/j.etap.2019.03.006
Received 27 September 2018; Received in revised form 12 February 2019; Accepted 5 March 2019
Available online 07 March 2019
1382-6689/ 

Interleukin 1-β is a pro-inflammatory protein having a key role in stroke, cardiovascular diseases, and heart failure (Szekely and Arbel, 2018). Notably, Interleukin 1-β mediates the Doxorubicin-induced cardiotoxicity in preclinical models (Zhu et al., 2011), suggesting it as a therapeutic target in the prevention of heart diseases (Everett et al., 2018).

Although the endocrine effects of BPA have been investigated, there is no information about its role in the cardiac susceptibility to cardiotoxic anticancer treatments like Doxorubicin. Therefore, the aim of the present study was to assess the effects of BPA, at low doses, on cardiomyoblasts metabolism during DOXO exposure exploring its mechanisms of action, with particular reference on its pro-inflammatory and pro-oxidative effects.

## 2. Materials and methods

### 2.1. Materials

Bisphenol-a and the MTT assay were purchased from Sigma Aldrich (Milan) and Dojindo Molecular Technologies Inc. (Rockville, MD, USA), respectively. Water was purified by distillation and reverse osmosis (Milli-Q Plus). Doxorubicin, Interleukin-6, Interleukin-1β Rat ELISA Kits, Fluo-3, AM (Calcium Indicator) Kit and Nitric Oxide Assay Kit were purchased from Thermo Fisher Scientific, Milan, Italy. Bisphenol A, pure ethanol solution 99,9%, Lipopolysaccharides and Malondyaldeide ELISA kit were purchased from Sigma Aldrich Inc, Italy). Interleukin-8 ELISA kit was purchased from My Bio Source's S.r.l (San Diego, USA). DCFDA / H2D CFDA,Cellular Reactive Oxygen Species Detection Assay Kit was purchased from AbCam plc, Italy. 4-Hydroxynonenal (HNE) ELISA Kit was purchased by Biomatik S.r.L (Wilmington, Delaware, USA). H9c2 cell line (ATCC® CRL-1446™) was purchased from the American Type Culture Collection (ATCC-Rockville, MD).

### 2.2. Methods

#### 2.2.1. Cell viability

We used MTT [3-(4,5-dimethyldiazol-2-yl)-2,5- diphenyl tetrazolium bromide] method (Dojindo Mol.Tech. Inc.,Rockville, MD) to measure the dehydrogenase activity of mitochondria viability of H9c2 cardiomyoblasts, as reported in our previous work (Quagliariello et al., 2018)

H9c2 cells were grown in the complete medium composed by the Dulbecco modified Eagle medium (DMEM) added with 10% (v/v) of heat inactivated Fetal Bovine Serum (FBS), antibiotics penicillin G (at 100 U/mL) and streptomycin (at 100 mg/mL). Cells were grown in 96-well plates at 10,000 cells per well in standard conditions (at 37 °C and 5% v/v of $CO_2$). After one day of growth we tested the following solutions suitably dissolved in the complete medium described before: control (only cell medium); DOXO (ranged from 0.01 to 40 μM); DOXO (ranged from 0.01 to 40 μM) co-incubated with BPA (at 0.002, 0.02, 0.2 or 2 μM). For all experiments, cells were incubated for one day under standard growth conditions; after, cells were washed three times with PBS and incubated with 0.1 mL of a MTT solution (at 0.5 mg/mL) for 4 h at standard cell growth conditions. Absorbance was acquired at a wavelength of 450 nm by using the Tecan Infinite M200 plate-reader (with an I-control software). The relative cell viability (%) was calculated by the following formula $[A]_{test}/[A]_{control} \times 100$, where "$[A]_{test}$" is the absorbance of the test sample, and "$[A]_{control}$" is the absorbance of cells exposed to only cell medium. After, we quantified the total cell protein content using the Micro BCA protein assay (Pierce, Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's instructions. Absorbance related to the protein content was acquired at 562 nm. The measured absorbance obtained from MTS assay is proportional to both the number and the metabolic activity of the cells; in order to obtain values strictly linked to the mitochondrial activity, the absorbance readings were normalized against the total cellular protein content, as described in literature (Almalik et al., 2013). The cytotoxicity was then normalized by total protein content in each well.

#### 2.2.2. Quantification of intracellular reactive oxygen species (iROS)

The quantification of intracellular reactive oxygen species (iROS) was performed by using the fluorescent probe DCFH-DA, as reported elsewhere (Barbarisi et al., 2017). In brief, H9c2 cells were grown in the same medium described in paragraph 2.2.1.

