An Investigation into the Adverse Effects of Oxidative Stress from Exposure to Bisphenol A and Its Analogues

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Abstract

Oxidative stress is a physiological event caused by an overaccumulation of reactive oxygen species (ROS) within the body. While ROS are a natural by-product of oxygen metabolism, too many can lead to cell and tissue damage and contribute to many etiologies. Bisphenol A (BPA), a component of many plastic products, has been shown to induce oxidative stress. While the industrial usage of BPA usage has lessened, the safety of its replacements is unknown. This paper will primarily discuss ROS and mechanisms of oxidative stress, the usage of BPA and its analogues, etiologies associated with oxidative stress resulting from exposure to BPA and analogues, and a proposed experimental design to determine oxidative stress from exposure to BPA and its analogues.

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Introduction

Oxidative stress is a physiological event that occurs when the production of reactive oxygen and nitrogen species (RONS) and the ability of the body to detoxify those reactive products is shifted out of balance (Preiser, 2012). While RONS play an important part in cell signaling pathways and are generated as a natural metabolic by-product, when RONS accumulate within the body, cell and tissue damage can occur leading to negative physiological effects (Meli et al., 2020). Bisphenol A (BPA)—a component of many polycarbonate plastics and epoxy resins—is a known endocrine disruptor and has also been shown to cause oxidative stress (Meli et al., 2020). The usage of BPA has been limited in some industries due to legislature stemming from health concerns, but it and its structural analogues, such as Bisphenol AF (BPAF), Bisphenol B (BPB), Bisphenol F (BPF), and Bisphenol S (BPS), are still used in a wide range of industrial applications such as dental fillings, plastic products, and food packaging (Rosenmai et al., 2014; Zhao et al., 2018; Huang et al., 2020). The safety and efficacy of these BPA analogues is still being determined. This paper will predominantly discuss RONS, mechanisms of action for oxidative stress, usage of BPA and BPA analogues, health concerns regarding the usage of BPA and its analogues, and a proposed experimental design for the determination of oxidative stress via exposure to BPA and its structural analogues. While there is correlation between these chemicals and their ability to be androgenic as well as carcinogenic, these topics lie outside the scope of this paper and will thus be excluded.

Background and Significance

The proposed research topic will investigate the origins, deleterious physiological effects, mechanisms of action regarding the induction of oxidative stress, and governmental regulation of BPA and its structural analogues. This topic is of great importance for research and industrial reform surrounding the usage of BPA and its analogues as the oxidative stress stemming from the use of these chemicals has been shown to contribute to the formation of many etiologies. Reform regarding the use of these chemicals is required because these chemicals are still in products used every day by consumers.

Oxidative Stress

Reactive Oxygen and Nitrogen Species (RONS)

Reactive oxygen and nitrogen species, referred to as RONS, are two types of chemically reactive molecules that contain oxygen (reactive oxygen species or ROS) and nitrogen (reactive nitrogen species or RNS) (Weidinger & Kozlov, 2015). RONS participate as oxidants or oxidizing agents in chemical reactions, which means they remove one or more electrons from another reactant (Merriam-Webster, n.d.). Most molecules considered to be RONS carry unpaired electrons and are called free radicals (Weidinger & Kozlov, 2015). However, not all RONS are free radicals. There are non-radical products that can act as powerful oxidizing agents and participate in free radical reactions within the body (Weidinger & Kozlov, 2015).

RONS are released into the extracellular space from neutrophils, macrophages, and dendritic cells in response to an inflammatory stimulus by the innate immune system to kill bacteria (Weidinger & Kozlov, 2015; Ozcan & Ogun, 2015). The intracellular release of RONS is caused by mitochondrial respiration and the monooxygenase activity of cytochrome P450

(Weidinger & Kozlov, 2015). RONS have been found to have important cellular functions such as the regulation of intracellular signaling cascades that control physiological functions such as the regulation of vascular tone, cell proliferation, differentiation, migration, and insulin synthesis (Weidinger & Kozlov, 2015). However, an overproduction of RONS could potentially be harmful to cells. Cellular levels of RONS are controlled via molecules called antioxidants (Nelson & Cox, 2012). An imbalance between RONS and antioxidants within the cell—referred to as oxidative stress—can induce deleterious effects, causing damage to necessary biological structures. (Weidinger & Kozlov, 2015).

Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are generated by mitochondria as a by-product of oxygen metabolism and have roles in many important cellular processes, such as cell signaling and maintenance of homeostasis (Preiser, 2012; Ozcan & Ogun, 2015). The path of oxygen reduction within the mitochondria has the possibility of forming highly reactive free radicals, such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) (Nelson & Cox, 2012; Pizzino et al., 2017). Another ROS is hydrogen peroxide (H_2O_2) (Pizzino et al., 2017).

