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RESEARCH ARTICLE

Evaluation of the Cytotoxic Effect of Bisphenol A and Its Analogs in MCF-7 and HSeC Cell Lines *in vitro*

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SUMMARY

Endocrine-disrupting chemicals like bisphenol A (BPA) and its analogs have negative effects on human health. This research aims to determine the cytotoxic effects of BPA and its four different analogs bisphenol S (BPS), bisphenol F (BPF), bisphenol Z (BPZ), bisphenol AF (BPAF) on both cancer and healthy cell lines simultaneously by performing an MTT test. In this study, human breast cancer cells (MCF-7) and human Sertoli cells (HSeC) were used for cell culture. MCF-7 and HSeC were exposed to BPA, BPS, BPF, BPZ, BPAF for 24 h. After that, the inhibitory effect of Bisphenols (IC50) was determined by measuring the absorbance. While BPF was the least cytotoxic alternative depending on the highest IC50 values in both cell lines, BPZ was found to be the most cytotoxic alternative in HSeC cell line. In the MCF-7 cell line, BPA and BPZ were found to have equally cytotoxic effects.

Key Words: *Endocrine-disrupting chemicals, bisphenols, cytotoxicity, MTT, MCF-7 cell lines, HSeC cell lines*

Bisfenol A ve Analoglarının MCF-7 ve HSeC Hücre Hatlarında in vitro Sitotoksik Etkisinin Değerlendirilmesi

ÖZ

Bisfenol A (BPA) gibi endokrin bozucu kimyasallar ve benzerleri insan sağlığı üzerinde olumsuz etkilere sahiptir. Bu araştırma, BPA ve dört farklı analogunun bisphenol S (BPS), bisfenol F (BPF), bisfenol Z (BPZ), bisfenol AF (BPAF) hem kanser hem de sağlıklı hücre hatları üzerindeki sitotoksik etkilerini MTT testi yaparak aynı anda belirlemeyi amaçlamaktadır. Bu çalışmada, hücre kültürü için insan meme kanseri hücreleri (MCF-7) ve insan sertoli hücreleri (HSeC) kullanılmıştır. MCF-7 ve HSeC, 24 saat BPA, BPS, BPF, BPZ, BPAF'ye maruz bırakılmıştır. Daha sonra, bisfenollerin (IC50) inhibitör etkisi absorbans ölçümü ile belirlenmiştir. En yüksek IC50 değerleri nedeniyle her iki hücre hattında BPF en az sitotoksik alternatif iken, BPZ'nin HSeC hücre hattında en sitotoksik alternatif olduğu bulunmuştur. MCF-7 hücre hattında, BPA ve BPZ'nin eşit sitotoksik etkilere sahip olduğu bulunmuştur.

Anahtar Kelimeler: *Endokrin bozucu kimyasallar, bisfenoller, sitotoksisite, MTT, MCF-7 hücre hatları, HSeC hücre hatları*

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INTRODUCTION

A great number of chemicals in the environment are toxic to human health. One of the most important effects of many chemicals such as food additives, pesticides, herbicides, cigarette smoke is on the endocrine system. Such chemicals impact the endocrine systems and so are called 'Endocrine Disrupting Chemicals (EDCs)', also known as xenoestrogenic (Zemheri and Uğuz, 2018). Endocrine-disrupting chemicals can be categorized as i) natural compounds, ii) pharmaceuticals, iii) environmental pollutants, and iv)industrially relevant chemicals (Zacharewski, 1998). In the aquatic environment such as surface water, wastewater, and sewage water, these chemicals are found in varying concentrations (Bhatnagar and Anastopoulos, 2017), and also they have various usage areas in the industry and homes (Zemheri and Uğuz, 2018). These chemicals and their decomposition products can be mutagenic, estrogenic, toxic, or carcinogenic (Zemheri and Uğuz,2018). Endocrine-disrupting chemicals cause overproduction or underproduction of hormones by mimicking the endocrine system or blocking a natural hormone (Bhatnagar and Anastopoulos, 2017).

