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Effect of bisphenol A on P-glycoprotein-mediated efflux and ultrastructure of the sea urchin embryo

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ABSTRACT

Usage of bisphenol A (BPA) in production of polycarbonate plastics has resulted in global distribution of BPA in the environment. These high concentrations cause numerous negative effects to the aquatic biota, among which the most known is the induction of endocrine disruption. The focus of this research was to determine the effects of two experimentally determined concentrations of BPA (100 nM and 4 μM) on cellular detoxification mechanisms during the embryonic development (2-cell, pluteus) of the rocky sea urchin (*Paracentrotus lividus*), primarily the potential involvement of multidrug efflux transport in the BPA intercellular efflux. The results of transport assay, measurements of the intracellular BPA and gene expression surveys, for the first time indicate the importance of P-glycoprotein (P-gp/ABCB1) in defense against BPA. Cytotoxic effects of BPA, validated by the immunohistochemistry (IHC) and the transmission electron microscopy (TEM), induced the aberrant karyokinesis, and consequently, the impairment of embryo development through the first cell division and retardation.

1. Introduction

Bisphenol A (BPA, 4,4'-(propane-2,2-diyl)diphenol), a synthetic monomer, is used as the main building component of polycarbonate plastic products and epoxy resins, as it provides hardness, flexibility and the resistance toward shape changes. Because of these characteristics, in the past 60 years BPA has become one of the highest production-volume chemicals (5.5 million tons per year) (Alonso-Magdalena et al., 2012; Rubin, 2011; Vandenberg et al., 2009; Flint et al., 2012). Vast usage of BPA is in production of beverage packaging, adhesives, building materials, electronic components, and paper coatings, thermal receipts, and in conjugated halogens for use as flame retardant (Rubin, 2011; Staples et al., 1998).

BPA molecule has relatively short half-life time (2.5–4 days) in the environment but has "pseudo-persistent" characteristics due to its constant release and leakage from plastic products (Flint et al., 2012; Rubin, 2011; Staples et al., 1998). It is an endocrine disrupting compound that mimics the estradiol molecules by size and shape, blocking or binding to the estrogen receptors and, consequently, causing the endocrine disrupting effect (Flint et al., 2012; Lyons, 2000, Rubin, 2011). Although it is thousand times less active than the natural estrogen, BPA concentration of 2–3 ppb in the cell's cytoplasm is enough to induce high hormone activity (Alonso-Magdalena et al., 2012). According to the European Food Safety Authority (EFSA), the tolerable daily intake (TDI) that can be introduced in the human organism is 0.05 mg/kg of body weight and the maximum no observed adverse effect level (NOAEL) is 5 mg/kg of body weight per day (EFSA, 2010).

The release of BPA into the environment is mainly through the authorized discharges of treated industrial waste water (West et al., 2001). By 2003, global production of BPA was 3.2 million metric tons, from which one-third was manufactured in the United States (Flint et al., 2012). The European Commission estimated the annual effluent of BPA to receiving waters to be 21.2 metric tons (European Comission, 2003). While BPA released in the air is expected to break down fairly rapidly, BPA in water is likely to be more persistent and

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leads to reasonable concern over potential impact on aquatic biota (Klečka et al., 2009; Lyons, 2000). Surface-water concentrations of BPA in the USA range from 0.081 to 12 μg/L (0.35–52.26 nM) (Flint et al., 2012), 6.44 to 15.6 ng/L (28.2–68.33 pM) in coastline of China (Ozlem and Hatice, 2008) and 0.01 μg/L (43.8 pM) in Europe (Klečka et al., 2009).

The European Commission and the United States Environmental Protection Agency (US EPA) consider BPA as "moderately toxic" or "toxic" to aquatic biota, reporting EC_{50} values ranging from 1 to 10 mg/L (or equivalent LC_{50} values ranging from 4.4 to 43.8 μM) (Alexander et al., 1988; Flint et al., 2012). Numerous toxicity studies on effects of BPA on aquatic biota have focused mainly on endocrine-related measurement endpoints. For example, BPA induces vitellogenin synthesis in male fathead minnows (*Pimephales promelas*) at concentrations of 640 and 1280 μg/L (2.8 and 5.6 μM) (Sohoni et al., 2001). In invertebrates, BPA effect on development and reproduction has been observed in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae) (Watts et al., 2001), while in the sea urchin (*Paracentrotus lividus*) concentrations of 300–3500 μg/L (1.3–15.3 μM) induced the larval malformations, developmental arrest, and the embryonic/larval mortality (Ozlem and Hatice, 2008).

Although toxicity effects of BPA on aquatic organisms are known, the more profound focus should be on understanding its half-life in the organism. The pending question is whether certain detoxification transport mechanism can eliminate BPA. Several recent studies have offered functional and molecular evidence for deployment of protective multixenobiotic resistance (MXR) mechanism as an important cellular defense against toxic compounds during the sea urchin embryonic development (Bošnjak et al., 2009, 2011, 2013; Goldstone et al., 2006; Hamdoun et al., 2004; Shipp and Hamdoun, 2012). The MXR mechanism is mediated through the transport activity of specific members of ATP-binding cassette (ABC) proteins, members of ABCB (P-glycoprotein; P-gp), ABCC (multidrug resistance-associated proteins or MRPs) and ABCG (and possibly ABCA and solute-carrier SLCs) subfamilies (Vasiliou et al., 2009). These proteins have the ability to traffic a diverse array of environmental compounds, including even moderately hydrophobic xenobiotics, and thus, reduce their intracellular accumulation and toxic effects (Bodó et al., 2003; Epel et al., 2008; Smital et al., 2004). ATP-dependent efflux transporters are an important part of the "chemical defense pathways", along with the cytochromes P450 and other oxidases, various conjugating enzymes, oxidative detoxification proteins, and the transcription factors that regulate these genes (Goldstone et al., 2006).

Sea urchins are an ecologically relevant animal group, and valuable models frequently used for determination of toxic potential of contaminants present in marine environments (Goldstone et al., 2006; Schipper et al., 2008). Given the potential of BPA accumulation and persistence in the environment, our main goal was to assess its impacts toward cellular detoxification mechanisms by exposing the rocky sea urchin (*P. lividus*) embryos to two concentrations of BPA (100 nM and 4 μM) and measuring: (1) the expression of target genes involved in MXR mechanism, endocrine disruption and the cell cycle regulation by quantitative PCR (qPCR); (2) the level of intracellular BPA accumulation; and (3) the ultra-structural changes of treated embryos by transmission electron microscopy (TEM) and the immunohistochemistry (IHC).