Beneficial roles of reactive oxygen species include protein phosphorylation, apoptosis, immunity, and activation of transcription factors (Pizzino et al., 2017). When ROS levels are regulated, cells function as they should. However, damage can occur to different cellular structures when ROS are overproduced and accumulate within a cell (Xin et al., 2016a). High concentrations of ROS have been linked to the damage of lipids, nucleic acids, carbohydrates, and proteins due to their high reactivity with these structures (Ozcan & Ogun, 2015; Pizzino et al., 2017). The overproduction of ROS and the ensuing oxidative stress has been implicated in

the development and/or progression of several diseases such as cancer, diabetes, cardiovascular disease, and metabolic disorders (Pizzino et al., 2017).

ROS can be produced through both endogenous and exogenous sources. Free radical production from endogenous sources include immune cell activation, inflammation, cancer, excessive exercise, aging, mental stress, and infection (Pizzino et al., 2017). Exogenous sources of ROS can occur from exposure to environmental contaminants, such as BPA and its analogues (Pizzino et al., 2017). Other exogenous sources include chemical solvents, cigarette smoke, alcohol, ionizing radiation, sulfa drugs, antimalarial medication, and heavy metals (Nelson & Cox, 2012; Pizzino et al., 2017).

Reactive Nitrogen Species (RNS)

Similar to ROS, reactive nitrogen species (RNS) are formed as by-products of metabolic processes. Nitric oxide (NO•) and its derivatives—nitrate (NO_3^-), nitrogen dioxide (NO_2•), peroxynitrite ($ONOO^-$), and 3-nitrotyrosine—are all considered to be RNS (Di Meo et al., 2016; Ozcan & Ogun, 2015). RNS are a two-edged sword, just like ROS, in that they are both harmful and beneficial to living systems (Di Meo et al., 2016). These molecules have roles in cellular signaling, the immune response, and vasodilation (Ozcan & Ogun, 2015).

RNS are known to cause protein nitration through multiple different physiological pathways, which results in protein dysfunction and neuronal loss (Di Meo et al., 2016). NO• has been implicated in its role in neurogenerative diseases when found in high concentrations (Di Meo et al., 2016). Another RNS, NO_2•, is an indoor and outdoor pollutant found in car emissions, cigarette smoke, heaters, gas stoves, and fossil fuels, and even short exposure can lead to inflammation, cellular injury, and pulmonary edema (Di Meo et al., 2016).

Antioxidants

Molecules referred to as antioxidants can react enzymatically and nonenzymatically with reactive oxygen species and other free radicals, providing protection from the deleterious effects of oxidative stress (Nelson & Cox, 2012). The role of antioxidants within the body is to scavenge for RONS and to eliminate the oxidant burden by transforming the ROS and their by-products into stable, nontoxic molecules (Finkel, 2003). Enzymatic antioxidants include superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase (Hassani et al., 2017). Superoxide dismutase and glutathione peroxidase are produced by the mitochondria and prevent oxidative damage against $O_2^{\bullet-}$ and H_2O_2, respectively (Nelson & Cox, 2012). Some nonenzymatic antioxidants are ferritin, transferrin, albumin, and ceruloplasmin, as well as antioxidants from dietary sources, like vitamin E, vitamin C, and carotenoids (Mirończuk-Chodakowska et al., 2018; Nelson & Cox, 2012). The presence of transition metal ions—such as iron and copper—is required for the activation of most nonenzymatic antioxidants, as these ions catalyze the antioxidants in order to inhibit the formation of new reactive species (Mirończuk-Chodakowska et al., 2018).