One of the endocrine-disrupting chemicals is bisphenol A(BPA), which is widely used in the production of plastic materials (Urriola-Muñoz, 2018). Its chemical name is 2,2-bis (4-hydroxyphenyl) propane. In 1981, it was synthesized for the first time by Russian chemist Aleksandr P. Dianin (Xiao, 2020). BPA is found in the structure of many industrial products, including thermal paper, food containers, flame retardants, building materials, electronic devices, medical equipment, and is a weak estrogenic chemical (Urriola-Muñoz, 2018). It is also used in making PVC, compact discs, automotive parts, bottles, and feeding bottles (Zemheri and Uğuz, 2018). The main route of exposure to BPA is food, and canned foods are the main sources of it (Murata and Kang, 2018).

Increasing health concerns (Moreman, 2017) due to the effects of BPA on the reproductive, endocrine, immune and nervous system have led to the restriction of BPA production and the search for alternative chemicals (Wu, 2018). Bisphenol compounds such as bisphenol S (BPS), bisphenol F (BPF), bisphenol Z (BPZ), bisphenol AF (BPAF) are chemicals used as alternatives to BPA. BPS contains a sulfone group and 2 hydroxyl groups (Wu, 2018) and it is the structural analog of BPA (Qiu, 2018). It is used in the production of epoxy resins and polycarbonate plastics, and it is more resistant to high temperatures and more stable than BPA (Wu, 2018). Also, it has endocrine-disrupting effects similar to BPA (Qiu, 2018). BPF is another bisphenol derivative used as an alternative to BPA (Qiu, 2018). It is in the structure of materials such as lining and flooring materials, coating, plastics, pharmaceuticals (Mu, 2019), and also widely used in the production of lacquer, varnishes, liners, adhesives, water pipes, dental sealants, road and bridge deck toppings, and coatings for food packaging. Also, according to both *in vivo* and *in vitro* studies, BPF has estrogenic properties similar to BPA (Mu, 2019). The extensive use of BPF leads to be found both in the human body (Qiu, 2018). Bisphenol Z (BPZ) whose chemical nomenclature is 1,1-bis (4-hydroxyphenyl) cyclohexane has been detected in many human and environmental samples (Kovačič, 2019). It can be used in the synthesis of anesthetic chemicals such as phencyclidine (PCP) (Schmidt, 2013). Besides, many personal care products, paper products, and food packaging materials contain BPZ. As well as enhancing plastics and electrical insulation, it is also used in high heat-tolerant materials (Lee, 2019). On the other hand, BPAF has recently been developed, and it is used in foodstuffs, as well as in electronic devices (Yang, 2016). Among its uses are cross-linking reagents in fluoroelastomers, and many plastic optical fibers (Yang, 2016). It can be more harmful than BPA because it consists of an electronegative CF3 moiety that is more reactive than CH3 of BPA (Lee, 2013).

To detect cell viability after exposure to bisphenols, cytotoxicity assays such as the LDH leakage assay, the neutral red, and the MTT test are carried out. Among those cytotoxicity assays, the MTT test is based on the ability of viable cells to reduce 3-(4,5-di-

metyhlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into an insoluble purple formazan through succinate dehydrogenase within mitochondria, and formazan accumulates in the viable cells. In our study, to determine cytotoxic effects of BPA, BPS, BPF, BPAF, BPZ on both MCF-7 and HSeC cell lines, the MTT test was performed. Using an MTT assay, we aimed to evaluate the cytotoxicity of BPA and four different analogs (BPS, BPF, BPZ, BPAF) on both cancer and healthy cell lines, simultaneously.

MATERIALS AND METHODS

Reagents

The reagents were as follows: Bisphenols (BPs) (purity ≥99%); BPA, BPS, BPF, BPAF, BPZ, Dulbecco's Modified Eagles Medium F-12 (DMEM F-12), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), tyripsin, phosphate-buffered saline (PBS), trypan blue, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO). Bisphenols (BPs), DMEM, MTT, tyripsin, PBS, trypan blue used in this research were from Sigma-Aldrich, Germany. FBS and DMSO were purchased from Serva, Germany.

Cell Culture

To conduct cell culture and MTT assay, MCF7 (ATCC® HTB-22™) and HSeC (MM-HSE-2305) were used. After taking the cells out of -80°C, they were thawed less than 2 minutes in the 37°C water bath. The cells were immediately transferred to a 15 mL tube containing DMEM in the laminar flow cabinet. They were centrifuged at 1000 rpm for 6 minutes. After centrifuge, the supernatant was removed. And then, they were transferred to the T25 flask. Finally, the flasks were placed in a %5 CO_2 and 37°C incubator. For the next passage, the confluence of cells was observed every two days. Figure 1 and Figure 2 show the view of the confluence of cells.