Subsequently, $5 \times 10^3$ cells/well were seeded in a 24-well plate for one day under standard growth conditions. After washing three times with PBS, cells were pretreated, or not, with BPA at different concentration (ranging from 0.002 to 2 μM) for 4 h and then incubated with DCFH-DA (at 5μM in PBS solution) for 0,5 min. After, cells were stimulated with a lipopolysaccharide (LPS) solution at 40 ng/mL or DOXO at 50 nM and 100 nM (both for 12 h). Notably, we decided to use these doses of DOXO because both reflect the tissue exposure after intravenous administration of the drug, considering the mean plasma concentration of the DOXO after in cancer patients during therapies (Darrabie et al., 2012). After exposure, cell fluorescence was detected by using a spectrofluorometer (xMark Microplate, Spectrofluorometer Biorad, Milan-Italy) as reported in our previous work (Barbarisi et al.,2017).

#### 2.2.3. Lipid peroxidation studies

Pro-oxidative effects of BPA were determined by using a conventional method described in literature (Bradley-Whitman and Lovell, 2015). H9c2 cells were grown as described in paragraph 2.2.1. Subsequently, $5 \times 10^3$ cells/well were seeded in 24-well plate for one day. In brief, H9c2 cells were treated with LPS (at 40 ng/mL) or DOXO (at 50 and 100 nM, corresponding to subclinical plasma concentration of the drug in cancer patients) for 6 h or pretreated for 4 h with BPA at different concentrations (from 0.002 to 2 μM). After, cells were washed three times with cold PBS, harvested with trypsin, and centrifuged for 10 min (at 1000 ×g). After, the cellular quantification of the two principal lipid peroxidation products, such as the Malondialdehyde (MDA) and 4-hydroxionenal (4-HNA) was performed by using ELISA methods according to the manufacturer's protocols. The cellular protein content was performed as described in paragraph *2.2.1* (by using the Micro BCA protein assay kit) according to the kit instructions.

#### 2.2.4. Quantification of nitric oxide release

Nitrite, as described elsewhere (Nagasaki et al., 2014), is a stable product of nitric oxide, quantified using the Griess Reagent System method, as reported in literature (Bryan and Grisham, 2007). In brief, $5 \times 10^3$ cells were seeded in a 24-well plate for one day under standard growth condition. Cells were then treated with LPS (at 40 ng/ml) or DOXO (at 50 and 100 nM) for 6 h or pretreated for 4 h with BPA at different concentrations (from 0.002 up to 2 μM). After, the culture medium was mixed at 50% v/v with a sulfanilamide solution (1% v/v in 5% v/v phosphoric acid) and of N-1- naphtylethylenediamine dihydrochloride solution (0.1% v/v in water). Absorbance of the solution was measured at the wavelength of 540 nm. Cellular concentration of nitrite was calculated by using a calibration curve of standard solution of 0.1 M of sodium nitrite concentrations 0.5–50 μM against absorbance.

#### 2.2.5. Quantification of intracellular $Ca^{2+}$

Doxorubicin increases the intracellular calcium concentration in cardiac cells leading to the initiation of necrosis (Pecoraro et al., 2017; Mele et al.,2016). This effect is mainly based on the overproduction of ROS (Görlach et al., 2015). We quantified the intracellular calcium concentration in H9c2 cardiomyoblasts by using Fluo-3 AM as fluorescent probe, as described in literature (Daily et al., 2017). In brief, H9c2 cells were treated with LPS (at 40 ng/ml) or DOXO (at 50 or 100 nM) for 6 h or pretreated for 4 h with BPA at different

concentration (ranged from 0002 up to 2 μM). After incubation periods, H9c2 cardiomyoblasts were loaded with Fluo-3 AM at standard growth condition and then washed with PBS. The Fluo-3 binding to the intracellular calcium produces fluorescence quantified by using microplate spectrofluorometer (xMark Microplate, Spectro fluorometer Biorad, Italy) at 525 nm.