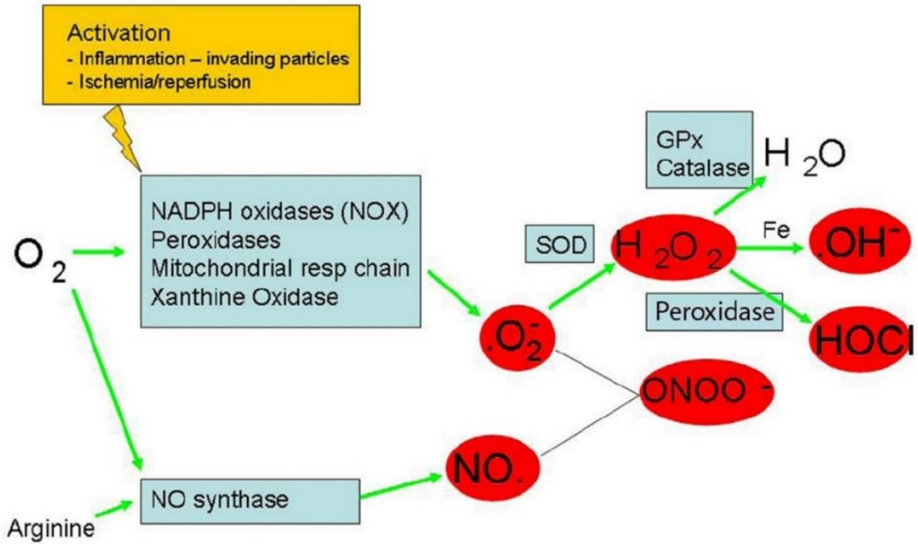
Mechanisms of Oxidative Stress

The production of ROS is vital for many physiological pathways. However, it is when the balance between prooxidant and antioxidant chemicals occurs that oxidative stress occurs (Figure 1). In aerobic cellular respiration, there are several steps during the electron transport chain in the mitochondria where ROS have the potential to form as seen in Figure 1 (Nelson & Cox, 2012; Preiser, 2012). The radical $^{\bullet}Q^-$ is an intermediate involved in the passage of electrons

from QH_2 to cytochrome b_1, thorough Complex III, and the passage of electrons from Complex I to Q.

Figure 1

Mechanisms of oxidative stress



Note. Preiser, J. C. (2012). Oxidative stress. *Journal of Parenteral and Enteral Nutrition*, *36*(2), 147-154. https://doi.org/10.1177/0148607111434963. Reprinted with permission.

(Nelson & Cox, 2012). This $^{\bullet}Q^{-}$ intermediate has the ability to pass an electron to O_2 in the reaction and generate the free radical $^{\bullet}O_2^{-}$, which is extremely reactive and can damage enzymes, nucleic acids, and membrane lipids (Nelson & Cox, 2012). While this harmful free radical only forms 0.1-4% of the O_2 used by actively respirating mitochondria, this can still cause significant cellular damage unless it is quickly removed (Nelson & Cox, 2012). The antioxidant superoxide dismutase catalyzes the transformation of two $^{\bullet}O_2^{-}$ molecules plus two protons into the products H_2O_2 and O_2 (Nelson & Cox, 2012). H_2O_2, considered to be a ROS, is then rendered harmless through the action of glutathione peroxidase and catalase (Nelson & Cox, 2012). NADPH is

required for the breakdown of H_2O_2 to H_2O and O_2 (Nelson & Cox, 2012). The RNS, NO•, is formed through the calcium-dependent action of NO synthase, which combines O_2 and arginine for its formation (Nelson and Cox, 2012).

When in kept in balance by antioxidants, RONS are beneficial to cells. For example, RONS have a role in cell signaling, as they are required for growth factor induced receptor tyrosine phosphorylation and the resulting activation of several key signal transduction pathways (Schieber & Chandel, 2014). Another role of RONS is that they act as second messengers in innate and adaptive immunity and in response to inflammation (Schieber & Chandel, 2014). Generation of RONS are needed in order for lipopolysaccharide to activate inflammatory cytokines to fight against pathogens and repair tissue damage, as well as for Toll-like receptor-initiated pathways and the optimal bactericidal activity of macrophages (Schieber & Chandel, 2014). Slight elevation in RONS levels in the immune system may enhance normal immune function through these pathways (Schieber & Chandel, 2014).

When RONS are not kept in the correct prooxidant/antioxidant ratios, that is when cellular damage and the development of etiologies can begin to occur. The same RONS that are required for cell signaling can also activate tumorigenic signaling pathways like PI3K and MAPK pathways (Schieber & Chandel, 2014). The transcription factor NF-κB is also a target of RONS, which controls the survival of tumor cells (Schieber & Chandel, 2014). In the inflammatory pathways, slightly elevated levels of ROS can enhance the immune system while high levels of ROS can promote the development of pathological inflammation (Schieber & Chandel, 2014).

