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RESEARCH ARTICLE

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The effects of different bisphenol derivatives on oxidative stress, DNA damage and DNA repair in RWPE-1 cells: A comparative study

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Abstract

Bisphenol A (BPA) is a well-known endocrine disruptor and it is widely used mainly in the plastics industry. Due to recent reports on its possible impact on health (particularly on the male reproductive system), bisphenol F (BPF) and bisphenol S (BPS) are now being used as alternatives. In this study, RWPE-1 cells were used as a model to compare cytotoxicity, oxidative stress-causing potential and genotoxicity of these chemicals. In addition, the effects of the bisphenol derivatives were assessed on DNA repair proteins. RWPE-1 cells were incubated with BPA, BPF, and BPS at concentrations of 0–600 μM for 24 h. The inhibitory concentration 20 (IC_{20}, concentration that causes 20% of cell viability loss) values for BPA, BPF, and BPS were 45, 65, and 108 μM, respectively. These results indicated that cytotoxicity potentials were ranked as BPA > BPF > BPS. We also found alterations in superoxide dismutase, glutathione peroxidase and glutathione reductase activities, and glutathione and total antioxidant capacity in all bisphenol-exposed groups. In the standard and modified Comet assay, BPS produced significantly higher levels of DNA damage vs the control. DNA repair proteins (OGG1, Ape-1, and MyH) involved in the base excision repair pathway, as well as p53 protein levels were down-regulated in all of the bisphenol-exposed groups. We found that the BPA alternatives were also cytotoxic and genotoxic, and changed the expressions of DNA repair enzymes. Therefore, further studies are needed to assess whether they can be used safely as alternatives to BPA or not.

KEYWORDS

Bisphenol A, bisphenol F, bisphenol S, cytotoxicity, DNA repair ezymes, oxidative stress

1 | INTRODUCTION

Bisphenol A (2, 2-Bis-(4-hydroxyphenyl)-propane; BPA) is a phenolic compound that is widely used in industrial, commercial and consumer applications to manufacture polycarbonate plastics and epoxy resins (Staples, Dorn, Klecka, O'Block, & Harris, 1998). Since 2013, ~15 billion pounds of BPA is used annually in the field of plastics, such as in food packaging, dental sealants, adhesives, and plastic beverage containers. This makes BPA one of the most abundantly produced chemicals globally (Hanioka, Jinno, Tanaka-Kagawa, Nishimura, & Ando, 2000).

BPA is a well-known endocrine disrupting chemical. This bisphenol derivative is structurally similar to diethylstilbestrol (DES) and has estrogenic properties. BPA has the ability to bind and activate estrogen receptor (ER) subtypes (i.e., ERα, ERβ and particularly ERγ) (Teng et al., 2013). It has also been suggested to disturb the normal functioning of ER receptors, although it has lower affinity for nuclear ERs than estradiol (Rehan et al., 2015). Due to abundant exposure,

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the general population has higher circulating concentrations of BPA when compared to estradiol. BPA can also disrupt androgenic signaling pathways, which play important roles in male sex organ development and reproductive functions (Rehan et al., 2015; Sidorkiewicz, Zaręba, Wołczyński, & Czerniecki, 2017; Teng et al., 2013).

BPA has become a major concern throughout the world due to its possible harmful effects on human health. Many studies have suggested that BPA is associated with many molecular events and pathological conditions, including: altered expression of certain genes (Ashby, Tinwell, & Haseman, 1999; Cagen et al., 1999; Chitra, Rao, & Mathur, 2003; Richter et al., 2007; Timms et al., 2005), changes in the synthesis of many steroidogenic enzymes (including testosterone and estrogen) (Akingbemi, Sottas, Koulova, Klinefelter, & Hardy, 2004), accelerated/increased mammary gland and prostate development (Prins & Ho, 2010), disrupted estrous cycling and delayed estrous cycles (Ema et al., 2001; Kato, Ota, Furuhashi, Ohta, & Iguchi, 2003; Nikaido et al., 2004;Ryan & Vandenbergh, 2006; Tyl et al., 2008), alterations in postnatal growth (Rezg, El-Fazaa, Gharbi, & Mornagui, 2014; Rochester, 2013; Ryan & Vandenbergh, 2006), early onset of puberty (Ryan & Vandenbergh, 2006; Tyl et al., 2008), reproductive disorders in both sexes (Rezg et al., 2014; Rochester, 2013; Vandenberg et al., 2012; Vandenberg, Maffini, Sonnenschein, Rubin, & Soto, 2009), and cancer (particularly breast cancer) (Wetherill et al., 2007). Due to such effects, alternatives to BPA are now widely used. The most commonly used alternative bisphenol derivatives are bisphenol F (BPF) and bisphenol S (BPS). BPF provides thickness and durability for epoxy resins and coatings, and is used in industrial floors and roads. This compound is also present in lacquers, varnishes, liners, adhesives, dental sealants, and food packaging (Environmental Health Hazard Assessment, 2012). BPS is mostly used as a wash fastening agent in cleaning products, as an electroplating solvent, and it is a constituent of phenolic resins (Clark, 2000). The European Chemicals Agency (ECHA) has reported that the amount of BPS being manufactured is 1000–10 000 million metric tons annually (ECHA, 2018). However, their toxic effects have not been widely studied (Rochester & Bolden, 2015).

The prostate gland is a hormone-dependent reproductive organ, and androgens and estrogens play a key role in prostate growth, function, homeostasis, and disease (Hsing & Chokkalingam, 2006; Siegel, Miller, & Jemal, 2015, 2016). Prostate diseases are prevalent in different populations. Advancing age, race/ethnicity, socio-economic status and family history are strongly linked to their development. Prostate cancer is the most common cancer amongst men in Western countries and it has been suggested to be the second leading cause of cancer-related deaths in the USA, just after lung and bronchus carcinoma (Siegel et al., 2016). During the development of the prostate, inappropriate estrogenic substances, such as bisphenol analogues (particularly BPA), can cause the reprogramming of the gland. This reprogramming can lead to an increase in gland size, alter the gene expression, and predispose the individual to an increased risk of prostate cancer (Prins & Ho, 2010).