#### 2.2.6. Inflammation studies

The quantification of IL-6, IL-8 and IL-1β in H9c2 cells was evaluated by using ELISA methods, as described in our previous work (Quagliariello et al., 2017a; 2017b). In brief, H9c2 cells were grown in the same medium described in paragraph 2.2.1. After 24 h of incubation, cardiomyoblasts were treated for 4 h with or without BPA at different concentrations (from 0.002 to 2 μM) before exposure to 40 ng/mL of LPS or DOXO (at 50 and 100 nM) for 12 h. After treatments, without any dilution, we quantified the IL-8, IL-6 and IL-1β production of cardiomyoblasts, according to the manufacturer's instructions, as described in literature (Freund et al., 2003). The sensitivity of ELISAs was < 10 pg/mL; the methods can quantify with precision interleukins at concentration ranged from 1 to 32,000 pg/mL.

### 2.3. Statistical analysis

The difference between groups was determined by a one-way analysis of variance (ANOVA) and by a subsequent Turkey's multiple comparison test in Sigma Plot Software. For all experimental data, a p-value < 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Cardiotoxicity studies

As reported in Fig. 1, DOXO decreases the viability of cardiomyoblasts in a concentration- dependent manner with an IC50 around 10 μM after 24 h of incubation. Co-incubation with BPA increased the DOXO-related cardiotoxicity. Specifically, co-incubation of DOXO with BPA at 0,2 μM always decreased the cell viability of around 15–24% compared to only DOXO treated cells ($p < 0001$ for all). Notably, co-incubating cells with DOXO and BPA at 0002, 002, 0,2 and 2 μM, the IC50 was reduced of around 50, 75, 85 and 100% compared to only DOXO exposure cells indicating cardiotoxic effects of BPA also at very low concentrations.

### 3.2. Quantification of the intracellular reactive oxygen species (i ROS)

We used the H9c2 cell lysate fraction to measure the pro-oxidative effects of BPA under the pro- inflammatory conditions (LPS) and the anticancer therapeutic agent DOXO (Fig. 2A and B, respectively). As reported by others authors LPS increased the intracellular ROS production of H9c2 cells, presumably by its binding to and stimulation of the pathway Toll-Like-Receptor type 4 - NADPH oxidase type 1 (Liu et al., 2015). Co-incubation of LPS or DOXO with BPA induced a more pro-oxidative intracellular microenvironment. Indeed, the intracellular ROS levels were 1.4, 1.75 and around 2 times in 0.002, 0.02, 0.2 μM of BPA, greater than in cells treated with LPS alone, respectively. Moreover, the intracellular ROS levels were 1.3, 1.5 and 1.73 times in 0.002, 0.02, 0.2 μM of BPA, higher than in cells treated with DOXO alone (as example at 50 nM), respectively. In both cases, there was a not statistically significant difference between 0.2 and 2 μM of BPA, which indicates a plateau phase, possibly due to a biochemical saturation mechanism.

### 3.3. Lipid peroxidation studies (quantification of intracellular MDA and 4-HNA)

As shown in Fig. 3, BPA significantly increases both MDA and 4-HNA production by cardiomyoblasts under LPS (Fig. 3,A) and DOXO (Fig. 3 B) exposure, both at 50 and 100 nM. In fact, as example, the production of 4-HNA by cardiomyoblasts was 15, 38, 54 and 70% higher at 0.002, 0.02, 0.2 and 2 μM, respectively, of BPA than in cells treated with only LPS (Fig. 3 A). A similar behavior was observed in DOXO-exposed cells: as example, at 50 nM, cardiomyoblasts produced 6, 33, 47 and 73% more 4-HNA at 0.002, 0.02, 0.2 and 2 μM of BPA versus DOXO alone (Fig. 3 B).

### 3.4. Quantification of intracellular nitric oxide

Nitric oxide plays a key role in cardiovascular diseases and atherosclerosis processes due to the induction of matrix metalloproteinase and protein kinases expression (Dhalla et al., 2010; Takimoto and Kass, 2007). As shown in Fig. 4 A and B, H9c2 cells increases the nitric oxide production under pro-inflammatory conditions and chemotherapy of around 71% and 98%, respectively, compared to only LPS and DOXO (at 50 nM) exposed cells. When co-incubated with BPA, cardiomyoblasts significantly produced more nitric oxide. For example, BPA at 0.002 μM co- administrated with LPS and DOXO (at 50 nM), increased nitric oxide production by 23% and 66%, respectively, versus cells treated only with LPS or DOXO ($p < 0.05$ and 0.001, respectively). When cardiomyoblasts were co-exposed to BPA (at 2 μM) and LPS or DOXO, the production of Nitric Oxide was 69% and 55% greater than cells exposed without BPA ($P < 0.001$ for both).