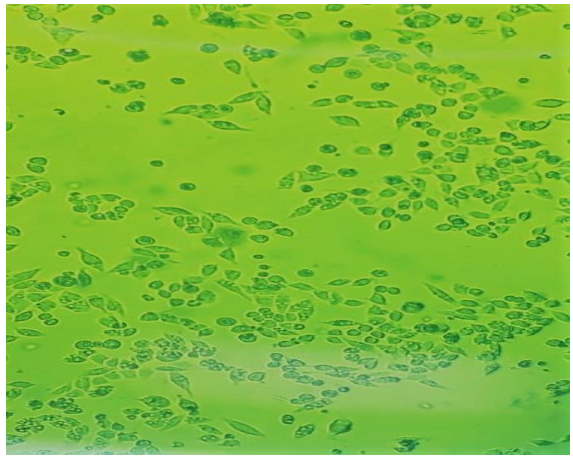

Figure 1. HSeC cell line. After passage, we checked the confluence of cells for the next passage. Since the cells do not fill the flask surface completely, it must be waited for the next passage.

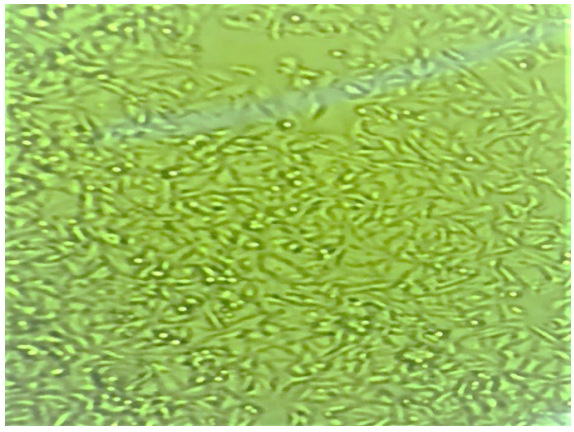

Figure 2. Image of MCF-7 cell line under the microscope. The cells ready for the next passage due to their confluence.

Determination of cytotoxicity

Cell viability was determined using the MTT assay as previously described (Zhang et al.2017), and then the inhibitory effect of Bisphenols (IC_{50}) value was analyzed. Cells (4000 cells/200 µl %10 DMEM) were seeded in 96-well plates, cultured for 1 day, and treated with different concentrations of BPA, BPS, BPF, BPAF, and BPZ (0.1, 0.5, 1, 5, 10, 50, 100, 500 µM) for 24 h. The cells within DMSO were used as a solvent control group. The medium containing BPs was removed, and 200 µl 0,5 mg/mL MTT solution in DMEM was added

to each well. The plate was then incubated at 37 °C for 4 h. The formazan was dissolved with dimethyl sulfoxide (DMSO) after removing the MTT solution. The amount of formazan dye product formed was quantified by measuring absorbance using a Thermo Scientific Multiskan Go microplate reader at 560 nm.

Statistical Analysis

By measuring absorbance, the cell viability was determined, and the results were presented as mean + standard deviation. A sigmoidal curve fitting approach was used to calculate cytotoxicity based on cellular viability vs bisphenol concentration and the IC_{50} values were taken from the concentration-response curves.

RESULTS and DISCUSSION

Cytotoxic effects of Bisphenol A (BPA) and its analogs (BPS, BPF, BPZ, BPAF) in MCF-7 cells and HSeC cells

BPA concentration that reduces cell viability by 50% (IC_{50}) for MCF-7 cell line was calculated as 45 µM whereas the IC_{50} BPA concentration for the HSeC cell lines was 35 µM. There was a significant decrease in cell viability in both MCF-7 and the HSeC at 100 µM. As a result of BPS exposure, the IC_{50} value for MCF-7 was 450 µM while the IC_{50} value for the HSeC was found to be 105 µM. Even at high concentrations of BPF, no decrease in cell viability was seen in the MCF-7 cell line. However, the IC_{50} value for the HSeC was calculated as 435 µM. BPZ exposure resulted in a decline in the viability of both MCF-7 and the HSeC cell lines. The IC_{50} value for MCF-7 was 45 µM whereas the IC_{50} value for the HSeC cell lines was calculated to be 25 µM. IC_{50} values for BPAF that cause cell viability of both MCF-7 and the HSeC cell lines to decrease significantly at high concentrations were calculated to be 56 µM and 48 µM for MCF-7 and HSeC, respectively. Calculated IC_{50} values for MCF-7 and Sertoli cell lines are shown in *Table 1*.