Oxidative stress is the imbalance between cellular antioxidants and oxidants, in the favor of oxidants. During oxidative stress, alterations in the activities and levels of certain antioxidant enzymes, as well as antioxidants, may occur in order to protect the cell from the cellular insult (Halliwell, 1996). Moreover, alterations in apoptosis and cell cycle-related proteins [e.g., tumor protein 53 (Tp53, p53)] and DNA repair enzymes [8-oxoguanine glycosylase (OGG1), DNA polymerase β (PolB), and apurinic/apyrimidinic (AP) endonuclease 1 (Ape-1)] may also be found after high levels of reactive oxygen species (ROS). OGG1 is the primary enzyme responsible for the excision of 8-oxoguanine (8-oxoG), a mutagenic base byproduct that occurs from exposure to ROS (Nishioka et al., 1999). PolB performs base excision repair (BER) required for DNA maintenance, replication, recombination, and drug resistance (Prasad, Horton, Liu, & Wilson, 2017). MutY Homolog (MyH) protein is involved in oxidative DNA damage repair and is part of the BER pathway. The enzyme excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired with guanine, cytosine, or 8-oxo-7,8-dihydroguanine (8-oxodG), a common form of oxidative DNA damage (Oka, Leon, Tsuchimoto, Sakumi, & Nakabeppu, 2015). Ape-1 is an enzyme that is involved in the DNA BER pathway. Its main role in the repair of damaged or mismatched nucleotides in DNA is to create a nick in the phosphodiester backbone of the AP site created when DNA glycosylase removes the damaged base (Marenstein, Wilson, & Teebor, 2004).

Authorities and scientists should only suggest alternative compounds after the toxicological profile of this chemical is established. Unfortunately, some alternative compounds have replaced many toxic analogues on the market, without proper toxicological evaluation. This may lead to inevitable human exposure to such chemicals and contaminate the environment, without knowing the future outcomes. Considering the toxicity potentials of BPF and BPS and their high presence in biological samples and the environment, the present study aimed to compare the cytotoxic, genotoxic and oxidative stress-causing potentials of these chemicals to BPA in RWPE-1 cells. Moreover, the effects of bisphenol derivatives were assessed on DNA repair proteins, particularly responsible for the BER pathway.

2 | MATERIALS AND METHODS

2.1 | Chemicals

BPA (>99% pure), BPF (98% pure), BPS (98% pure), keratinocyte serum-free medium (K-SFM), fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), agarose, low-melting-point agarose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), protease inhibitor cocktail, all cell culture materials and all Comet assay chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine pituitary extract (BPE), human (recombinant) epidermal growth factor (EGF) was purchased from Gibco® (Waltham, MA, USA). Primers for p53, OGG1, PolB, MyH, Ape-1 and glyceraldehyde-3-phosphate dehydrogenase (GADPH) were obtained from Eurogentec SARL (Angers, France).

2.2 | Kits

Colorimetric assay kits for glutathione peroxidase (GPx) and glutathione reductase (GR), and superoxide dismutase (SOD) were obtained from Cayman Chemical (Ann Harbor, MI, USA). Commercial kits for the measurement of total glutathione (GSH) and total antioxidant capacity (TAOC) levels were also from Cayman Chemical. The GenElute™ Mammalian Total Ribonucleic acid (RNA) Miniprep Kit was from Sigma-Aldrich. SuperScript II Reverse Transcriptase kit was from Invitrogen (Carlsbad, CA, USA). Bio-Rad Mix [for real-time polymerase chain reaction (PCR)] was from Bio-Rad (Hercules, CA, USA).

2.3 | Primers

Gene specific oligonucleotide primers used for real-time quantitative PCR; p53: GCT-TTC-CAC-GAC-GGT-GAC (forward), GCT-CGA-CGC-TAG-GAT-CTG-AC (reverse); OGG1: TGG-AAG-AAC-AGG-GCG-GGC-TA (Forward), ATG-GAC-ATC-CAC-GGG-CAC-AG (reverse); PolB: GAG-AAG-AAC-GTG-AGC-CAA-GC (forward), CGT-ATC-ATC-CTG-CCG-AAT-CT (reverse); MyH: CCA-GAG-AGT-GGA-GCA-GGA-AC (forward),TTT-CTG-GGG-AAG-TTG-ACC-AC (reverse); Ape-1: GCT-GCC-TGG-ACT-CTC-TCA-TC (forward), GCT-GTT-ACC-AGC-ACA-AAC-GA (reverse); GAPDH: CCA-CTC-AAA-GTC-AGC-ACA-GCG (forward), TGG-TCC-TGG-GGT-TCC-AC (reverse).

2.4 | Cell culture and bisphenol treatment

The RWPE-1 cell line was established in 1997 from a histologically normal prostate, which is a human papilloma virus 18 (HPV18) immortalized, non-tumorigenic prostatic cell line (Bello, Webber, Kleinman, Wartinger, & Rhim, 1997). The cells were a generous gift from Dr Xavier Gidrol (Interdisciplinary Research Institute of Grenoble [IRIG], Biomics Lab, Commissariat à l'Énergie Atomique et aux Énergies Alternatives [CEA], Grenoble, France) and maintained in K-SFM supplemented with BPE (25 mg/mL), EGF (2.5 μg/mL) and penicillin/streptomycin (1%). The RWPE-1 cells were grown in flasks and after reaching 80% confluency, cells were trypsinized, washed with sterile PBS, and centrifuged at 1500 ***g*** for 5 min and later subcultivated.

2.5 | Preparation of bisphenol analogues

Stock solutions of BPA, BPF and BPS (all 100 mM) were prepared in DMSO (1%). Fresh stock solutions were prepared before each individual experiment and fresh dilutions were prepared by using culture medium to achieve final concentrations at 0, 50, 100, 200, 300, and 600 μM.

2.6 | Determination of cell viability

Cell viability was determined by a modified MTT assay (Cory, Owen, Barltrop, & Cory, 1991), and trypan blue exclusion. A total of 20 000 cells/well were plated onto 96-well microtiter plates in 200 μL medium with or without BPA, BPF or BPS. After incubation for 24 h at 37 °C in a humidified incubator, the medium was removed and the cells were washed with PBS, and incubated for 2 h by adding 20 μL MTT (5 mg/mL in PBS) to each well. The medium was removed 2 h later; formazan crystals were dissolved in 200 μL DMSO and cell viability was determined by reading the absorbance at 570 nm using a Multiskan Ascent microtiter plate reader (Labsystems, Paris, France). Cell viability was calculated from the mean absorbance values of three replicates. The control cells were accepted to have 100% cell viability. The viability of cells was determined by comparing relative formazan concentrations (OD570-OD690) of the treated cells with those of the untreated control cells. The results were expressed as the mean percentage of cell viability vs the control group. Inhibitory concentration 50 (IC_{50}, concentration that causes 50% of cell viability loss) and inhibitory concentration 20 (IC_{20}, concentration that causes 20% of cell viability loss) were later calculated for each bisphenol derivative. IC_{20} doses instead of IC_{50} doses were used in order to reflect daily exposures to these chemicals, as we are not exposed to very high amounts of these chemicals in everyday life. The IC_{20} values for each bisphenol derivative were used in the subsequent assays (enzymatic and non-enzymatic antioxidants, Comet assay and quantitative real-time PCR). The experiments were repeated four times. The mean of all the experiments were calculated.