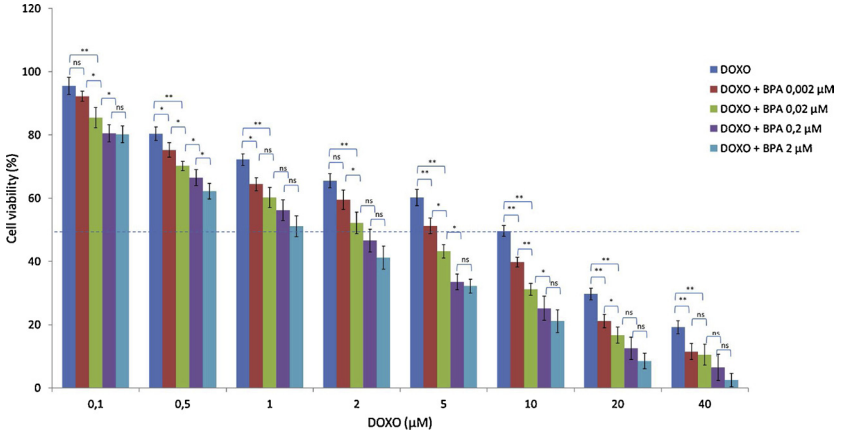


**Fig. 1.** Cardiomyoblasts viability (values ± SEM) after 24 h of exposure to DOXO (from 0.01 up to 40 μM) alone or in combination with BPA (ranged from 0.002 up to 2 μM). *$p < 0.05$; **$p < 0.001$; ns: not significant.

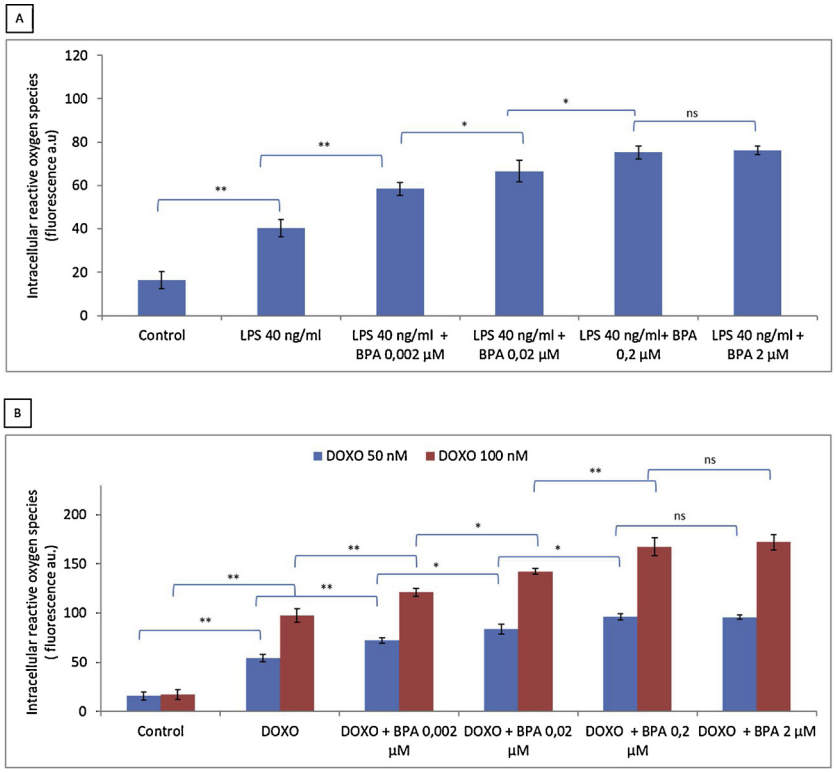


**Fig. 2.** Quantification of intracellular reactive oxygen species (expressed as a.u) in H9c2 cells pretreated with or without BPA (from 0.002 up to 2 µM) for 4 h before incubation with LPS (A) or DOXO (B) for one day. *p < 0.05; **p < 0.001; ns: not significant.