2. Materials and methods

2.1. Reagents

Bisphenol A (4,4′-isopropylidenediphenol) was obtained from the Acros Organics (Morris Plains, NJ). Potassium chloride (KCl), verapamil (VER) and dimethyl sulfoxide (DMSO) were purchased from the Sigma–Aldrich (St. Louis, MO). MK571 was purchased from Cayman Chemical Company (Ann Arbor, MI) and calcein-acetoxymethylester (calcein-AM, C-AM) was purchased from Invitrogen Molecular Probes (Eugene, OR). DMSO was used as a solvent control for MK571 stock solutions. The BPA stock solution was prepared in 0.2 μM millipore filtered seawater (FSW) while the VER stock solution was prepared in 96% ethanol (EtOH).

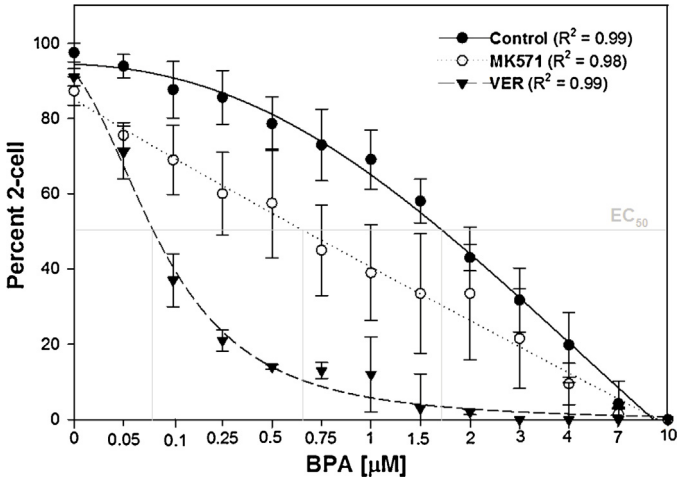

Fig. 1. Embryotoxicity assay (three to five females contributed to embryo batches, consisting of 1000 eggs/mL): BPA potency to inhibit cell division of the sea urchin embryos is observed in the absence (solid line) or presence of competitive inhibition of MRP activity by 5 μM MK571 (dotted line), or P-gp activity by 5 μM VER (broken line). Data are expressed as percentage of embryos reaching 2-cell stage 130 min postfertilization and represent the average ± SDs of nine (BPA), and three (BPA + MK571 and BPA + VER) independent experiments. Gray axes meet in the EC_{50} concentration points.

2.2. Animals

The adult rocky sea urchins (*P. lividus*) were provided by the Aquaculture of Marine Organisms Core Facility at the Zoological Station Anton Dohrn (Naples, Italy). Sea urchins were kept in flow-through tanks, at 15 ± 2 °C seawater and fed algae (*Ulva* sp.) weekly. Spawning was induced by intracoelomic injection of 0.5 M KCl. Fertilization and embryo cultures were held at 16 °C in 0.2 μM filtered seawater (FSW). The success of fertilization was checked under the microscope and only batches of embryos with >90% successful fertilization were used.

2.3. Embryotoxicity assay

Embryotoxicity assay was performed to determined BPA concentrations to be used in downstream experiments. Final concentrations of BPA used for the assay were 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 7 and 10 μM, and final concentrations of the inhibitors was 5 μM for both MK571 and VER. Egg culture preparation, fertilization and exposure of embryos to solvent controls, BPA and/or inhibitors (MK571 and VER) were performed as previously described (Bošnjak et al., 2009, 2011, 2013). EC50, e.g. the concentration of BPA at which only 50% of embryos are successfully finishing the first cell division, was 1.7 μM (388 μg/L) of BPA, while low and high concentrations were 100 nM (22.82 μg/L) and 4 μM (913.16 μg/L), respectively (Fig. 1).

Table 1
Primer pairs used for qPCR.

Gene	Primer name	Primer sequence (5′–3′)	Amplicon length
Abcb1	qP-gp-1	F: GATATCCTAACGACCTTCCTTGCTG	137 bp
	qP-gp-2	R: ATGGTGGGGATCTGGTCATGA	
Abcc9	qC9-1	F: CTTCCTTCATAGTCAACGCGATCG	138 bp
	qC9-2	R: ATCTCGTCACACTCTCTATGCGCTGTA	
Cyclin B	qCb-1	F: ATCCATCAGAGATTGCTGCTGG	142 bp
	qCb-2	R: TTGGCCATCTTGCGCACAATAG	
CDK	qCDK-1	F: CTCAGAACCTCCTCATCGACAAC	129 bp
	qCDK-2	R: CCTCAGGTGCACGATACCATAGAG	
SHR2	qShr2-1	F: AGTGTCATGGCAGGAGGTGATATG	117 bp
	qShr2-2	R: CGAACTCACCATCAGCTTCAACT	
Housekeeping gene			
Ubiquitin (Ubq)	qUBQ-F	F: CCGATCAGCAGCGTCTTATCTTC	127 bp
	qUBQ-R	R: ACACTCTGCATACCACCACGAAGTC	

F: forward primer sequence; R: reverse primer sequence.

2.4. Quantification of target gene mRNA upon BPA exposure of embryos

Rocky sea urchin embryos (*P. lividus*) were exposed to BPA 30 min post fertilization (PF) as it has been previously established that MXR transporters are active at 25 min PF (Hamdoun et al., 2004). The main goal was to determine the expression levels of five different target genes: *abcb1* – ATP-binding cassette, sub-family B, member 1; *abcc9* – ATP-binding cassette, sub-family C, member 9; *shr2* – orphan steroid hormone receptor 2; *cdk* – cyclin-dependent kinase; and *cb* – cyclin b, during two different embryo stages (2-cell and pluteus) in the presence of two different BPA concentrations – low (100 nM; 22.82 μg/L), and high (4 μM; 913.16 μg/L). Real-time PCR (qPCR) analysis of target genes was done as previously described (Bošnjak et al., 2013; Livak and Schmittgen, 2001): exposed and unexposed (control) embryos were sampled for the qPCR analysis; egg cells (before fertilization), 2-cell (1 h and 30 min PF) and plutei (96 h PF). Target genes (*abcb1*, *abcc9*, *shr2*, *cdk* and *cb*; Table 1) and housekeeping gene ubiquitin (*ubq*; Table 1) expressions were measured in 1:10 diluted cDNA samples. The results of the qPCR for *abcb1*, *abcc9*, *shr2*, *cdk* and *cb* gene were calculated relative to the control condition (unexposed embryos) and normalized to ubiquitin. Induction of target genes was considered as biologically significant when overpassed a 2-fold minimum threshold.