2.7 | Enzymatic and non-enzymatic antioxidants

After 24 h incubation with bisphenol derivatives, the cells in each group were scrape-harvested in cold PBS on ice and centrifuged. Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail, and then centrifuged at 2000 *g* and 4 °C for 10 min. After further centrifugation at 20 000 *g* and 4 °C for 20 min, antioxidant enzyme activities, total GSH and TAOC levels were measured in the supernatant.

The activity of cytosolic GPx (GPx1) was measured in a coupled reaction with GR, as described earlier (Flohé & Günzler, 1984), using a commercial kit. The assay is based on the instant and continuous reduction of oxidized glutathione (glutathione disulfide [GSSG]) formed during GPx reaction by an excess of GR activity providing for a constant level of GSH. As a substrate, *tert*-Butyl hydroperoxide was used and concomitant oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) was monitored spectrophotometrically at 340 nm. One unit of enzyme was defined as the amount of GPx that transformed 1 μmol NADPH to NADP per minute at 37 °C. GPx actvitiy was expressed as U/mg protein.

GR activity was assessed based on the reduction of GSSG to reduced glutathione GSH. Samples were added to a mixture of 100 mM phosphate buffer, pH 7.5, and 1 mM GSSG. The reaction was started by the addition of 0.1 mM NADPH. The oxidation rate of NADPH was followed at 340 nm for 5 min. Controls were performed to correct for nonspecific NADPH oxidation. One milliunit of GR was calculated as the quantity of enzyme that reduced 1 mM GSSG per minute, a reaction that induced the oxidation of 1 mM NADPH.

GR activity was expressed as mU/mg protein (Goldberg & Spooner, 1983).

The total SOD activity was measured by colorimetric assay using a commercial kit. Xantine oxidase produces superoxide ion, while converting xanthine and water to uric acid and hydrogen peroxide (H_2O_2). This kit uses the water-soluble tetrazolium salt that produces a water-soluble formazan dye upon reduction with a superoxide anion. The reduction rate of superoxide was linearly associated with xantine oxidase activity and was inhibited by SOD. The 50% inhibition activity of SOD (IC_{50}) was determined by this colorimetric method. As the absorbance at 440 nm was proportional to the amount of superoxide anion, the inhibition of SOD activity was quantified by measuring the decrease in color development at 440 nm. The SOD activity was expressed as U/mg protein (Sun et al., 1988).

Total GSH measurement was performed using a total GSH assay kit, based on an enzymatic recycling method. The sulfhydryl group of GSH reacts with 5,5′-dithiobis-2-nitrobenzoic acid and produces a yellow 5-thio-2-nitrobenzoic acid (TNB). The disulfide GS-TNB that is simultaneously produced is reduced by glutathione reductase to produce GSH and TNB. The TNB level is directly proportional to the GSH concentration in the sample. Measurement of the absorbance at 405 nm indicates the GSH level in the sample and the results were expressed as nmol/mg protein (Akerboom & Sies, 1981).

To measure the TAOC levels, an assay relying on the ability of antioxidants in the sample to inhibit the oxidation of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) ABTS+ by metmyoglobin was used. The amounts of ABTS+ produced were monitored by reading the absorbance at 405 nm. The capacity of the antioxidants in the sample to prevent ABTS+ oxidation was compared with that of Trolox and was quantified in mM Trolox equivalents (Miller et al., 1993).

2.8 | Alkaline single-gel electrophoresis (Comet assay)

After 24 h incubation with bisphenol derivatives, the RWPE-1 cells were collected. The alkaline Comet assay was carried out according to the method of Singh, McCoy, Tice, and Schneider (1988) with some modifications. For the modified alkaline Comet assay, the formamido pyrimidine glycosylase (Fpg) protein, which detects 8-OH guanine and other oxidatively damaged purines, was used to assess oxidative DNA base damage.

A thin layer of agarose gel was prepared by applying 1% normal melting agarose in 100 mL Ca^{2+}- and Mg^{2+}-free PBS; 100 μL agarose was loaded onto the Comet slide and allowed to solidify overnight. 100 μL of cell suspension (200 000 cells/mL) was mixed with 900 μL 0.6% low-melting agarose and maintained at 37 °C before the sample (1000 μL) was coated onto the slide and covered with a coverslip. The slides were placed on ice for ≥10 min, to allow the gel to solidify. After removal of the coverslips, all the slides were immersed in cold lysis solution (2.5 M NaCl, 10 mM Tris, 0.1 M ethylenediamine tetra-acetic acid [EDTA]) at room temperature for 1 h.

In the modified Comet assay, slides were rinsed three-times with 0.4 M Tris-HCl and for each condition three slides were incubated with or without 100 μL Fpg solution (5 U/slide) for 45 min at 37 °C. After the lysis procedure, the slides were placed on ice to stop the enzymatic reaction. The coverslips were removed and the slides were transferred to the electrophoresis tank and kept covered with an electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 30 min. Electrophoresis was performed in the dark, at 25 V/300 mA for 30 min. The slides were then washed by immersion three-times for 5 min in 0.4 M Tris-HCl (pH 7.5) and stained with 50 μL ethidium bromide (20 μg/mL) in PBS. The nuclei were stained by using 50 μL of 20 μg/mL ethidium bromide. Slides were stored at 4 °C in a humidified airtight container to prevent drying.

The slides were analyzed using an epifluorescence microscope (Leitz Laborlux Leica, Wetzlar, Germany; $\lambda_{excitation}$: 493 nm; $\lambda_{emission}$: 620 nm) and image analysis was performed using Komet 4.0 software (Kinetic Imaging-Andor Bioimaging, Nottingham, UK). The tail intensity (%, the percentage of DNA migrated from the head of the comet into the tail) values were used to estimate DNA damage (Singh et al., 1988). For each treatment, the average tail intensity (%) was determined from the analysis of 450 comets. Experiments were performed in triplicate and mean ± SD results were used to express DNA damage.