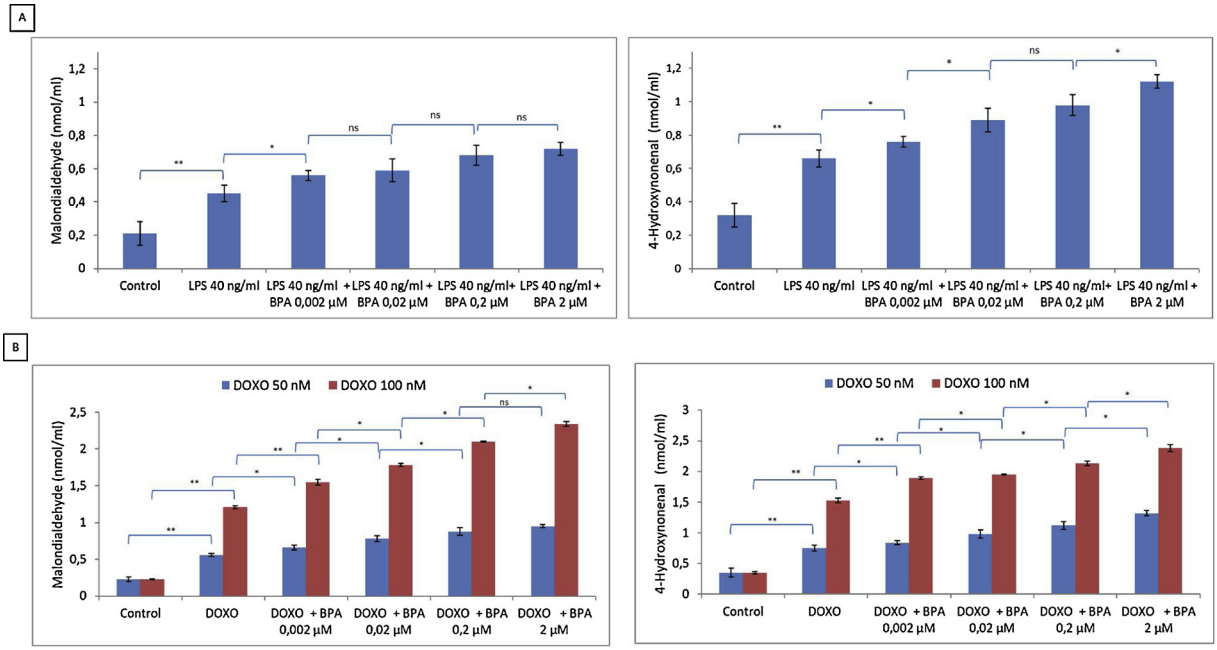


**Fig. 3.** Lipid peroxidation products (MDA and 4HNA) expressed in nmol/mL, produced by cardiomyoblasts under LPS and DOXO (at 50 and 100 nM) treatments alone combined with BPA (ranged from 0.002 up to 2 µM). *p < 0.05; **p < 0.001; ns: not significant.

### 3.5. Quantification of intracellular $Ca^{2+}$

As shown in Fig. 5 A and B, both LPS and DOXO increases the intracellular calcium accumulation in cardiomyoblasts, compared to untreated cells ($p < 0.001$ for all). BPA exposure increases the intracellular calcium content in cardiomyoblasts of 37.5, 56, 62 and 67% at 0.002, 0.02, 0.2 and 2 µM of BPA ($p < 0.001$) in co-incubation with LPS, compared to cells treated only with LPS (Fig. 5A). Indeed, co-incubation with DOXO (at 50 nM) and BPA at 0.002, 0.02, 0.2 and 2 µM increases the intracellular calcium content of around 27, 36.5%, 54% and 59% ($p < 0.001$ for all) compared to only DOXO treated cells (Fig. 5B). Moreover, co-incubation with DOXO (at 100 nM) and BPA at different concentration leads to a similar differences, compared to only DOXO treated cells, seen for treatments with 50 nM, indicating a concentration dependent behavior.