2.5. Calcein-AM (C-AM) assay

C-AM is a non-fluorescing acetomethoxy derivate of calcein. Only when transported through the cellular membrane into live cells, intracellular esterases remove the acetomethoxy group that obscures the part of the molecule that chelates Ca^{2+}, Mg^{2+}, Zn^{2+} and other ions, inducing strong fluorescence within the cell. Calcein-AM (C-AM) assay was performed at the 2-cell and pluteus stages as previously described in detail (Bošnjak et al., 2013; Hamdoun et al., 2004): 2-cell embryos and plutei were exposed to transporter inhibitor (5 μM MK571 or 5 μM VER), to BPA alone (4 μM; high concentration), or in the presence of a specific transporter inhibitor (4 μM BPA; 4 μM BPA + 5 μM MK571; 4 μM BPA + 5 μM VER) in the presence of fluorescent substrate C-AM for 45 min. Final concentrations of both DMSO solvent in MK571 stock solution and ethanol solvent in VER stock solution was 0.05% in all experiments.

2.6. Immunohistochemistry (IHC) of the cytoskeleton and transmission electron microscopy (TEM) of BPA-treated 2-cell stage embryos

In order to evaluate BPA-toxic effect on cytoskeletal elements of the 2-cell stage embryos, monoclonal anti-β tubulin-Cy3 antibody (Sigma–Aldrich) was used for tubulin, and Atto 488 Phalloidin dye (Sigma–Aldrich) for actin visualization, while 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) labeled DNA. The rest of the procedure as well as TEM samples processing were done as previously described (Venuti et al., 2004).

2.7. Quantification of BPA by HPLC

High performance liquid chromatography (HPLC) was applied to quantify the free BPA in exposed methanol-extracted cells samples. Cell samples were extracted in 1 mL methanol, vortexed 8 min and centrifuged for 10 min at 4000 rpm and 4 °C (Yi et al., 2010). The extracts were analyzed by HPLC with fluorescence detection, using no resin in the column, by Varian ProSTAR 230 HPLC analytical system coupled with ProStar 363 fluorescence detector (excitation wavelength set to 227 nm and emission to 313 nm) and ProStar 410 autosampler.

2.8. Data analysis and presentation

The percentage of developed 2-cell embryos was used as a measure of BPA toxicity. Data from each set of individual BPA experiments were first analyzed with the Kolmogorov–Smirnov test for normality and then with Bartlett test for homogeneity of variances. All numeric data, represent the 3–12 replicates (i.e. 3–12 individual batches of embryos obtained from 3 to 5 female specimens, final eggs concentration = 1000 eggs/mL); its mean, the standard deviation, and the EC_{50} values were calculated and plotted using Sigma Plot software (Systat Software Inc., San Jose, CA). The trend-lines of mean values were plotted using four-parameter logistic nonlinear regression model. The changes in EC_{50} concentrations of BPA, in the presence or absence of inhibitors, were compared by paired *t*-test, with p values < 0.05 as statistically significant. All data are represented as mean ± standard deviation (SD).

3. Results

3.1. Effect of BPA on urchin first cell division

In this assay we measured the effective concentration of BPA that delays or inhibits the first cell division. Embryos were exposed to BPA 30 min PF. The effective concentration which inhibited division in 50% of embryos (EC_{50}) of BPA was 1.7 μM (388 μg/L) (Fig. 1). Next we used the assay to evaluate if the activity of MXR efflux transporter proteins is involved in the protection against BPA during the first cell division. When incubating urchin embryos in the presence of 5 μM MK571 or VER inhibitors alone, we did not observe any interference with the first cell division and >90% of

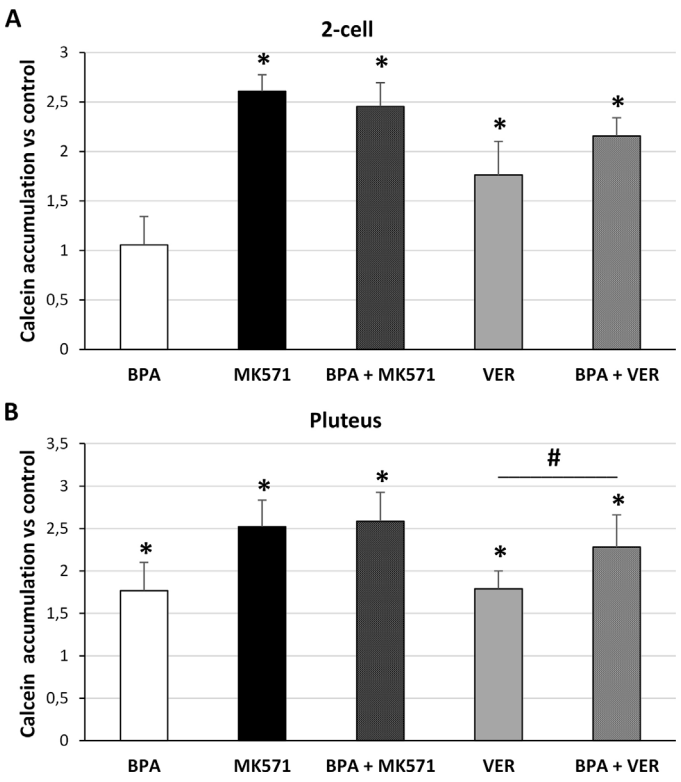

Fig. 2. Change in intracellular fluorescence in the 2-cell embryos (A) or pluteus (B) treated with 4 μM BPA in the presence or absence of an MRP-like inhibitor – 5 μM MK571, and P-gp like inhibitor – 5 μM VER, in relation to untreated controls of the identical developmental stage. Bars represent mean ± SD of three batches of embryos. Asterisk (*) indicates values that are significantly different ($p < 0.05$) compared to the control value. # indicates value that is significantly different ($p < 0.05$) compared to the inhibitor (VER) value. Three to five females contributed to embryo batches, consisting of 1000 eggs/mL.

