Identification of Concentration Dependent in *vitro effect* of Bisphenol F on H295R Cell Viability, Membrane Integrity and Lysosomal Function

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Abstract

In recent years, the use of Bisphenol A (BPA) has been regulated in many countries because of its potential adverse effects on human health. As a result of the restriction, structural analogues such as bisphenol F (BPF) have already been used for industrial applications as alternatives to BPA. Although much information on the endocrine activity of BPA is available, a proper human hazard assessment of analogues that are believed to have a less harmful toxicity profile is lacking. The aim of our in vitro study was to assess the potential effect of BPF on H295R cell viability, membrane integrity and lysosomal function. Adrenocortical carcinoma cells were cultivated during 24 h in the presence of BPF (0.1, 0.5, 1, 10, 25, 50, 75, 100, 300, 500 μM). Metabolic activity decreased with increasing dose of BPF - from 10 μM (84.33 ± 4.31%). A significant increase in metabolic activity after 24 hours of exposure was observed after cultivation with 0.1 μM BPF (111.50 ± 3.89%) and a slight was observed after cultivation with 1 μM BPF (101,70 ± 1.61%). Exposure doses of BPF caused a slight increase in esterase activity at the lowest concentrations and a significant decrease at higher concentrations. We observed a slight increase in lysosomal function after cultivation with 0.1 and 1 μM, higher exposure doses (25 - 500 μM) caused decrease in lysosomal function. The obtained results confirmed that BPF at higher concentrations caused cytotoxicity. A substitution of BPA by BPF should be thus considered with caution.

Keywords: endocrine disruptors; BPF; H295R; viability; membrane integrity; lysosomal function

1. Introduction

Bisphenol compounds are found throughout the contemporary world in the form of plastics that are used extensively by consumers for food storage. These polymers are also widely used in the packaging of baby formula, baby bottles, the lining of canned food and drink, dental implants, and sales receipts. However, in 2007, the first indications were published that bisphenol polymers may leak monomers into food and drink and disrupt endocrine pathways by mimicking estrogen [1]. Since then, BPA (bisphenol A) has become one of the most well-known endocrine disrupting compounds with pronounced effects on the reproductive system, child development, metabolic disorders, obesity, endocrine disorders, and the nervous system; as well as being implicated in causing DNA damage, oxidative stress, and breast cancer [1-4]. Therefore, the industry quickly formulated new BPA-free plastics to be used as food packaging and food storage containers instead. These BPA-free plastics are made using bisphenol analogues with very similar structural and chemical properties [1]. Since bisphenol A analogues are structurally and chemically similar to BPA, many researchers have recently been looking into the effects of these BPA alternatives. The study of BPA itself is relatively new, the study of bisphenol analogues

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that have replaced BPA is an even newer field of study [1, 4].

BPF epoxy resins are used for several consumer products such as lacquers, varnishes, liners, adhesives, plastics, water pipes, dental sealants, and food packaging [5]. BPF have been detected in many everyday products, such as personal care products - body wash, hair care products, makeup, lotions, toothpaste, [6], paper products - flyers, tickets, mailing envelopes, airplane boarding passes, [7] and food - dairy products, meat and meat products, vegetables, canned foods, cereals [8]. BPF have also been detected in surface water, sediment, and sewage effluent, generally at lower concentrations than BPA [9-11, 13]. BPF have been detected in indoor dust at the following concentration: BPF, 0.054 μg/g [7, 13]. In humans, BPF have been detected in urine at concentrations and frequencies comparable to BPA [7, 12, 13]. In urine samples from 100 American, nonoccupationally exposed adults, Liao et al. (2012) found BPF in 55% of samples at concentrations up to 212 ng/mL, BPA was found in 95% of the samples, with concentrations up to 37.7 ng/mL [7, 13, 17, 18].