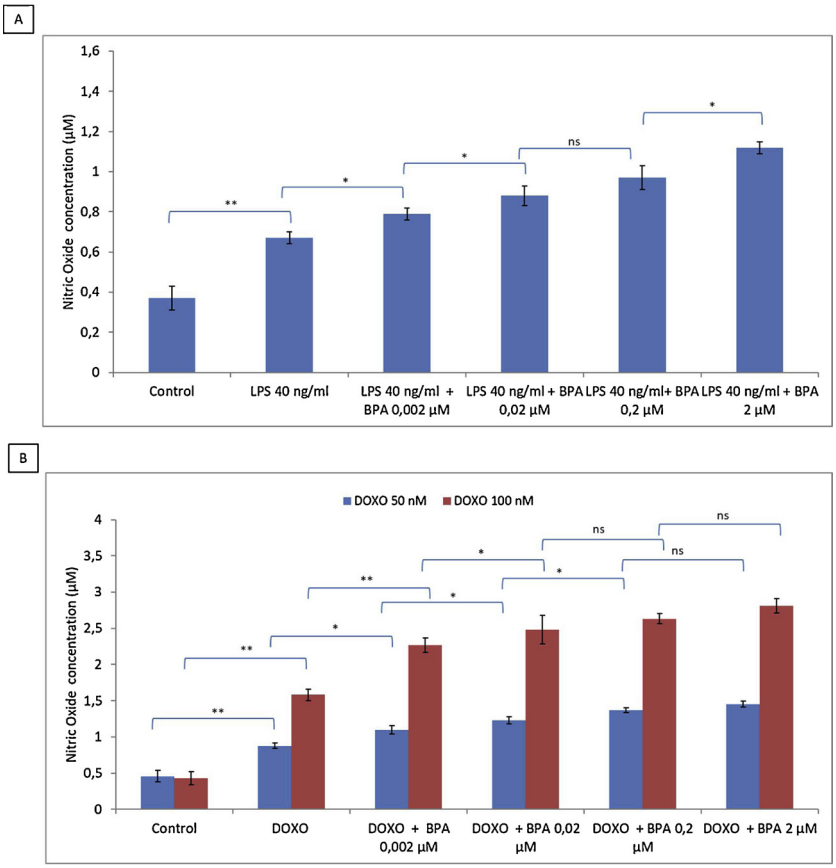


**Fig. 4.** Quantification of nitric oxide production by H9c2 cells un-pretreated or pretreated with BPA (from 0002 to 2 µM) for 4 h before stimulation with LPS (A) or DOXO at 50 and 100 nM (B) for 24 h. *$p < 0.05$; **$p < 0.001$; ns: not significant.

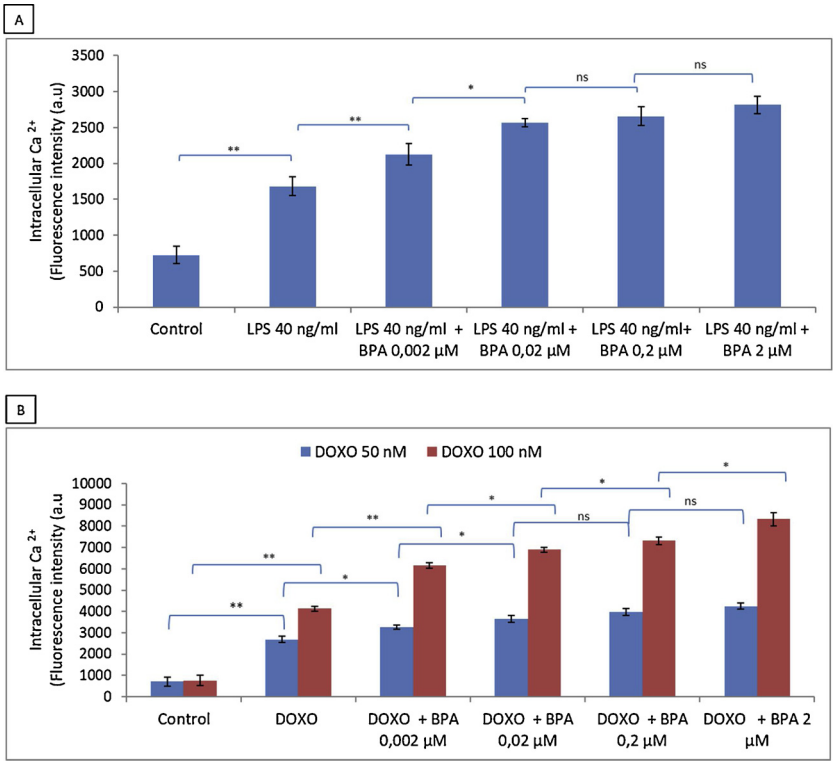


**Fig. 5.** Intracellular calcium quantification in H9c2 cells pretreated with or without BPA (ranged from 0002 up to 2 µM) for 4 h and subsequent incubation with LPS (A) or DOXO at 50 and 100 nM (B) for one day. *$p < 0.05$; **$p < 0.001$; ns: not significant.