2.9 | Quantitative real-time PCR

Total RNA was isolated after RWPE-1 cells were treated for 24 h with IC_{20} doses of the different bisphenol analogues, using the GenElute™ Mammalian Total RNA Miniprep Kit.

The RNA quality was estimated using electrophoretic separation on a high-resolution agarose gel (look for sharp ethidium bromide-stained ribosomal RNA bands). The purity and concentration of isolated RNA were quantified spectrophotometrically (A_{260}/A_{280} > 1.8 and A_{260}/A_{230} > 2.0) by using NanoDrop 1000 instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA was quantified using A_{260} values.

Total RNA (2 μg) was used for first-strand complementary DNA (cDNA) synthesis using a SuperScript II Reverse Transcriptase kit according to the manufacturer's instructions. Each cDNA template (5 μL) was used in the PCR reactions with gene-specific primers, p53, OGG1, PolB, Ape-1 and MyH. The *GAPDH* gene was used as an endogenous control. Real-time PCR was performed using a CFX96 Real-Time PCR System (Bio-Rad) using Bio-Rad Mix according to the protocol supplied by the kit's manufacturer.

2.10 | Statistical analysis

Statistical analysis was performed using GraphPad Prism® (version 8.0, GraphPad Software, San Diego, CA, USA). All data were presented as the mean ± the standard error of the mean (SEM). Differences were considered to be statistically significant when the *P* value was <0.05. The pairwise comparisons were made using parametric or non-parametric tests based upon the Normality tests' results.

3 | RESULTS

3.1 | Cell viability

In Figure 1, the cell viability data produced by MTT assay for the BPA, BPF and BPS-treated RWPE-1 cells are presented. The cytotoxic effect of BPA, BPF and BPS on the viability of RWPE-1 cells was dose-dependent. When comparing the cytotoxicity effects of each chemical, it was clear that BPA was more toxic than BPF, and BPF was more toxic than BPS on RWPE-1 cells. BPA had the highest cytotoxic effect with an IC_{50} dose of 113.74 μM, while BPF had an IC_{50} dose of 249 μM. BPS was less toxic than BPA and BPF, with an IC_{50} dose of 380.90 μM. The IC_{20} values for BPA, BPF and BPS were 45, 65 and 108 μM, respectively (Table 1). Therefore, the cytotoxicity order of these chemicals was BPA > BPF > BPS.

3.2 | Enzymatic and non-enzymatic antioxidants

The activities of enzymatic antioxidants and levels of non-enzymatic antioxidants after RWPE-1 cells were exposed to different bisphenol analogues are given in Figure 2.

GPx1 activities of BPA (29%, $P < 0.01$), BPF (41%, $P < 0.0001$) and BPS (11%, $P < 0.05$) exposed cells were significantly less compared to the control. GR activities in the BPA (4.5-fold) and BPS (6.7-fold) groups were higher vs the control ($P < 0.001$, and $P < 0.0001$, respectively). Although the activity in the BPF group (59%) was also higher vs the control, the difference between the groups was not statistically significant ($P > 0.05$). SOD activity in all the bisphenol-applied groups was lower vs the control. The SOD activity in the BPA group was 24% lower, while in BPF group the reduction was 59% (both $P < 0.05$ vs the control). The SOD activity in the BPS group was reduced by only 3% and this was statistically non-significant.

The total GSH levels were higher in the BPA (30%), BPF (73%) and BPS (65%) groups vs the control ($P < 0.05$). This is most probably due to the immediate response to a toxic insult and cells were exposed to these chemicals for only 24 h. TAOC levels were only significantly reduced in the BPA (20%, $P < 0.01$) and BPF (59%, $P < 0.0001$) exposed cells.

TABLE 1 Inhibitory concentration 50 (IC_{50}) and 20 (IC_{20}) for the bisphenol analogues

	IC_{20}, μM	IC_{50},μM
BPA	45	113.74
BPF	65	249
BPS	108	380.90

BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; IC_{20}. inhibitory concentration 20; IC_{50}, inhibitory concentration 50

3.3 | Comet assay

The IC_{20} concentrations of the different bisphenol analogues were used in the different Comet assay protocols. Tail intensities (%) of the study groups are given in Figure 3. In the modified Comet assay, Fpg caused significantly higher tail intensities in all groups when compared to the standard (without Fpg) Comet assay. For this reason, it can be suggested that oxidative stress might be one of the underlying reasons responsible for the DNA damage caused by bisphenol derivatives. In both methods, all of the bisphenol analogues produced high levels of DNA damage, as evidenced by higher tail intensities compared to the untreated RWPE-1 cells. In the standard Comet assay, BPA (2.5-fold, $P < 0.05$), BPF (3.1-fold, $P < 0.0001$), and BPS (3.20-fold, $P < 0.05$) produced significantly higher levels of DNA damage vs the control. In the modified Comet assay, all tested bisphenols caused significantly higher levels of tail intensity when compared to the untreated RWPE-1 cells (all $P < 0.001$).

3.4 | Changes in gene expressions

Gene expression changes were analyzed after the RWPE-1 cells were exposed to the different bisphenol analogues for 24 h (Figure 4). Gene expression of p53 decreased in the BPF ($P < 0.05$) and BPS groups

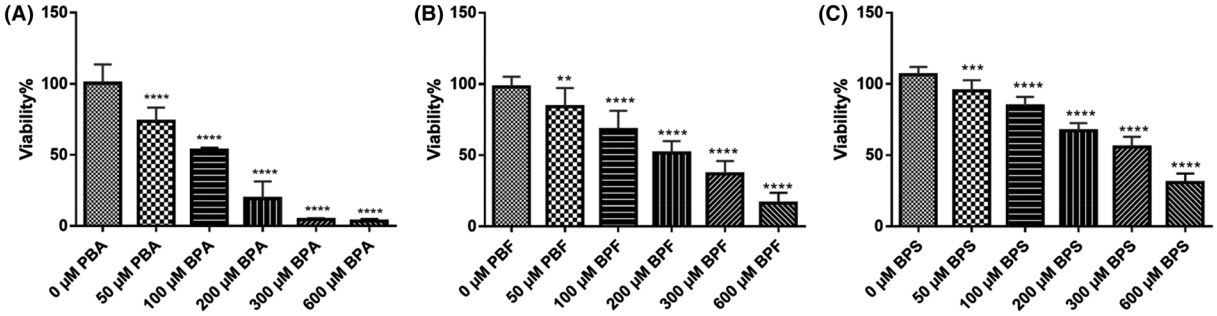

FIGURE 1 Cell viability after exposure to bisphenol derivatives for 24 h. RWPE-1 cells were exposed to BPA (a), BPF (b), and BPS (c) in concentrations ranging from 0 to 600 μM. Cell viability was determined using the MTT assay. The cytotoxicity order of these chemicals were BPA > BPF > BPS. The results are given as the mean ± SEM of four separate experiments. Statistically different from negative control at (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$. Statistical analysis was conducted using the unpaired *t*-test. BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S