2. Materials and Methods

Cell culture and treatment

The NCI-H295R cells were obtained from the American Type Culture Collections (ATCC CRL-2128; ATCC, Manassas, VA, USA). The cells were cultured in a Good Laboratory Practice (GLP) certified laboratory (National Institute of Chemical Safety, Budapest, Hungary; OGYI/45151-4/2012) according to previously established and validated protocols. After the initiation of the H295R culture from the original ATCC batch, the cells were cultured throughout four passages, split and frozen down in liquid nitrogen. The cells used in the scheduled experiments were cultured for a minimum of two additional passages to achieve an optimal hormone production using new H295R batches from frozen stocks. The H295R cells were grown in 25 cm2 plastic tissue culture flasks (TPP, Trasadingen, Switzerland) in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham 1:1 mixture (Sigma, St. Louis, MO, USA) supplemented with 1.2 g/L NaHCO3 (Molar Chemicals Halasztelek, Hungary), 12.5 mL/L of BD Nu-Serum (BD Bioscience, Bath, UK) and 5 mL/L of ITSC Premix (BD Bioscience) in a CO2 incubator at 37°C with a 5% CO2 atmosphere. The culture medium was changed 3 times/week, and after obtaining an acceptable cell density, it was removed from the culture flasks. The H295R cells were detached from the bottom of the culture flasks with 0.25% trypsin-EDTA for 3 min (Sigma). The cells were subsequently centrifuged (10 min., 125 x g) and re-suspended in fresh cell culture medium. The cell number was counted using a Burker chamber and adjusted to required cell concentration. The cell suspension was plated into sterile 96-well cell culture plates ($6*10^4$ cells/100 μL/well) for cytotoxicity and hormone measurements. The cells were incubated for 24 h in a CO2 incubator at 37°C under a humidified atmosphere of 95% air and 5% CO2. To explore the effect of bisphenols, cells were cultured for 24 h in medium containing specific concetrations of each bisphenol (0.1, 0.5, 1, 10, 25, 50, 75, 100, 300, 500 μM) (Sigma - Aldrich, St. Louis, USA). The specific concentration range of bisphenols was selected according to the results of our pilot range-finding experiments.

Triple assay

For cell viability, three cellular activities were monitored with three indicator dyes: metabolic activity with alamarBlue (ThermoFisher Scientific, USA), plasma membrane integrity with 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Sigma-Aldrich, USA), and lysosomal activity with neutral red (NR; Sigma-Aldrich, USA). AlamarBlue assay is known as resazurin reduction test. Resazurin is a nontoxic, cell-permeable, blue nonfluorescent redox indicator which can be used to determine the number of viable cells based on conversion to resorufin by mitochondrial and other enzymes such as diaphorases. After entering cells, resazurin is reduced to resorufin of red, highly fluorescent color. Viable cells convert resazurin to resofurin continuously, increasing fluorescence of the culture medium. Ratio of cell metabolic activity can be quantified using a microplate reader fluorometer. CFDA-AM is another fluorogenic dye, which indicates the integrity of the plasma membrane. It is a nontoxic esterase substrate that can be by nonspecific esterases of viable cells converted from a membrane-permeable, nonpolar, nonfluorescent substance to polar, fluorescent