Taken together, BPA and its analogs have cytotoxic effects on both cancer cells and non-cancer cells.

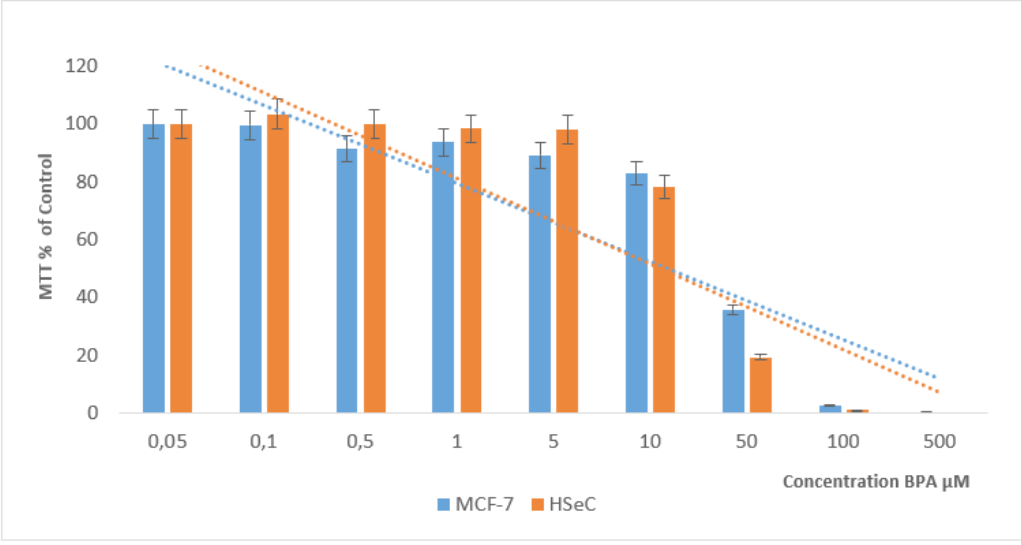

Figure 3. The graphic of concentration of BPA µM (x-axis) versus MTT % of Control.

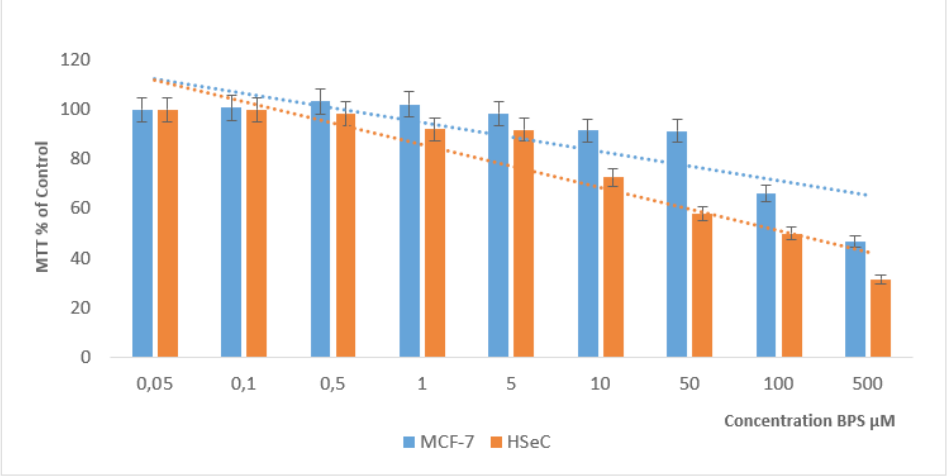

Figure 4. The graphic of concentration of BPS µM (x-axis) versus MTT % of Control.