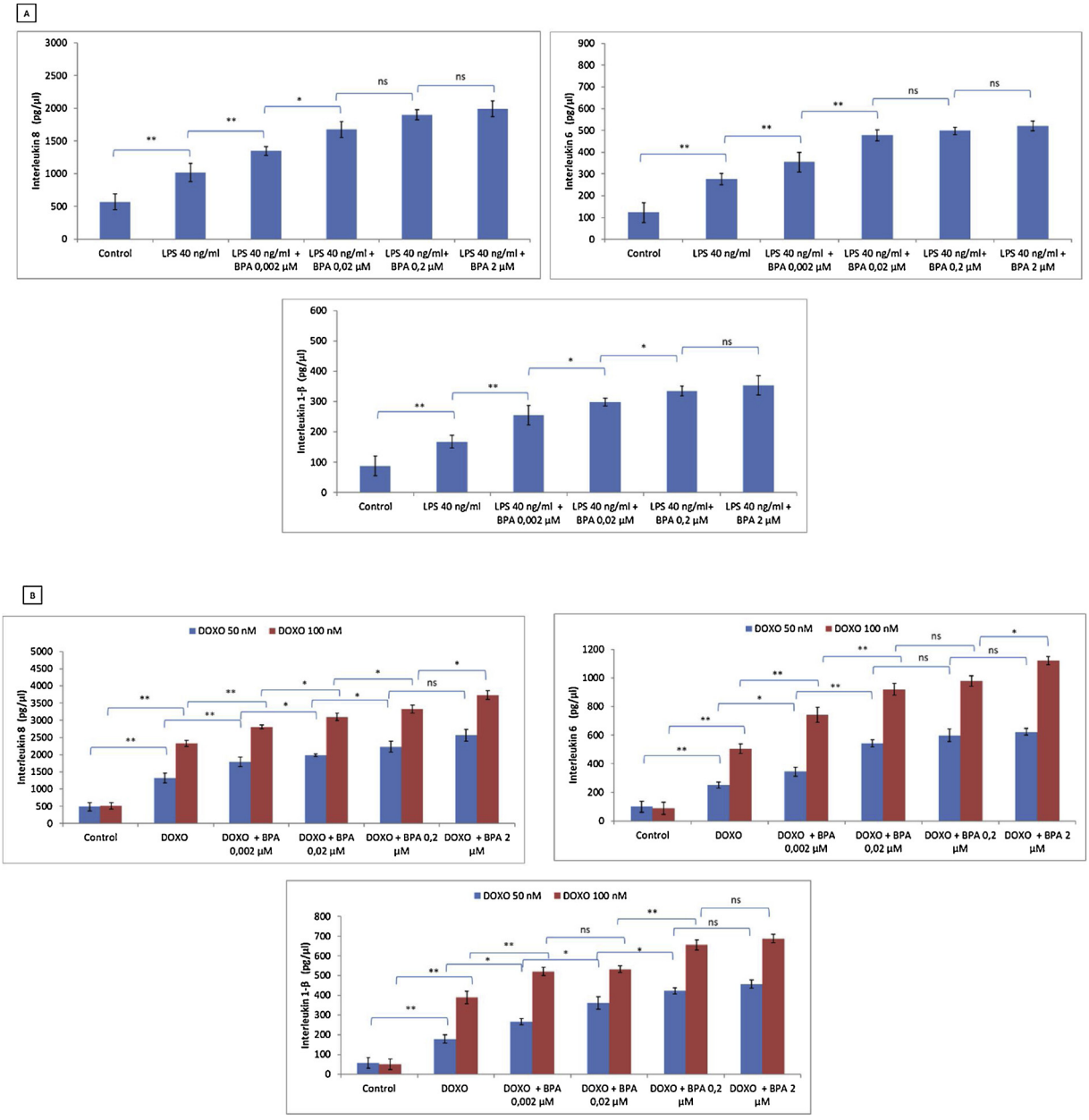


**Fig. 6.** IL-8, IL-6 and IL-1β quantification in cardiomyoblasts (at a density of $1.2 \times 10^5$ cells/well) treated with or without BPA (ranged from 0.002 up to 2 µM) for 4 h before incubation with LPS at 40 ng/mL (A) or DOXO at 50 nM or 100 nM (B) for 12 h. *p < 0.05 ; **p < 0.001; ns: not significant.