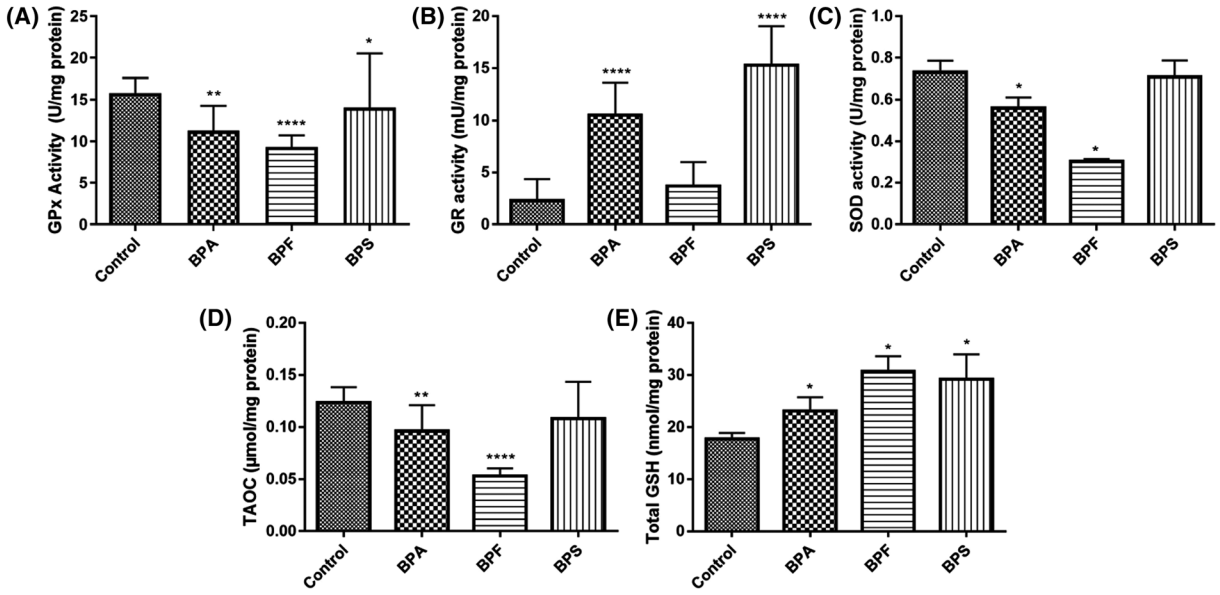

FIGURE 2 Effect of BPA, BPF and BPS on antioxidant enzyme activities and cellular antioxidants. Glutathione peroxidase (GPx) activity (a); glutathione reductase (GR) activity (b); superoxide dismutase (SOD) activity (c); total antioxidant capacity (TAOC) levels (d); and glutathione (GSH) levels (e). The results of three independent experiments were given as the mean ± SEM. Statistically different from negative control at (*) $P < 0.05$, (**) $P < 0.01$, (****) $P < 0.0001$. Statistical analysis was conducted using the Mann–Whitney test

($P > 0.05$) compared to the control group. Ape-1 expression was lesser vs the control group but the difference between the groups was not statistically significant. MyH expressions were significantly reduced in the BPA, BPF, and BPS groups ($P < 0.05$, $P < 0.01$, and $P < 0.05$ vs the control, respectively). OGG1 expressions were reduced in all of the bisphenol-exposed groups when compared to the untreated cells; however, the differences were not significant vs the control group. Although we did not observe marked reductions, even insignificant reductions in OGG1 expression may result in a decrease in the BER pathway and may cause unrepaired bases, the importance of this should be studied for longer periods of exposure. On the other hand, PolB expressions were markedly higher in all of the study groups vs the controls, BPF showed significant differences vs the control group ($P < 0.05$).

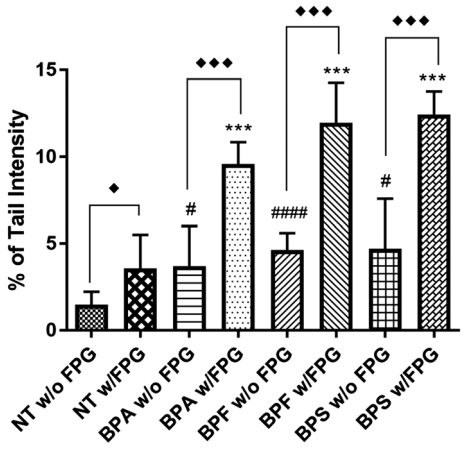

FIGURE 3 Tail intensity (%) values of the study groups obtained from Comet assays. Cells were treated with different bisphenol derivatives at IC_{20} doses in the presence or absence of Fpg. The results of three independent experiments were as given mean ± SEM. Statistical analysis was conducted using the unpaired *t*-test. (#) $P < 0.05$, and (####) $P < 0.0001$ vs not treated (NT) without (w/o) Fpg. (***) $P < 0.001$ vs NT with (w) Fpg. (◆) $P < 0.05$, and (◆◆◆) $P < 0.001$ samples w/Fpg vs samples w/o Fpg. BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; Fpg: formamidopyrimidine glycosylase

4 | DISCUSSION

BPA is an endocrine disrupting chemical that is extensively used in industry (Kortenkamp, 2007). The global consumption of BPA was ~7.7 million metric tons in 2015. According to the last legislations of the USA Food and Drug Administration (FDA), BPA was banned from some consumer products; particularly products used by children (e.g., baby feeding bottles, sippy cups, etc.) (FDA (U.S. Food and Drug Administration)., 2015). Other than the USA, Canada, France, and other European Union countries, Australia, Japan, and Turkey have also banned the use of BPA from child products (Health Canada (HC)., 2012). According to the latest report of the European Chemical Agency, BPA was listed in the 'Candidate List of Substances of Very High Concern' in 2017 (ECHA, 2018). This ban pushed the plastic industry to seek alternatives to BPA and structurally similar chemicals, such as BPF and BPS, were introduced swiftly in the industry. These bisphenols are now mostly used for the manufacturing of phenolic resins and polycarbonate plastics (Cunha & Fernandes, 2010).