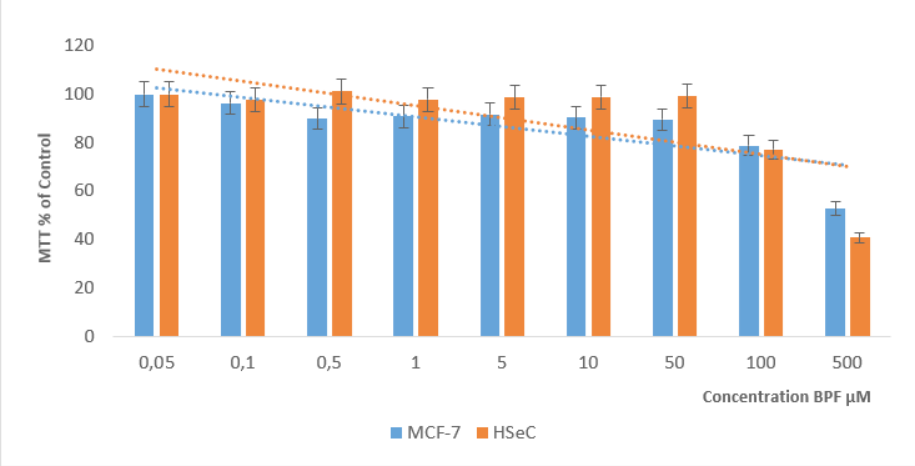

Figure 5. The graphic of concentration of BPF µM (x-axis) versus MTT % of Control.

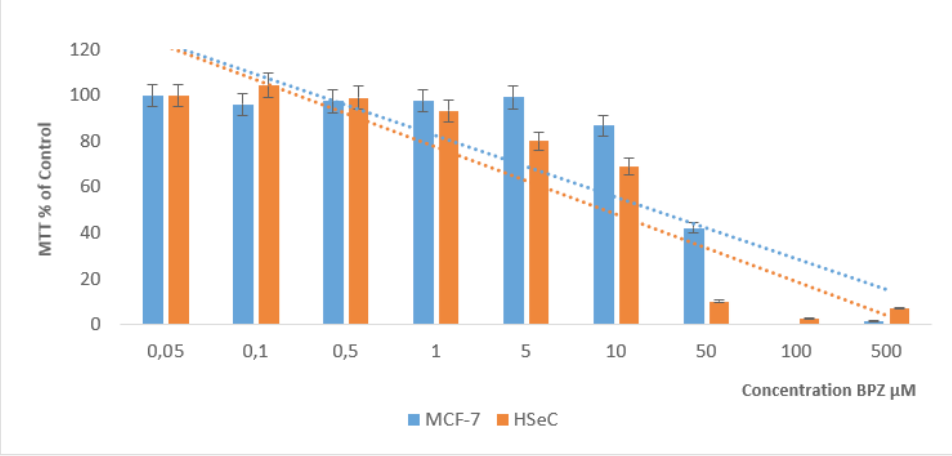

Figure 6. The graphic of concentration of BPZ µM (x-axis) versus MTT % of Control.

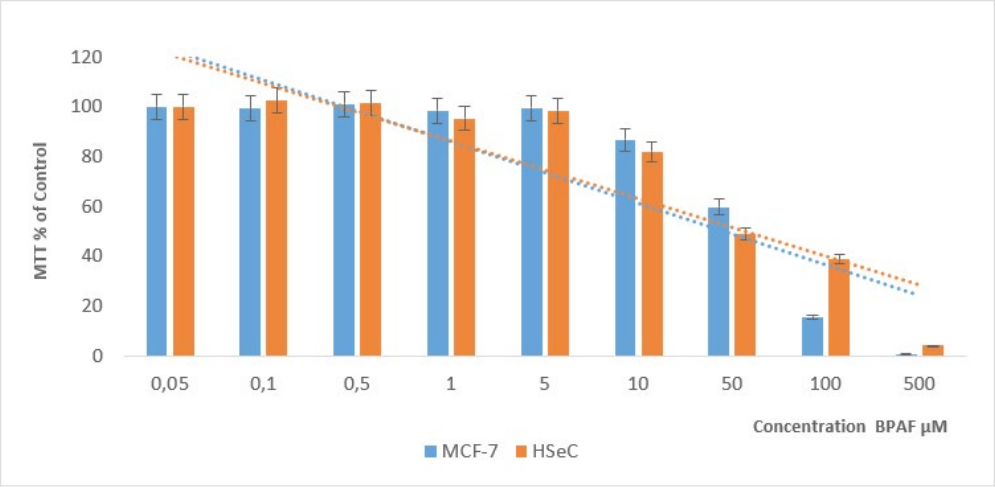

Figure 7. The graphic of concentration of BPAF µM (x-axis) versus MTT % of Control.