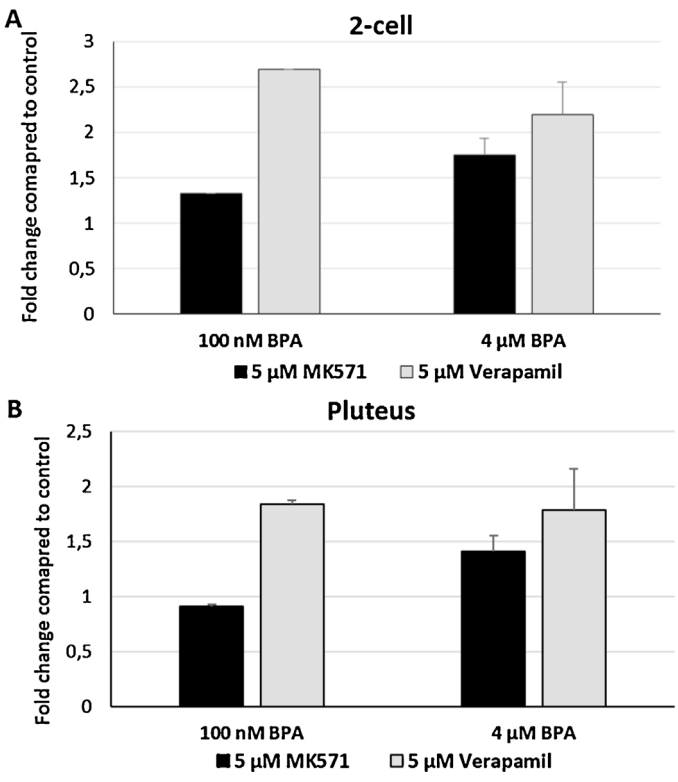

Fig. 3. Intracellular accumulation of BPA after exposure to low (100 nM) and high (4 μM) BPA in the presence of MXR specific inhibitors – 5 μM MK571 (black bars) and 5 μM Verapamil (gray bars) in 2-cell (A) and pluteus stage (B). Bars represent fold change ± SDs of four separate measurements. Three to five females contributed to embryo batches, consisting of 1000 eggs/mL).

embryos successfully entered the next cell division, while the presence of inhibitors did influence the toxicity of BPA (Fig. 1). The inhibition of MRP-like efflux with MK571 specific inhibitor caused 2.6 fold increase in BPA toxicity (EC_{50} = 0.65 μM BPA), while the inhibition of P-gp-like efflux with VER specific inhibitor caused 22 fold increase (EC_{50} = 0.075 μM BPA).

3.2. Characterization of MXR activity in response to BPA exposure

Based on the obtained dose response curve from the embryotoxicity assay for BPA two concentrations of this synthetic compound were selected for further experiments: low (100 nM or 22.829 μg/L) and high (4 μM or 913.16 μg/L) BPA (Fig. 1). Low BPA had almost no effect as >90% of embryos have successfully finished the first cell division, while high BPA was toxic causing delay or arrest of cell division in approximately 80% of exposed embryos. Low BPA was used for all experiments, except for C-AM assay were only high BPA was tested. We focused further on exposing the embryos to designated concentrations during early (2-cell stage; 90 min) and longer (pluteus; 96 h) embryo development period. Using the calcein-AM assay, we determined the level of efflux activity of MXR proteins. *P. lividus* embryos were exposed only to high 4 μM BPA concentration in the absence or presence of each of MXR specific inhibitors, and in the presence of non-fluorescent substrate 250 nM calcein-AM. Both 2-cell and pluteus, in the presence of MXR specific inhibitors alone showed a significant increase of calcein accumulation. In the 2-cell embryos exposed to BPA alone, no significant increase in calcein accumulation was observed (Fig. 2A), in contrast to pluteus (Fig. 2B). BPA in the presence of MXR inhibitors (MK571 + BPA or VER + BPA) did not cause significant increase in calcein accumulation in 2-cell embryos cultures (Fig. 2 A). In pluteus, there is no difference in calcein accumulation between the culture exposed to specific MRP inhibitor (MK571) alone or treated in presence of BPA (MK571 + BPA). However, the difference in accumulated calcein was significantly higher when plutei were exposed to mixture of P-gp specific inhibitor and BPA (VER + BPA), in respect to the inhibitor alone (VER).

To confirm the mechanism of BPA transport out of embryos, obtained based on the embryotoxicity and C-AM assay results, we measured intracellular accumulation of BPA in the 2-cell and pluteus embryos by HPLC method. The embryos were exposed to two BPA concentrations alone (low of 100 nM or high of 4 μM) or in a combination with MRP (MK571) or P-gp specific inhibitor (VER) (Table 2). The intracellular concentration of BPA in the 2-cell embryos exposed to BPA without inhibitors were 0.015 μM for 100 nM and 0.780 μM for 4 μM BPA. In the pluteus, intracellular concentration amounted to 0.062 μM for 100 nM and 1.257 μM for 4 μM BPA. As it was predicted by the embryotoxicity assay, MXR inhibitor VER induced higher accumulation of BPA compared to MK571. Similarly, both BPA concentrations, in the presence of VER had a significant increase in intracellular accumulation in both embryo stages compared to the control. Likewise, intracellular BPA accumulation in the 2-cell after exposure to low BPA concentration and VER caused 2.68, or 2.19 fold increase when treated with high BPA, compared to the control (Fig. 3). In pluteus VER caused 1.83 and 1.78 fold increase in intracellular accumulation of BPA treated with low or high BPA, respectively, compared to the control. In accordance to our C-AM assay results, MK571 inhibitor did not have significant impact on the increase in intracellular accumulation of BPA treated by low concentrations in both tested embryo stages. However, MK571 did cause significant accumulation of BPA

Table 2
Intracellular accumulation of BPA in the 2-cell and pluteus *P. lividus* embryos.

BPA	2-cell		Pluteus	
	Low (100 nM)	High (4 μM)	Low (100 nM)	High (4 μM)
Control	0.015 ± 0.006	0.780 ± 0.443	0.062 ± 0.018	1.257 ± 0.351
5 μM MK571	0.020 ± 0.001	1.364 ± 0.312*	0.057 ± 0.009	1.771 ± 0.205*
5 μM VER	0.040 ± 0.009*	1.714 ± 0.769*	0.115 ± 0.076*	2.248 ± 0.673*

MK571: specific MRP inhibitor; VER: specific P-gp inhibitor. Data are represented as the average μM concentration ± SDs of four separate measurements.
* Values that are significantly different ($p < 0.05$) compared to the control values.

in both cultures exposed to high concentrations, although these values were lower compared to VER exposed cultures.