BPA and its derivatives are suggested to have high affinity for binding to nuclear ERs and they might also have potency to alter the function of the male reproductive system. The prostate gland is a hormone-dependent organ. Androgen receptors play a key role in

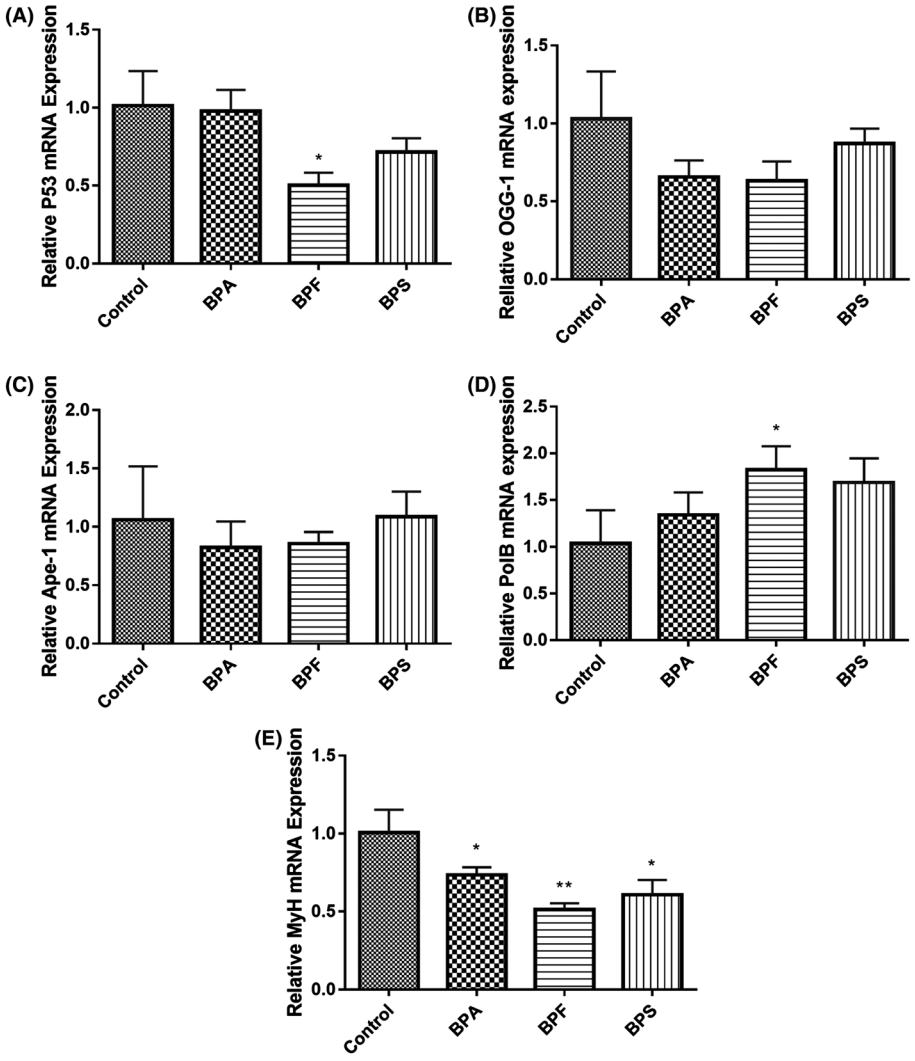

FIGURE 4 The alterations in oxidative stress- and apoptosis-related genes after exposure to bisphenol derivatives. p53 (a); OGG1 (b); Ape-1 (c); Pol B (d); and MyH (e) expressions in the study groups. Results are given as fold-changes vs not treated cells. Statistically different from negative control at (*) $P < 0.05$, and (**) $P < 0.01$. Statistical analysis was conducted using the unpaired *t*-test. Ape-1, apurinic/apyrimidinic endonuclease 1; MyH, MutY Homolog; OGG1, 8-oxoguanine glycosylase; PolB, DNA polymerase β

functional activities of prostate and control its normal growth (Cunha et al., 1987; Taplin & Ho, 2001). Inappropriate hormone exposure can alter the gland's function, reprogram the gland, and may lead to prostate cancer. In the literature, there are many in vivo and in vitro studies about the effects of BPA on the prostate (Prins et al., 2017; Prins & Ho, 2010; Teng et al., 2013).

There are many studies that have shed some light on the possible unwanted effects of BPA; however, there are very few investigations on the effects of BPA alternatives, such as BPF and/or BPS (Herrero et al., 2018). In the present study, we assessed the cytotoxic and genotoxic effects of BPA and its alternatives (BPF and BPS) on RWPE-1 cells. Moreover, we analyzed the oxidant/antioxidant status alterations and bisphenol-induced gene expression changes after exposure to these bisphenol derivatives.

The results of the present study can be discussed in four different parts:

4.1 | Cytotoxicity

Cytotoxicity is a basic parameter for assessing the toxicity of chemical, physical and biological agents. The present MTT results showed that BPA exposure decreased cell viability, at lower concentrations when compared to its analogues, in RWPE-1 cells. In fact, the differences between the IC_{50} values of BPA and BPF, and of BPA and BPS, was more than two and three orders of magnitude, respectively. Because of the high cytotoxicity of BPA, the RWPE-1 cells were not able to survive above concentrations >300 μM (Figure 1). Overall, BPA and its analogues have concentration-dependent cytotoxicity (BPA > BPF > BPS) in RWPE-1 cells. In accordance with our present results, many studies have also shown that BPA had the highest cytotoxic effect when compared to BPF and BPS, in different cell lines. However, in hormone-dependent primary cells and cell lines (breast cancer cells, HL-60 cells, mouse embryonic stem cells, primary cultured hepatocytes, etc.), BPA enhanced cell proliferation at low concentrations (especially at the nanomolar level) (Aghajanpour-Mir et al., 2016; Pritchett, Kuester, & Sipes, 2002; Yin, Yao, Qin, Wang, & Faiola, 2015).