Table 1. IC_{50} (µM) values for MCF-7 and HSeC versus Bisphenol analogs. The IC_{50} value of BPF was not calculated for MCF-7 cell line.

BPs	MCF-7	HSeC
BPA	45	35
BPS	450	105
BPF	-	435
BPZ	45	25
BPAF	56	48

In MCF-7 cell line IC_{50} (µM) values are respectively; BPF>BPS>BPAF>BPZ=BPA; in HSeC cell line IC_{50} (µM) values are respectively; BPF>BPS>BPAF>BPA>BPZ.

DISCUSSION

In this experiment, BPA and its analogs (BPS, BPF, BPZ, BPAF) with different concentrations were prepared. Considering the fact that bisphenols affect the male reproductive system and cause breast cancer in females, MCF-7 and HSeC cell lines were used in our study. The prepared bisphenols were exposed to MCF-7 and HSeC for 24 h and then, the amount of formazan dye product was evaluated by measuring absorbance. The objective of this study was to assess the cytotoxicity profiles of BPA and its analogs on both MCF-7 and HSeC cell lines at the same time.

Several studies have demonstrated that bisphenols are related to several pathological conditions such as endocrine disorders, type II diabetes, reproductive system disorders, cancer (Rahmani, 2020, Wang, 2020). Accordingly, it was claimed that BPA reduces daily sperm production and causes infertility (Geens, 2012, Gerona, 2013, Aris, 2014, Chen, 2016). Moreover, female offspring's mammary gland formation is affected (Muñoz-de-Toro, 2005), and testosterone, LH, and FSH hormone balance are disrupted by BPA (Nakamura, 2010). Additionally, it can alter signal pathways that regulate cell growth and proliferation, leading to increased proliferation of cancer cells (Murata and Kang, 2018). In one study conducted by Neri et al. (2015), cytotoxic effects of BPA on monocytes cell line was evaluated by using trypan blue assay. As a result, they found that cytotoxic effects have increased based on the higher concentrations of BPA. In a study investigating the cytotoxic effect of Bisphenol A on MCF-7 cell lines and amniocytes, cell damage induced

by BPA was determined by performing the MTT test. This study, in which genotoxic damage was also determined, revealed that BPA caused a significant cytotoxic effect on the MCF-7 cell line (Aghajanpour-Mir, 2016). Our study also confirms this situation. In our study, we found the IC_{50} value of BPA in the MCF-7 cell line as 45 μM (Table 1 and Figure 3). A study evaluating the cytotoxic effect resulting from BPA exposure on MCF-7 cell line found that the IC_{50} value was approximately 65 μM (Hernández-Hernández, 2019). By Hernández-Hernández, 3T3-L1 was used as a healthy cell line. Accordingly, the IC_{50} value of 3T3-L1 cell line was higher than MCF-7 cell line. In our study, we used the Sertoli cell line (HSeC) as a healthy cell. However, we calculated IC_{50} value in HSeC to be lower (Figure 4). This may be due to the fact that the healthy cell lines used in two different studies are morphologically and physiologically different from each other. While the healthy cell line we used is a cell belonging to the male reproductive system, the 3T3-L1 used in this study is the mouse fibroblast cell.