3.3. Exposure of *P. lividus* embryos to the BPA and target gene expressions (*abcb1, abcc9, shr2, cb, cdk*)

To further investigate the effect of BPA on MXR mechanism, urchin cell cycle and endocrine balance, we measured whether BPA exposure up regulated genes involved in the targeted mechanisms. The qPCR indicated no changes of target gene *abcc9* in either the 2-cell or pluteus stages exposed to both BPA concentrations (data not shown). Considering *abcb1* gene expression, the qPCR indicated no changes at the 2-cell stage exposed to both BPA concentrations (Fig. 4). In contrast, expressions of *abcb1* measured in plutei treated with two BPA concentrations were significantly upregulated: 5.2-fold in 100 nM BPA and 6.2-fold for 4 μM BPA treatment compared to non-treated embryos (Fig. 4). In order to evaluate if BPA is influencing endocrine disruption in embryonic sea urchins, we focused on the *shr2* gene which encodes for orphan steroid hormone receptor 2 (SHR2). Our results evidenced that plutei exposed to 4 μM BPA had significantly induced up-regulation of the *shr2* gene (2.7-fold; Fig. 4). Finally, we looked at the expression of *cb* gene encoding cyclin B (CB) protein and for *cdk* gene encoding cyclin-dependent kinase (Cdk1). Our qPCR data showed that only the *cdk* gene had significant up-regulation in pluteus after the exposure to 4 μM BPA (2.2-folds), while *cb* gene remained unchanged (Fig. 4).

3.4. Effects of BPA on mitosis

In order to evaluate the effects of BPA on mitosis we tested if 5 μM BPA concentration (enabling ~20% of embryos to finish the first cell division, Fig. 1) affects cytoskeletal proteins tubulin and actin of the 2-cell stage embryos (Fig. 5A). IHC demonstrated that in the 2-cell stage high BPA concentration induced formation of a globular or scattered-polymerized spindle that has been lacking astral polarity (Fig. 5B and C). Cleavage furrow appeared asymmetrical and disarranged (Fig. 5B), while in few cases polynuclear cells in the early 2-cell stages were discerned both by IHC and TEM (Fig. 5C), and a delay in cycle timing and progression compared to the control 2-cell stages (Fig. 5A). When assessing the ultra-structural changes induced by BPA in the 2-cell stage, striking changes were observed only during early post-fertilization stage and before the end of 2-cell division. Polynucleate embryos showing extensive irregular vacuolization or multiple nucleoli within nucleus were observed, along with a number of development-arrested but fertilized embryos.

4. Discussion

We determined the toxicity and effect of BPA on the sea urchin *P. lividus* early development. Toxicity of BPA was assessed following previous protocols (Bošnjak et al., 2009, 2011) and effective concentrations showed to be similar to previously reported study (Ozlem and Hatice, 2008), where an EC_{50} value of 300 μg/L (1.31 μM) BPA induced spermiotoxic and embryotoxic effects in the sea urchin. Furthermore, Roepke et al. (2005) previously studied in two sea urchin species, *Strongylocentrotus purpuratus* and *Lytechinus anamesus*, the toxicity effect of BPA and several other endocrine disrupting compounds, on the success of development till the pluteus larval stage (96 h post-fertilization). EC_{50} of BPA was 226.5 ng/mL (0.99 μM), suggesting therefore the existence of species-specific sensitivity, where *S. purpuratus* and *L. anamesus* were less resistant compared to *P. lividus*. Such species-specific sensitivity of other toxic compounds (mercuric chloride, $HgCl_2$ and trybutiltin, TBT) has been previously reported in the Mediterranean urchin species *Arbacia lixula* and *P. lividus* (Bošnjak et al., 2011).

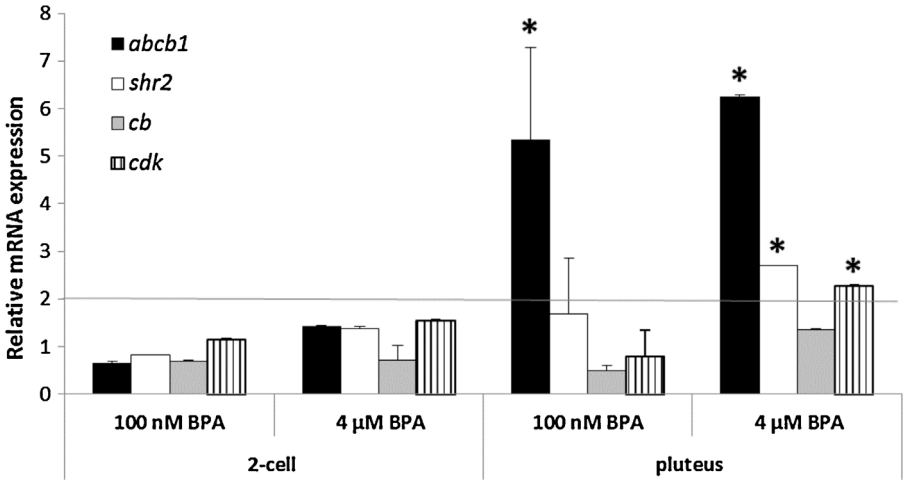

Fig. 4. qPCR plots, the mRNA expression level of *abcb1*, *shr2*, *cb* and *cdk* in *P. lividus* 2-cell and pluteus after exposure to 100 nM or 4 μM BPA. Expression data were obtained from three independent RNA isolations of each egg culture, normalized to ubiquitin mRNA and control (unexposed) samples. Bars represent mean ± SD. Asterisk (*) indicates values that are significantly different ($p < 0.05$) compared to the control value. Gray line represent cell baseline gene expression level.