In a recent study, Russo et al. (2018) investigated the cytotoxic effects of seven different bisphenol compounds in vitro using human breast adenocarcinoma cells (MCF-7 cells), human cervical epithelial cancer cells (HeLa cells), mouse fibroblasts (3 T3-L1 cells) and rat

glioma cells (C6 cells). The cells were treated for 48 h with a range of micromolar concentrations (10, 25, 50, 100, 200, 300 μM) of BPA, bisphenol A diglycidyl ether, BPS, BPF, bisphenol B, bisphenol E, bisphenol M, and bisphenol AF (BPAF). Their IC_{50} values for BPA in 3 T3-L1, MCF-7, C6, and HeLa cell lines were >100, 50, 160 and 209.1 μM, respectively. The IC_{50} values for BPF were 110.6, >100, 239.4 and 274.3 μM; whereas for BPS, IC_{50} values were >100, >100, 168.4 and 299.3 μM, respectively. Although the researchers used different cell lines and longer exposure periods compared to our present study, BPA was found to be more toxic then BPF and BPS in accordance with our present results. Furthermore, considering all cell lines, BPF and BPS had moderate toxicity in this research. In another study, Michałowicz, Mokra, & Bąk (2015) investigated the toxic effects of BPA, BPF and BPS in human peripheral blood mononuclear cells after 4-h exposure. BPA caused a statistically significant reduction in cell viability at 220 and 440 μM. The researchers also reported that BPA was more cytotoxic than BPF and BPF was more cytotoxic than BPS, which is similar to our present results. Fic, Žegura, Sollner Dolenc, Filipič, and Peterlin Mašič (2013) reported that significant cytotoxicity was not observed at concentrations of up to 100 μmol/L for BPA, BPF and BPS in HepG2 cell lines. Feng et al. (Feng et al., 2016) conducted experiments on human adrenal carcinoma cell line (H295R cells) and observed that after 72 h of exposure, IC_{50} values of BPF, BPA, and BPS were 208.0, 103.4, and 159.6 μM, respectively (BPA > BPS > BPF), differing from our present results. Overall, BPA was found to be the most cytotoxic chemical when compared to its two analogues, BPF and BPS. Different cell lines and different incubation periods may have resulted in different biological responses to each chemical.

4.2 | Oxidative stress

Under oxidative stress, superoxide is produced as a primary ROS and it is dismutated by SOD. This process has a crucial importance for living cells. While superoxide is dismutated by SOD, H_2O_2 is produced (Schafer & Buettner, 2001). Catalase (CAT) catalyzes the decomposition of H_2O_2 to water and oxygen, while GPx enzymes effectively reduce H_2O_2 and lipid peroxides to water and lipid alcohols, respectively, and in turn oxidizes GSH to GSSG. GR restores intracellular GSH levels by reducing GSSG. In the absence of GPx activity and normal GSH levels, H_2O_2 might not be detoxified and can cause an increase in hydroxyl radical and lipid peroxyl radical levels. In the present study, GPx, GR and SOD activities and total GSH and TAOC levels were measured. Bisphenols caused reductions in GPx and SOD activities, whereas elevated GR activity; meaning that prostate cells could not efficiently decrease intracellular H_2O_2 and superoxide levels in the presence of bisphenols. The total GSH levels increased in all bisphenol groups. This may be due to an adaptive response to short-term exposure to bisphenols (24 h). On the other hand, there may also be a significant increase in GSSG levels and in turn GR activities increased in all of the study groups in order to the overcome the high amounts of GSSG. In an in vivo study by Chitra, Latchoumycandane, and Mathur (2003), the researchers investigated the effects of BPA on the antioxidant system of rats. BPA was administered at 0.2, 2 and 20 μg/kg body weight (day) per day for 45 days. The weight of the ventral prostate significantly increased, whereas the weights of the testis and epididymis markedly decreased. The SOD, CAT, GR and GPx activities were reduced in the epididymal sperm of the treated rats' vs the controls. It was also stated that BPA induced oxidative stress in epididymal sperm. Zhang, Liu, and Zong (2016) evaluated the toxicity of BPS using mouse hepatocytes and renal cells. Both of the cells were exposed to BPS for 12 h at different doses (0.1–1 mM). The researchers found that BPS reacted directly with CAT and changed the structure and activity of the enzyme by binding to the Gly117 residue of CAT. The viability of hepatocytes and renal cells decreased in a dose-dependent manner. In both cell types, increases in ROS were not detected at <0.1 mM BPS; while between 0.1 and 1 mM BPS, they observed a dose-independent increase in ROS production. The results also showed that oxidative stress might have occurred in the presence of high concentrations of BPS. BPS treatment (1 μM–0.1 mM) did not change CAT activity. Higher doses of BPS caused high ROS production and CAT activities in both of the cell types increased significantly compared to the control group.

In a study by Khan et al. (2016), Wistar rats were orally administered 150, 250 and 500 mg/kg day BPA/day for 14 days. At the end of the study, the mitochondrial electron transport chain was deteriorated in the liver. Liver enzyme levels, superoxide formation, protein oxidation and lipid peroxidation showed increases, while GSH levels and SOD activity decreased vs the control. The researchers stated that BPA could destroy mitochondrial energy mechanisms and led to serious toxicity in the liver.

Hassan et al. (2012) administered BPA (0.1, 1, 10, 50 mg/kg day/day) to rats for 4 weeks. In the highest-dose group, significantly low levels of GSH were found compared to the control group. Moreover, the SOD, GPx, GST, GR and CAT activities were significantly lower than the control. The data obtained from that study showed that BPA led to oxidative stress in vivo, similar to our present in vitro study. In a study by Ozaydın et al. (2018), Wistar rats were exposed to BPA at three different doses (5, 50 and 500 μg/kg day/day) for 8 weeks. Liver GSH levels and SOD, GPx and CAT activities decreased markedly, while plasma thiobarbituric acid reactive substances and nitric oxide levels were elevated significantly when compared to the control rats.

4.3 | DNA damage

Considering the cytotoxicity and oxidative stress caused by bisphenols, we evaluated the possible DNA damaging effects of these compounds with a modified Comet assay, by using the lesion-specific enzyme Fpg (Boiteux, O'Connor, & Laval, 1987). In many studies, the oxidative stress produced by bisphenols has been correlated with the oxidative DNA damaging effects of these chemicals (Huc, Lemarié, Guéraud, & Héliès-Toussaint, 2012; Leem et al., 2017; Ooe, Taira, Iguchi-Ariga, & Ariga, 2005). Our present findings showed that without Fpg, BPF-applied cells showed significantly higher tail

intensity when compared to the control; while with Fpg, BPS has generated more oxidative DNA damage when compared to the control in RWPE-1 cells. DNA damage for bisphenols was seen in the order of BPS > BPF > BPA. Other researchers have also reported that BPA caused oxidative DNA damage both in vitro (Fic et al., 2013; Skledar et al., 2016; Xin et al., 2014) and in vivo (Chen et al., 2016; Tiwari et al., 2012). There are not many studies comparing the genotoxic effects of bisphenol derivatives. Fic et al. (2013) performed a comparative study that had different results compared to our present data. HepG2 cells were exposed to BPA, BPF, BPAF, bisphenol Z, 3,3-dimethyl bisphenol A), and BPS at 0.1, 1, and 10 μM (which are lower than our present exposure concentrations). They found that after 24 h of exposure, 0.1 and 10 μM BPA- and BPS-treated cells showed increases in the DNA strand breaks compared to the control group. However, a dose–response relationship was not observed.