carboxyfluorescein (CF). This conversion suggests the integrity of the plasma membrane since only intact membrane can maintain the cytoplasmic milieu which is needed to support esterase activity. Neutral red staining belongs to the colorimetric assays. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes. The dye is then released from the viable cells using a solution of acidified ethanol and the absorbance of the dye is measured spectrophotometrically. The use of these 3 dyes to provide an overview of the cytotoxicity/cytoprotectivity of treatments to cells in 96-well plates was first described by Schirmer et al. [14]. This protocol was followed in this study with minor changes. Briefly, with this method, three parameters of cell viability are measured simultaneously on the same set of cells without interference. In the first step, after 24 h treatment, to the cells seeded in 96-well plate a solution of almarBlue and CFDA-AM in MEM medium (minimum essential medium eagle; Sigma-Aldrich, St. Louis, USA) was added. The cells were incubated for one hour followed by measurement at individual wavelengths. The cells were then washed with PBS and neutral red dye in MEM medium was added to the cells for 1 h. After incubation cells were washed twice with PBS (phosphate- buffered saline; Sigma-Aldrich, St. Louis, USA) and exposed for another 30 minutes to the lysis buffer followed by measurement at a specific wavelength. The multiple endpoint assay is based on measurements, determined here using a Glomax Multi + Combined Spectro-Fluoro Luminometer (Promega Corp., USA) at respective excitation/emission wavelengths of 525/580-640 nm for alamarBlue, 490/510-570 nm for CFDA-AM and 525/660-720 nm for NR. Data were obtained from four independent experiments and expressed in percentage of the control groups, which was set to 100%. This protocol was followed in this study with minor changes. Briefly, with this method, three parameters of cell viability are measured simultaneously on the same set of cells without interference. In the first step, after 24 h treatment, to the cells seeded in 96-well plate a solution of almarBlue and CFDA-AM in MEM medium (minimum essential medium eagle; Sigma-Aldrich, St. Louis, USA) was added. The cells were incubated for one hour followed by measurement at individual wavelengths. The cells were then washed with PBS and neutral red dye in MEM medium was added to the cells for 1 h. After incubation cells were washed twice with PBS (phosphate- buffered saline; Sigma-Aldrich, St. Louis, USA) and exposed for another 30 minutes to the lysis buffer followed by measurement at a specific wavelength. The multiple endpoint assay is based on measurements, determined here using a Glomax Multi + Combined Spectro-Fluoro Luminometer (Promega Corp., USA) at respective excitation/emission wavelengths of 525/580-640 nm for alamarBlue, 490/510-570 nm for CFDA-AM and 525/660-720 nm for NR. Data were obtained from three independent experiments and expressed in percentage of the control group, which was set to 100%.

Statistical analysis

Obtained data were statistically analyzed using the GraphPad Prism 8 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical parameters (minimum, maximum, standard error, etc.) were evaluated at first. One-way analysis of variance (ANOVA) with Dunnett's posttest was used for statistical evaluations. The level of significance was set at ***($P < 0.001$), **($P < 0.01$) and *($P < 0.05$). Data were obtained from at least four sets of independent experiments (n=4). Results were presented as means (± SEM). Each experiment was performed three times independently with cells from different passages [5-10].

3. Results

Assessment of the cell viability using alamarBlue revealed that 0.1 μM of BPF led to significant increase *($P<0.05$) and 0.5* μM of BPF led to nonsignificant increase *($P>0.05$)* of cell viability compared with the control. Inversely, significant decrease *($P<0.001$)* of cell viability was detected after BPF administration at concentrations 10 *($P<0.01$)*, 25, 50, 100, 300 and 500 μM *($P<0.001$)* (Fig. 1, Table 1).

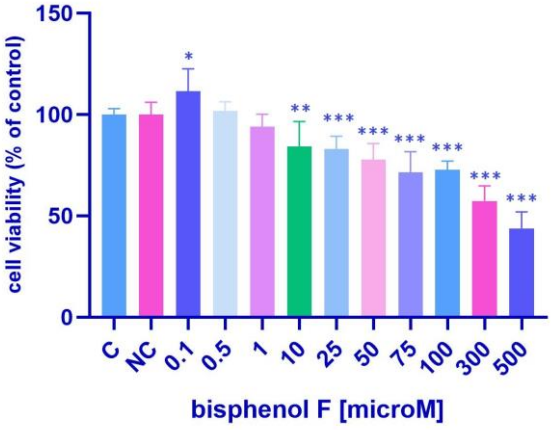

Figure 1. Cell viability after 24 hours of bisphenol F exposure

BPF at concentration 0.1 0.5 and 1 μM exhibited slightly improved (P>0.05) level of lysosomal function compared with the control group, while highef doses (25 - 500 μM) caused a significant decline (P<0.05 - P<0.001) of lysosomal activity after 24 hour treatment (Fig. 2, Table 1).