### 3.6. Quantification of pro-inflammatory interleukins

Considering the well known pro-inflammatory effects of endocrine disruptors (Rogers et al., 2013), we investigated on the abilities of BPA in the induction of IL-8, IL-6, and IL-1β production in cardiomyoblasts under pro-inflammatory conditions and chemotherapy. Exposure of cardiomyoblasts with LPS increased the production of all interleukins of around 2 times compared to untreated cells (p < 005 for all). Pre-treatments with BPA increased significantly the production of all pro-inflammatory interleukins (Fig. 6 A and B). For example, co-incubation with LPS and BPA at 0.02 µM leads to an overproduction of IL-8, IL-6, and IL-1β of around 60, 73 and 87.5%, respectively, compared to only LPS- treated cells. Notably, co-incubation with DOXO (at 50 nM) and BPA at 0.02 µM increased the production of IL-8, IL-6, and IL-1β of around 43, 120 and 100% compared to only DOXO-treated cells. Moreover, co-incubation with DOXO (at 100 nM) and BPA at 0.02 µM increased the production of IL-8, IL-6, and IL-1β of around 37, 80 and 38% compared to only DOXO-treated cells.

## 4. Discussion

Exposure to endocrine disrupters, at environmental doses, is associated to an increased risk of cardiovascular diseases and obesity (Kirkley and Sargis, 2014). It is widely demonstrated that systemic inflammation, obesity and a pro-oxidative status increased the risk of cardiovascular risk factors, heart failure and atherosclerosis (Mason and Libby, 2015).

Obesity is a well-known risk factor for the atherosclerosis and heart failure; as recently demonstrated, obese cancer patients have an increased risk of cardiotoxicity related to anthracyclines (like DOXO) and anti Erb2 drugs (as example to Trastuzumab) (Guenancia et al., 2016; Maurea et al., 2010). Recently, pro-inflammatory interleukins like Interleukin 1-β have been related to the pathophysiology of the DOXO-induced cardiotoxicity in cancer patients (Todorova et al., 2017;

Apostolakis et al., 2009) in fact antibodies against Interleukin-1 are currently under studying in clinical trials in order to decrease the risk of cardiovascular diseases, stroke, heart failure and atherosclerosis (Ridker et al., 2018).

Herein, we hypothesized that BPA, the most known endocrine disruptor, co-incubated with DOXO, could exacerbate the cardiotoxicity in cardiomyoblasts, increasing the release of pro-inflammatory interleukins. In our study, BPA significantly increased the IL-8, IL-6 and IL-1β release by cardiomyoblasts also at very low concentrations, corresponding to its human environmental exposure (0.001 -0.01 μM) (Wang et al., 2013; Welshons et al., 2006; Lin et al., 2013).

Lipid-peroxidation is a process having a key role in several diseases like cancer, heart failure and DOXO-Induced cardiotoxicity (Olson and Mushlin, 1990). BPA enhances the peroxidation process during pro-inflammatory condition and DOXO exposure thus exposing cardiomyoblasts to the mutagenic and toxic effects of these products (Niedernhofer et al., 2003).

A limitation of our work is based on the study of the BPA effects only in cardiomyoblasts cells and not in adult cardiomyocytes with contractile functions. Indeed, more biochemical studies are under studying in order to understand the effects of BPA on new targets involved in cardiovascular diseases like the pro-protein convertase subtilisin / kexin type 9 (Hadjiphilippou and Ray, 2017). We are currently planning preclinical studies on mice models to evaluate the effects of BPA on DOXO, as well as on Trastuzumab-Induced cardiotoxicities studying its metabolic effects (stimulation of cardiac fibrosis, necrosis/apoptosis and inflammation) and any changes of left ventricular ejection fraction and ejection fraction during anticancer treatments.

## 5. Conclusion

We showed direct metabolic effects of BPA on the cardiomyoblasts during exposure to DOXO stimulating new studies on the association between endocrine disrupters and cardio-oncology. In summary, BPA has pro-inflammatory activities, increased significantly the DOXO-induced cardiotoxicity. Overproduction of heart interleukin 1-β, 8 and 6 during anticancer treatments is a risk factor for cardiotoxic phenomena and our results reveals, for the first time, the contribution of BPA in the exacerbation of the cell damage induced by DOXO. Pleiotropic effects of BPA, not related to its pro-estrogenic activities, should be studied in preclinical models in order to understand the impact of environmental exposure to BPA on cardiovascular health.

## Conflict of interests

The authors declare no conflict of interest.

## Acknowledgments

This work was funded by the “Ricerca Corrente” grant from the Italian Ministry of Health which we thank for the research funding. We are also grateful to Jean Ann Gilder (Scientific Communication srl., Naples, Italy) for writing assistance and Dr. Gindulina V (Istituto Nazionale Tumori, IRCCS, Fondazione G. Pascale, Napoli) for data analysis management.

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