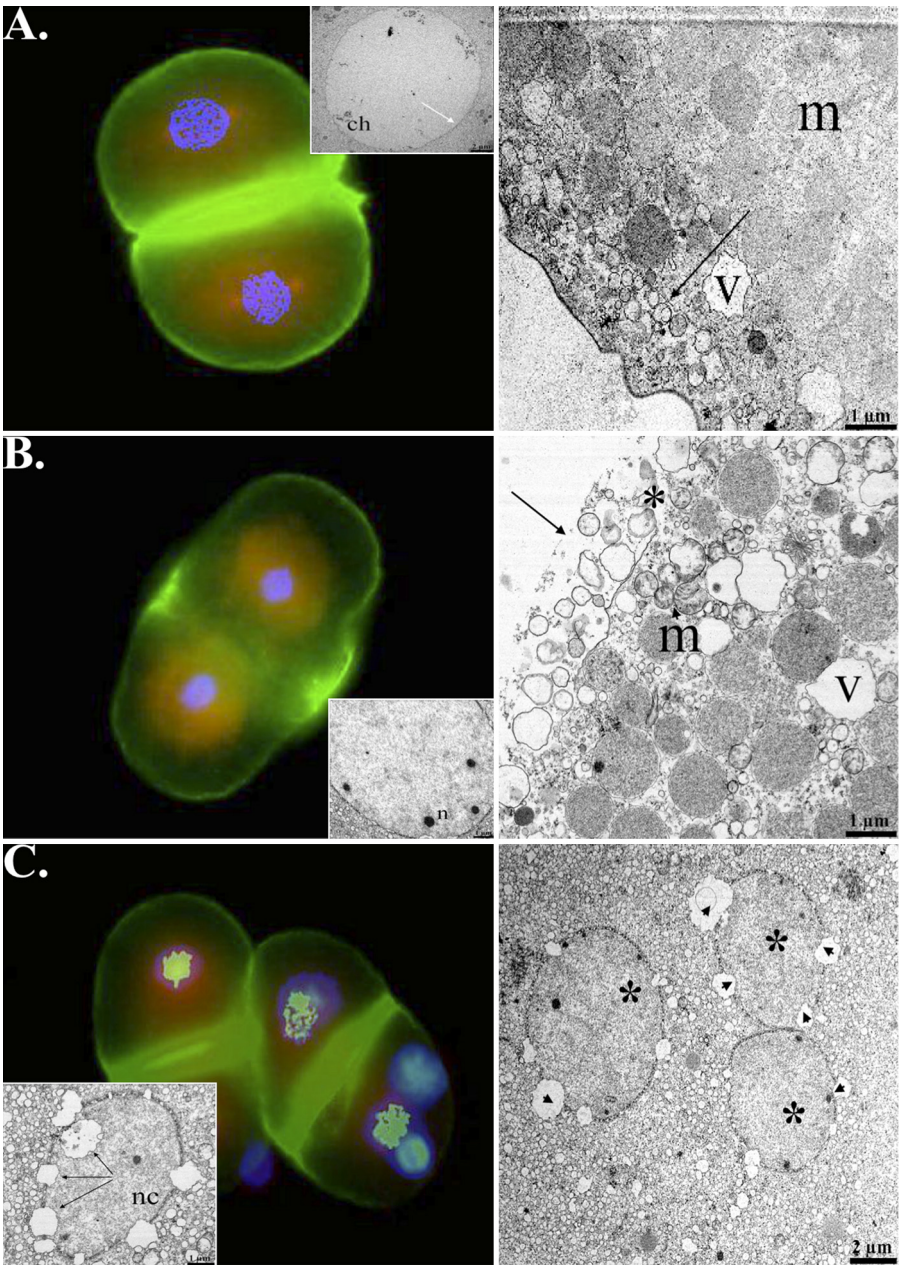

Fig. 5. Morphological and ultrastructural aberrations in the rocky sea urchin 2-cell stage. (A, left) Immunohistochemistry (IHC) of the control unexposed 2-cell stage in mitosis showing typical appearance of mitotic spindle labeled by monoclonal anti-β tubulin-Cy3 antibodies (red), actin labeled by Atto 488 Phalloidin dye (green) and nucleus in telophase labeled by DAPI (violet). Insert: TEM detail of the nucleus: white arrow, inner nuclear membrane; ch, chromatin. (A, right) TEM micrograph of control unexposed 2-cell stage showing mitochondria (m), vacuole (v) and spongioform cell membrane (arrow). (B, left) IHC of 5 μM BPA-exposed 2-cell stage in telophase showing polymerized spindle without astral polarity (red) and cell membrane and cleavage furrow of discontinuous and varying thickness, with shrinkage in some parts and lack of the bilateral symmetry (green). Insert: TEM detail of exposed nucleus showing multiple nucleoli (n). (B, right) TEM of exposed 2-cell stage showing small mitochondria (m), vacuole (v) and disrupted cell membrane (*) missing the external envelope (arrow). (C, left) IHC of treated 2-cell stage where forming of three nuclei in telophase was observed (violet). Insert: exposed nucleus (nc) showing vacuolizations of different size (arrows). (C, right) TEM of exposed 2-cell stage showing three nuclei (*) in the telophase with numerous nuclear vacuolizations (arrowheads).

Next we used our assay to evaluate if the activity of MXR efflux transporter proteins is involved in the protection against BPA during the first cell division. According to the predicted EC_{50} values of BPA ranging from 1 to 10 mg/L (equivalent LC_{50} values ranging from 4.4 to 43.8 μM) (Alexander et al., 1988; Flint et al., 2012) the chosen low BPA concentration was considerably below these predicted values, while the high BPA was just at the lower range limit in *P. lividus* embryos. In C-AM assay (Anselmo et al., 2012; Bošnjak et al., 2013; Hamdoun et al., 2004), non-fluorescent C-AM rapidly crosses cell membranes where it can be converted to fluorescent calcein by non-specific esterases or extruded by MXR efflux transporter proteins, P-gp or MRPs, before intracellular transformation to non-MXR substrate. High MXR transport activity can prevent the accumulation of free calcein in the cell, leading to minimal fluorescent accumulation. Both P-gp and MRPs are actively engaged in sea urchin efflux of different toxic compounds (Bošnjak et al., 2009, 2011; Goldstone et al., 2006). The presence of BPA, could compete with the elimination of calcein-AM if those transporters, P-gp or MRPs, are active in efflux of BPA, and this would result in accumulation of high levels of calcein in comparison to non-treated control. In *P. lividus* increased fluorescence upon specific transporter inhibition, was congruent to previous studies

in several echinoid species (Anselmo et al., 2012; Hamdoun et al., 2004) evidencing high expression of efflux transporters during whole urchin embryonic development (Shipp and Hamdoun, 2012).

Specific inhibition of MXR transport activity can be obtained using 5 μM MXR/MDR inhibitors Verapamil (VER) or MK571. VER inhibits P-gp mediated efflux of a variety of substrates, having Ki values (i.e. concentration that inhibits 50% of maximal transport) ranging from 0.2 to 10 μM (Holló et al., 1996; Miller, 2001). MK571 is the most potent MRP inhibitor (e.g. MRP1 and MRP2) and less potent inhibitor of several other MXR transporters (Miller, 2001). Calcein accumulation in presence of MXR-specific inhibitor (VER) showed to be significant only in BPA-exposed pluteus. Similar results of calcein-AM test in *P. lividus* 2-cell (no calcein accumulation) and blastula (high calcein accumulation) were reported recently for $HgCl_2$ and TBT (Bošnjak et al., 2013). This may indicate that the concentrations of the toxicants and the time of exposure are not enough for detection of inhibition/competition traffic between the calcein-AM and toxic compound (BPA, $HgCl_2$ or TBT) in early 2-cell stage, but become evident in the later developmental stages (blastula and pluteus).