On the one hand, the researches offered evidence that the toxicity profiles of BPA analogs are similar or higher than BPA. For example, BPS, which has similar estrogen and androgen receptor activity to BPA (Wu, 2018), has been demonstrated to have pro-inflammatory effects on the mouse macrophage cell line (Qiu, 2018). In addition, it may cause obesity as BPA (Wu, 2018). In a study comparing the toxic effects of five different BPA analogues including BPF and BPAF, it was shown that alternative compounds may have higher toxic effects compared to BPA (Sharin, 2021). A study was conducted in which the toxicity of BPA and BPS was compared on human bronchial epithelial cells (BEAS-2B). It was stated that both BPA and BPS have led to induce cytotoxicity and DNA damage (George and Rupasinghe, 2018). In a study in which the cytotoxic effect of BPAF, BPA, and other analogs was evaluated by Lei et al. (2016), it was stated that the cell viability of MCF-7 increased significantly between 0.01-1 μM concentration, while at high concentrations, cell viability was notably reduced between 25-100 μM (Lei, 2016). According to our results, BPS and BPF caused a decrease in cell viability between 100-500 μM (Figure 4 and 5), while BPZ and BPAF caused cell damage between 20-60 μM (Figure 6 and 7). Moreover, as the researchers stated that the cytotoxic effect of BPAF may be similar to or rather lower than BPA (Lei, 2016), in our study, we also found the cytotoxic effect of BPAF was similar to BPA. On the other hand, some studies revealed that BPA analogs have been less toxic compared to BPA. Hercog et al. (2020) evaluated the effect of cyanotoxins and bisphenols in HepG2 cells altogether. In their study, MTT assay was used to assess the cytotoxicity, and γH2AX assay that measures the induction of DNA double-strand breaks (DSBs) was conducted. Their results showed that the BPs (BPA, BPS, BPF) alone didn't reduce either cell viability or induced DSBs (Hercog, 2020). One study examined the cytotoxicity and genotoxicity of BPA and BPF using intestinal, hepatoma, and renal cell lines. Results revealed that BPA proved to be the most toxic compound, but BPF had intermediate cytotoxic effects (Audebert, 2011). In another study, Kose et al. (2020) have assessed the cytotoxicity, oxidative stress, and genotoxicity of bisphenol derivatives in RWPE-1 cells. They concluded that the highest level of cytotoxicity was found for BPA, followed by BPF and BPS. Ikhlas et al. (2019) have investigated BPA analogs' toxic effects due to the lack of data available on BPA analogs. These researchers used BPB and BPF as common BPA analogs. Their findings confirmed the fact that induction of cytotoxicity by BPB and BPF in human peripheral blood cells. Their studies suggested that BPA analogs have the capability of inducing cytotoxicity through genotoxicity and oxidative stress. Russo et al. (2018) tested the toxicity of seven analogs of BPA (bisphenol AF, bisphenol B, bisphenol M, bisphenol A diglycidyl ether, bisphenol S, bisphenol F, bisphenol E) to compare their toxicity with that of BPA. They have used a combination of in-silico and in-vitro techniques. They have suggested that four bisphenol A analogs (BPAF, BPB, BPM, BPA diglycidyl ether) caused the more toxic effect, while the rest of the analogs (BPS, BPF, BPE) resulted in less toxic effect compared to BPA (Russo, 2018).

CONCLUSION

BPS, BPF, BPZ, BPAF are structural analogs of BPA and are widely used in industries. Although they are used instead of BPA in many areas, few details exist about the toxicity of alternative bisphenols. Therefore to elucidate their toxic potentials, we conducted the MTT assay on MCF-7 and HSeC cell lines. It is known that bisphenols damage reproductive systems and cause cancer. We chose healthy and cancer cells to compare them because there are no sufficient studies on the toxic effect of Bisphenols on both cancer cells (MCF-7) and male reproductive cells (HSeC). In the MTT assay, we found that BPF was the least cytotoxic alternative because of its highest IC_{50} values in both cell lines compared to other alternatives *(Table 1)*. BPS was determined to be the second least cytotoxic analog. As for the BPAF, it was found to have higher cytotoxicity than BPF and BPS. BPAF, however, had lower cytotoxicity than BPA and BPZ. In the HSeC cell line, BPZ was found to be the most cytotoxic alternative while BPZ and BPA were found to be equally cytotoxic in the MCF-7 cell line. Taken together, our study will simultaneously bring about in comparison with MCF-7 and HSeC cell lines after bisphenols are exposed to them. Also, it is highly significant to examine the toxicological profiles of these compounds to estimate the relationship between exposure and toxic outcomes. However, other mechanisms such as oxidative stress, DNA damage, and other parameters can cause cell damage. As a consequence, further studies are still required.

CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

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AUTHOR CONTRIBUTION STATEMENT

Idea (İpek S., İyigündoğdu İ., Üstündağ A., Duydu Y.), manuscript design (İpek S.), performing experiments (İpek S., İyigündoğdu İ., Üstündağ A.), data analysis (İpek S., Üstündağ A.), data interpretation (İpek S., Üstündağ A.), literature review (İpek S.), writing article (İpek S.).

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