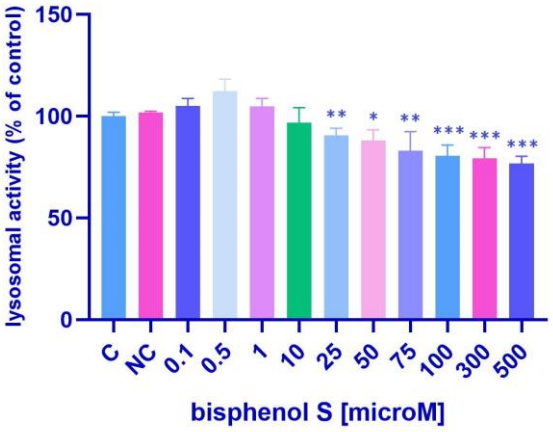

Figure 2. Lysosomal activity after 24 hours of bisphenol F exposure

Cell membrane integrity measurement in H295R cells treated with bisphenol F for 24 h showed significant changes (P<0.001) only in experimental groups treated with the higher doses of BPF (25, 50, 75, 100, 300, 500 μM), which resulted in the decline of values. 0.1 and 0.5 μM of BPF exhibited slightly improved (P>0.05) level of lysosomal function compared with the control group (Fig. 3, Table 1).

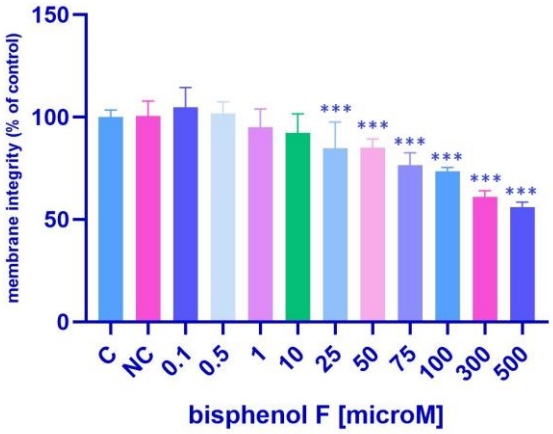

Figure 3. Membrane integrity after 24 hours of bisphenol F exposure

Table 1. Cell viability, lysosomal activity and membrane integrity after 24 hours of bisphenol F exposure

	Cell viability (%)	Lysosomal activity (%)	Membrane integrity (%)
C	100.00 ± 1.04	100.00 ± 0.73	100.00 ± 1.20
NC	100.10 ± 2.13	100.40 ± 0.98	100.40 ± 2.63
0.1μM	111.50 ± 3.89	104.20 ± 0.99	111.60 ± 3.48
0.5μM	101.70 ± 1.61	105.50 ± 1.51	101.80 ± 2.01
1μM	94.05 ± 2.19	105.40 ± 1.42	95.03 ± 3.15
10μM	84.33 ± 4.31	96.86 ± 2.60	92.37 ± 3.23
25μM	83.01 ± 2.23	92.04 ± 2.61	84.73 ± 4.52
50μM	77.72 ± 2.80	92.99 ± 1.08	84.96 ± 1.52
75μM	71.60 ± 3.59	92.14 ± 1.04	76.50 ± 2.18
100μM	72.80 ± 1.52	87.49 ± 1.21	73.52 ± 0.66
300μM	87.32 ± 2.67	81.63 ± 0.34	60.89 ± 1.11
500μM	43.87 ± 2.87	74.72 ± 1.39	56.07 ± 0.83