4.4 | DNA repair proteins

ROS can attack DNA continuously and may lead to DNA structural modifications, DNA-protein adducts, DNA cross-linking, DNA strand breaks, and the generation of oxidized bases (Jena, 2012). Among all the bases, guanine is mostly attacked by ROS, leading to generation of 8-oxodG (Krokan, Drabløs, & Slupphaug, 2002). This modified base is removed by the BER pathway, which is a well-coordinated repair system upon exposure to ROS (Krokan et al., 2002).

To further confirm bisphenol-mediated DNA damage, we investigated the expressions of some DNA damage-related genes. OGG1 initiates the highly conservative BER pathway by releasing the modified base, especially the 8-OHdG, resulting in an AP site. The abasic site is then cleaved by Ape-1, leaving a 50-deoxyribose phosphate residue. This residue is removed by the AP-lyase activity of DNA PolB, which then inserts a correct nucleotide. Finally, DNA ligase III seals the repaired DNA strand. X-ray repair cross-complementing protein 1 interacts with a complex of DNA repair proteins including poly (ADP-ribose) polymerase, ligase III and PolB, and coordinates the gap-sealing process in the short-batch BER (Petermann & Caldecott, 2006). MyH protein is also involved in oxidative DNA damage repair and is part of the BER pathway (Oka et al., 2015). On the other hand, the cellular response pathway regulates the transcription of effector proteins that have major roles in arresting the cell cycle (Hartwell & Weinert, 1989). In the presence of DNA damage, cell cycle checkpoints stop the progression of the cycle until the damage is repaired. If not, another cellular response might be activated resulting in programmed cell death. After DNA damage, p53, a transcription factor and a crucial tumor suppressor protein is activated (Kern et al., 1991). A variety of target genes, which play prominent roles in cell cycle arrest, repair and apoptosis, are activated by p53 (Bargonetti & Manfredi, 2002). We observed that in the presence of DNA damage, the expression of some of these genes changed. Our present results show that all of the bisphenols decreased *OGG1* gene, *MyH* gene and *Ape-1* gene expressions after 24 h compared to the untreated control cells. *OGG1* gene expression was significantly attenuated in both BPA- and BPF-treated cells. In the BPS-treated cells, *OGG1* expression decreased by ~20% compared to untreated cells. *MyH* gene expression also decreased after treating with different bisphenols: ~10% for BPA, ~50% for BPF and ~40% for BPS when we compared to the untreated cells. Decreases in *Ape-1* gene expression were more pronounced in cells treated with BPA (40%), followed by BPF (~30%) and BPS (15%). Only *PolB* gene expressions increased after treatment with all bisphenols (25% fold for BPA, ~50% for BPF and ~60% for BPS). However, further studies should be performed to identify the exact mechanism involved in PolB activation under stress conditions. We can propose that significant decreases in the BER pathway might activate a cascade leading to the increment of expression of PolB.

Chou et al. (2017) investigated whether BPA (10, 103 and 105 nM) exposure disrupts microRNA regulation and its gene expression in endometrial cancer. In that study, the researchers found that BPA exposure caused down-regulation of DNA repair gene ADP-ribosylation factor 6 (ARF6), p53 and upregulated cyclin E2 (CCNE2) to interrupt to the cell cycle. These results suggested that BPA exposure could affect the ARF6-p53-CCNE2 pathway to arrest the cell cycle for aberrant cell proliferation, endometrial cancer development and metastasis through decreasing expressions of apoptotic genes and increasing cyclins. In another study by Gassman et al. (2015), the effects of BPA exposure on oxidative stress in Ku70-deficient mouse fibroblasts were evaluated. Cells were treated with BPA alone or co-exposed to BPA and potassium bromate ($KBrO_3$). The researchers concluded that BPA promoted cell survival following oxidative DNA damage after exposure to $KBrO_3$. In another study, Chen et al. (2016) showed that cadmium exposure aggravated BPA-induced genotoxicity and cytotoxicity through OGG1 inhibition in mouse embryonic fibroblast cell line (NIH3T3).

Considering all studies and our present results, we can suggest that all three bisphenols might increase cellular apoptosis and enhance DNA damage, decrease DNA repair capacity, and cause genomic instability. Bisphenols can cause several pathological conditions, including cancer, with the inhibition of DNA repair genes like *OGG1* and *MyH*. These results were consistent with the idea that DNA repair initiation is suppressed by bisphenols (Chou et al., 2017).

5 | CONCLUSION

This present research showed that alternatives of BPA, namely BPF and BPS, were cytotoxic to RWPE-1 cells. These chemicals can also affect the cellular oxidant/antioxidant balance and lead to genotoxicity, like BPA. All of the bisphenol analogues produced high levels of DNA damage in RWPE-1 cells, while inhibiting the DNA repair pathway. The results suggest the likelihood that BPS might be 'safer' than BPA and BPF on prostate cells, as BPS was less toxic and generally had lesser effects on oxidant/antioxidant status compared to BPA and BPF. However, BPS induced DNA damage at approximately the same rate as BPF and its genotoxic effect was higher than BPA. These results may be important for further studies that will explore the underlying toxicity mechanisms BPF and BPS.

Although 'BPA-free products' are now available (from feeding bottles to receipts), these products contain either BPS or BPF, the toxicities of which should be carefully determined before they are considered as 'safer alternatives'. As both of these compounds, particularly BPS, have similar chemical structures to BPA, we can suggest that they may also have endocrine disrupting properties, the importance of which should be evaluated with both in vitro and in vivo studies. Because there is only limited data on the hazard identification for these bisphenol analogues, integrated assessment would be very beneficial and help the regulatory authorities to reconsider the use of BPF and BPS, instead of BPA. More mechanistic studies should be encouraged to identify the toxicity mechanism/s of these bisphenol analogues, as replacing one health hazard with another is not appropriate and will cause further damage to the environment and most importantly to humans. Scientists and authorities should urgently focus on the human health risk assessment of BPA substitutes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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