BPA inhibition of calcein efflux by MXR activity has been recently reported in 1 week old echinoid *Psammechinus miliaris* larvae (Anselmo et al., 2012), while this research presents first evidence of BPA effect on the calcein accumulation in early embryos (2-cell stage). Summarized, these results indicate that continuous exposure of embryos to BPA presumably leads toward the oversaturation of P-gp like efflux transport in older developmental embryo stages that seems more involved in the protection against this synthetic compound. However, we used the concentration of MK571 (5 μM) below the total inhibition level of MRP-like transport (Žaja et al., 2008), therefore we cannot firmly claim that MRP-like transporters are not as well active in the protection against this toxic contaminant. In concordance to the obtained results of calcein efflux, the HPLC results also indicate that used concentration of MRP-like-specific inhibitor have not reached oversaturation level. Namely, when both embryo stages were exposed to low BPA concentration and MK571 (MRP-specific inhibitor) or VER (P-gp-specific inhibitor), BPA within cell did not increase dramatically in the first scenario (BPA + MK571), indicating that other un-blocked transporter(s) are engaged in the efflux, or that specific transporters are not oversaturated with their inhibitors. In case of exposure to high BPA concentration however, it seems that part of the BPA that oversaturates Pg-p transporter and consequently cannot exit that way, is additionally effluxed through MRP mechanism as well. Furthermore, the MRP-like efflux might have more significant role in the pluteus than in the 2-cell stage, implying an ontogenic-related strengthening of the MXR mechanism where different efflux patterns become activated in older embryonic stages (Shipp and Hamdoun, 2012). Similarly, in blastulae stage a general decrease in the expression of ABCB1 and ABCC9 transporters has been previously shown (Bošnjak et al., 2013; Shipp and Hamdoun, 2012), further evidencing that the younger embryonic stages are more sensitive to environmental contamination compared to the pluteus. Hamdoun et al. (2004) have suggested that during the first *S. purpuratus* cell division, the efflux activity of ABCB1, being post-translationally controlled by maternal mRNA, even increases with exposure to inhibitors of transcription, while in older sea urchin embryos *abcb1* shows a sharp down regulation, most likely reflecting the successive waves of structural and biochemical reorganization undertaken in the embryos (Shipp and Hamdoun, 2012).

In general, a lower accumulation of BPA in plutues (with or without two inhibitors) in relation to the 2-cell stage, speaks in favor of more effective and developed MXR mechanism that puts additional challenge on the surviving strategies of urchin during the first developmental stages.

In order to study the interaction of MXR mechanism with sea urchin cell cycle and endocrine balance potentially affected by BPA, we measured whether toxicant exposure up regulated genes (*abcb1*, *abcc9*, *shr2*, *cb*, *cdk*) involved in the targeted mechanisms. MXR mechanism-related genes in embryonic sea urchin; *abcb1* gene encoding for P-gp efflux transporter ABCB1 and *abcc9* gene encoding for SUR-like ABCC9 protein (Shipp and Hamdoun, 2012), have been implicated in efflux of mercuric chloride, $HgCl_2$; trybutiltin, TBT; and oxybenzone, OXI (Bošnjak et al., 2013). In our study we have evidenced up regulation of *abcb1* but not *abcc9* gene. Interestingly, the role of *abcc9* gene is still vague in the sea urchin; it is up-regulated throughout its embryonic development, coding sulfonylurea-receptor-like protein (SUR2) (Shipp and Hamdoun, 2012), which is primarily responsible for mediating the closure of the ATP sensitive potassium channel (K_{ATP}-channel) in mammals (Panten et al., 1996). Only recently it was indicated that *abcc9* ortholog in sea urchin may be involved in transport of xenobiotics (Shipp and Hamdoun, 2012) and moreover that it is significantly up-regulated after exposure of the rocky sea urchin embryos to sublethal concentration of mercuric chloride ($HgCl_2$) (Bošnjak et al., 2013). At least in case of BPA in sea urchin, it is indicative that *abcc9* is less likely involved in its transport. In contrast, *abcb1* was up regulated in pluteus. These results are congruent to our HPLC and calcein-AM data where intracellular BPA concentration was higher when P-gp-specific inhibitor VER was incubated with embryo culture, suggesting that P-gp as ABC transporter might be involved in BPA efflux in *P. lividus*. It has been widely accepted that *abcb1* gene that encodes P-gp is involved in the MDR/MXR mechanism acting in phase 0 of cellular detoxification and prevents the accumulation of organic compounds in their unmodified form (Ambudkar et al., 1999). No changes in *abcb1* gene in the 2-cell stage support the thesis that ABCB1 is not the major efflux system in early *P. lividus* stages (Bošnjak et al., 2013), in contrast to other invertebrates (Roepke et al., 2006). It is also accepted that the main MXR activity in sea urchin eggs is mediated post-translationally after fertilization (Hamdoun et al., 2004), and that the increase in activity does not involve de novo transcription or translation, but rather that transporter proteins are present in eggs and moved within cell membrane in microfilament-dependent way (Shipp and Hamdoun, 2012). In our study this explains why P-gp protein activity is fully functional at this stage and thus able to efflux the calcein. Nevertheless, calcein-AM and HPLC data indicate that other specific members of the ABCC/MRP subfamily may be involved in BPA efflux, needing further investigation. Previously, the involvement of ABCG/BCRP transporter (breast cancer resistance protein) and ABCC/MRP members has been evidenced in efflux of metabolites in glutathione, glucuronic or sulfate conjugates form (Haimeur et al., 2004; Goldstone et al., 2006). In addition to unmodified BPA molecule, these transporters are able to transport its metabolite BPA-glucuronide (BPA-G), formed during phase II metabolism, via uridine diphosphate-glucuronosyltransferase conjugation (Kuester and Sipes, 2007). This may be especially important for later developmental stages of sea urchin embryos, as for both ABCC1 and ABCG2 proteins, high transporter transcription was reported in blastula and gastrula stages of *S. purpuratus* (Shipp and Hamdoun, 2012). Our assumption of involvement of other transporters in BPA efflux is further supported by recent research on human and rat cells (Mazur et al., 2012); MRP2, MRP3 and BCRP were evidenced in efflux of BPA, and MRP3 in BPA-G efflux in humans, while in rats, MRP2 and BCRP were involved in BPA, and MRP2 in BPA-G efflux. Such substrate specificity authors related to key differences in transporter amino acid sequences that reflected in putative biding sites composition.