4. Discussion

Over the past three decades, there has been increasing concern about the possible impacts of exposure to chemicals in the environment on endocrine and reproductive systems in humans and wildlife [15]. The presence of many bisphenol analogues other than BPA in environmental media, foodstuffs, consumer products, and humans from many regions of the world, suggests a large scale and potentially a global contamination trend. Humans are exposed to bisphenol analogues via the same pathways that have been demonstrated for BPA, including oral, dermal, handto-mouth transfer, as well as other mechanisms [16]. BPF is still being produced around the world, and there are still many BPF-containing products that humans contact daily. In addition, there is not much toxicity assessment of the substitutes of BPA, so it is not enough to evaluate the suitability as an alternative to replace BPA. Under these circumstances, we assessed the cytotoxicity of BPF. According to the literature, the intake of dietary BPF in the form of contaminated food and water is the main source of exposure. Mainly, exposure to BPA analogues comes from microwaving food in plastic containers made from these materials, from using plastic bowls and cups that are worn out and may be leaching monomers, or even from tap water in areas where bisphenols were used to coat the inside of water pipes [17, 18, 1]. The metabolism and biological fate of BPF have not been well studied, but *in vitro* and *in vivo* experiments indicated that BPF metabolism and distribution are similar to those of BPA. *In vitro*, BPA was metabolized by human and rat hepatic cells to many different metabolites, including nonbioactive sulfate and glucuronide conjugates [19; 20, 13]. *In vivo*, BPF administered to pregnant rats via gavage resulted in the excretion of BPF and several metabolites in the urine, including the nonactive sulfate-conjugated BPF. Active BPF was also distributed to many tissues, including the uterus, placenta, amniotic fluid, and fetuses. The ratio of the active parent compound to the metabolites/conjugates was similar to that of BPA [21, 22, 13]. The primary route of excretion for BPF appeared to be through the sulfatase conjugate, rather than the glucuronide conjugate (as with BPA). This may be due to the fact that BPF glucuronide may be more easily deconjugated to its bioactive state and reabsorbed in large quantities, which also appears to occur with BPA [21, 22, 13]. Nowadays, many studies prove the endocrine disrupting potencial of BPA analoques, BPF also showed other in vitro effects such as cytotoxicity, cellular dysfunction, DNA damage, and chromosomal aberrations [23-27]. According to our results, BPF at concentrations 25 - 500 μM significantly dicreased cell viability, metabolic activity, lysosomal activity and membrane integrity. On the other hand, low concentrations (0.1 and 0.5 μM) of BPF increased all of these parameters. Several *in vitro* studies also showed cytotoxic potencial of BPF, which is in agreement with our results [25, 26, 28, 29]. According to Russo et al., 2018 bisphenols IC_{50} values confirming their poor acute toxicity. As compared to BPA, bisphenol F was found as the less toxic congener. These results are partly consistent with the scale of phospholipid affinity showing that toxicity increases at increasing membrane affinity (30). According to our results, cell viability/metabolic activity appears to be the most sensitive parameter to the activity of bisphenol F, followed by membrane integrity and the least sensitive parameter was lysosomal activity. Cabaton et al. showed that BPF was effective on HepG2 cell DNA fragmentation at noncytotoxic concentrations. An *in vivo* study in gestating Wistar rats revealed that BPF decreased the 5α-reductase expression and dopamine (DA)−serotonin (5-HT) systems in the prefrontal cortex of juvenile female rats at postnatal day 21 [16, 24, 31]. Additional research is urgently needed to fill in knowledge gaps and deepen toxicity evaluations, given that the production and applications of bisphenol analogues are on the rise and that many of them have already been present in environmental compartments, foods, and humans.

4. Conclusions

The results of the cytotoxicity evaluation of BPF indicated that a significant level of cytotoxicity was observed at the following tested concentration: 25, 50, 75, 100, 300 and 500 μM, however its low concentrations led to improved cell viability, membrane integrity and lysosomal activity, which indicates the biphasic, hormetic response of BPF in biological systems.

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