SHR2 is a member of the nuclear receptor superfamily that has been classified in mammals as a member of the steroid–thyroid–retinoic acid receptor superfamily responsible for

activation of transcriptional factors of thyroid hormone and the vertebrate morphogen retinoic acid (Evans, 1998) and under influence of endocrine disrupting compounds (Lyons, 2000; Rubin, 2011). The ligands or activating hormones for these receptors remain mostly elusive, except for the COUP-TFs (chicken ovoalbumin upstream promoter transcription factors) that has shown extensive sequence conservation among different taxa (Chan et al., 1992). SHR2 is ubiquitously expressed in embryos, larvae, and adult tissues of sea urchin (Kontrogianni-Konstantopoulos and Flytzanis, 2001; Goldstone et al., 2006), also shown to bind estradiol in sea urchin embryos (Roepke et al., 2006), thus having a prominent position in activation of transcriptional factors necessary during the early development and cells harmonization. Up regulated *shr2* in our study indicates the potential toward abnormal formation of ectodermal cells in sea urchin larvae, because specifically, the SHR2 protein binds to an upstream element of the sea urchin CyIIIb actin gene that encodes a cytoskeleton type actin expressed in the cell lineage of the aboral ectoderm (Kontrogianni-Konstantopoulos et al., 1996). In early development, only maternal transcripts of *shr2* are present in the embryo in alternative splicing forms, vanishing before gastrulation without being replaced by embryonic RNAs. Later at the early cleavage stage, SHR2 is synthesized by translation of maternal RNA and contrary to the mRNA, SHR2 protein is detected throughout embryonic development (Kontrogianni-Konstantopoulos et al., 1998). Even though we are not able to discern if maternal or intrinsic expression of *shr2* takes place in the pluteus, we found biologically significant gene induction (considering a 2-fold induction as a minimum threshold for biological significance; Fig. 3) after exposure to high BPA concentration. As previously reported by Kontrogianni-Konstantopoulos et al. (1998), such *shr2* upregulation consequently triggers cytoskeleton type actin expression that in turn might induce aberration in development of ectodermal sea urchin cells. Although we have confirmed aberrations in the early development in sea urchin embryos, there is still to clarify on the mechanisms of *shr2* regulatory pattern toward cytoskeletal type actin and its output. Similarly, detection of orphan steroid receptors in the mouse – COUP-TFs, also revealed discrete spatial and temporal expression, affirming their role in the regulation of gene expression during embryogenesis. Interestingly, the simultaneous expression of COUP-TFs raised the possibility that COUP-TFs can act as negative regulatory factors during development and differentiation (Pereira et al., 1995), which can be related to the observed growth retardation in the sea urchin embryo.

Cyclins control a transition between the phases of eukaryotic cell cycle as regulatory subunits of the Cdks and are ubiquitous regulatory proteins (Ito, 2000). CB and Cdk1 control the eukaryotic cell division cycle at the checkpoint from G2 to M-phase. CB binds to Cdk1, forming a CB-Cdk1 complex also called mitosis promoting factor (MPF) (Masaki et al., 2000; Pines, 2006). The amount of CB and the activity of the CB–Cdk1 complex rises through the cell cycle until mitosis and then falls abruptly due to degradation of CB, while Cdk1 is constitutively present (Hershko, 1999). It is also known that in multiple cell organisms the CB–Cdk1 complex regulates microtubule dynamics (Stiffler et al., 1999). In contrast to our study, exposure of abalone *Haliotix diversicolor supertaxa* embryos to BPA has caused significant up-regulation of both *cb* and *cdk* genes that resulted in the metaphase delay in embryonic cells, related to the destabilization of microtubules and to the prolongation of time required to form a functional mitotic spindle (Zhou et al., 2011). However these results were obtained when the abalone embryos were exposed to significantly higher BPA concentrations, i.e. 2 and 10 μg/mL (equals to 8.8 and 43 μM concentrations, respectively). Therefore, we can assume that the higher concentrations of BPA would also cause similar destabilization of microtubules and impair the normal progress of cell cycle in the rocky sea urchin embryos. We have confirmed these assumptions by observation made through electron microscopy.

Finally, we have looked into the effects of BPA on mitosis. Previous studies indicated that in addition to aberrant activation of the estrogen receptor, BPA directly interferes with the mechanisms of cell division causing aneuploidy (George et al., 2008). Although some authors suggested that a major mode of BPA action on dividing cells is through its binding and depolymerization of tubulin filaments and effects on centrosomal proteins (Pfeiffer et al., 1997, Staples et al., 1998), a novel research contrasted these data (George et al., 2008). Our observations were similar to previous study of the influence of organic and inorganic mercury (Bošnjak et al., 2009). However, using IHC we have not observed multiple spindles formation, as described in another sea urchin species (*Lytechinus pictus*) and HeLa cell culture (George et al., 2008), where such condition later resulted in disorganized elongation of astral microtubules toward the cortex. Such effect seems to be dose-dependent and authors reported that the vast majority of cells observed (>90%) contained only one or two additional poles when treated with 100 μM BPA, while nearly 70% of multipolar spindles were observed in 200 μM treated cells. Compared to our treatment of 5 μM BPA, it is reasonable to conclude that the multipolar spindle effect could not be observed in large prevalence. In contrast, polykarya was observed by TEM, indicating that the formation of multiple spindles took place in smaller proportion of exposed embryos and that aneuploydic effect of BPA occurs.

In echinoderm embryos, after explosive outgrowth of astral microtubules, its tubulin reaches toward cortical actin cytoskeleton marking the position of the future contractile ring (Burgess and Chang, 2005). This suggests that malformations in the spindle and its tubulin are likely to reflect in malformations of the cleavage furrow and cell membrane. High BPA concentration in 2-cell embryos induced dysfunction of the polymerization of filamentous actin that, consequently, resulted in a cell membrane and furrow of discontinuous and varying thickness, showing shrinkage in some parts and lack of the bilateral symmetry. Such anomalies induced by BPA may have aggravating effect on the activity of transporter proteins that are “inherited” from mother at this embryonic stage and not zygotically-expressed. After sea urchin eggs fertilization, actin polymerization enables microfilament-mediated process of transportation of ABC transporter proteins enclosed in vesicles, which are further inserted in the cell membrane and activated (Hamdoun et al., 2004). This process of protein transportation is crucial for the integrity of actin in fertilized urchin eggs, because it enables activation of MXR mechanisms when the embryos relies only on maternal proteins. Although it seems that BPA effects on spindle morphology is extragenic (George et al., 2008) in the abalone, the authors have concluded that overexpression of cyclin B (*cb*) and cycline-dependent kinase (*cdk*) induced by BPA treatment, were responsible for destabilization of microtubules and delay of metaphase (Zhou et al., 2011). In our study overexpression of *cdk* (2.2-folds) was measured in the pluteus but not in the 2-cell stage, ruling out its potential effect on the cytoskeletal malformations in the early development. Overall, 5 μM BPA induces impairment in both, tubulin and actin, functioning in the 2-cell embryos, retarding the progress of the cell cycle and potentially impairing maternal MXR mechanisms activation. However, we were not able to discern if mortality in BPA-exposed embryos is related to the indirect impairment of MXR activation through actin dysfunction, or to the general errors in cytoskeletal functioning in mitosis.

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