Bisphenol A (BPA) and Its Analogues

History and Usage of BPA and Its Analogues

Bisphenol A

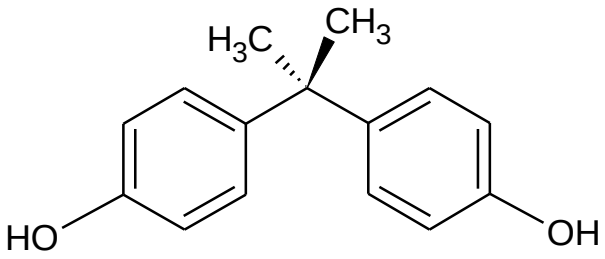
Bisphenol A (BPA, Figure 2) is one of the most widely used industrial compounds worldwide (K. Wang et al., 2019). One survey conducted in 2010 showed that an estimated 8 million tons of BPA was produced annually, and approximately 100 tons of BPA is released into the atmosphere each year (Vandenberg et al., 2010). BPA has been widely used in the production of polycarbonate plastics and epoxy resin linings for food and beverage containers (Lv et al., 2017). However, one of its prominent uses is as a color developer for carbonless copy paper and thermal paper used in receipts, faxes, luggage tags, and labels (Lv et al., 2017).

BPA has been detected in food, the environment, and even in human bodily fluids, like blood serum, urine, follicular fluid, placental tissue, and amniotic fluid (K. Wang et al., 2019; Vandenberg et al., 2007). BPA has been found in concentrations of 0.2-106 ng/g in food samples, 2-208 ng/m^3 in the air, and 54-79 $\mu g/cm^2$ in thermal paper (Y. Wang et al., 2019). Human blood samples have shown concentrations of BPA reaching 0.5-10 µg/L, and levels at 4.76 µg/L and 59.72 µg/L in placental and urine samples respectively (K. Wang et al., 2019).

The primary exposure for humans to BPA is through food and food packaging, and routes for secondary exposure include drinking water and dust (K. Wang et al., 2019). This chemical has also been known to leach from the linings of food containers and from dental fillings (Vandenberg et al., 2007). BPA leaching is caused by exposure to heat or contact with acidic or basic compounds. This exposure accelerates the hydrolysis of the ester bond linking BPA molecules with polycarbonate and epoxy resins, releasing it into the environment (Hassani et al.,

Figure 2

Structure of Bisphenol A



2017). Additionally, exposure to ultraviolet light has been shown to release BPA into the environment (K. Wang et al., 2019).

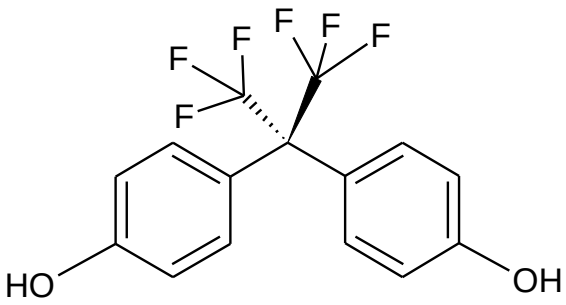
Much legislation has been instituted regulating the usage of BPA, as concerns about the presence of this chemical have increased. The Canadian government banned the import, sale, and advertisement of polycarbonate baby bottles containing BPA in 2010, and the European Union soon followed suit, by prohibiting the use of BPA in the manufacture of polycarbonate infant feeding bottles (Rosenmai et al., 2014). In 2012, the Food and Drug Administration in the United States banned the use of BPA in sippy cups, infant bottles, and in the epoxy resins that line infant formula cans (Ullah et al., 2018). The increased pressure to remove BPA in industrial uses has increased the usage of structural analogues of BPA such as BPAF, BPB, BPF, and BPS.

Bisphenol AF

Bisphenol AF (BPAF, Figure 3) is a fluorinated structural analog for BPA and is used as a crosslinker for fluoroelastomers, fibers, electronics, and other polymer applications such as plastic fibers and waveguides (Huang et al., 2020). It has been shown that BPAF can accumulate in various tissues, blood serum, and urine of rats (Maćczak et al., 2017).

Figure 3

Structure of Bisphenol AF (BPAF)

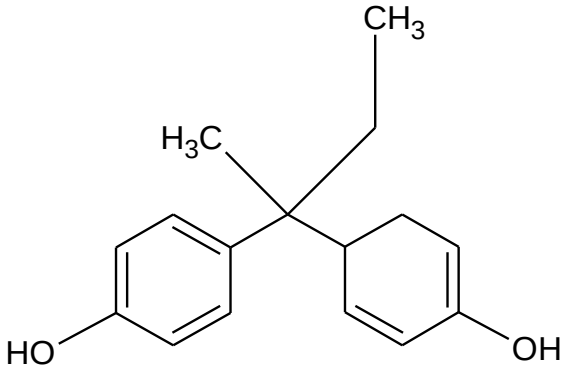


Bisphenol B

Bisphenol B (BPB, Figure 4) is another common BPA replacement and was first developed as a dye in 1869 (Ullah et al., 2018). After the use of BPA was reduced, BPB began to be used in the production of epoxy resins, infant feeding bottles, and thermal papers in 2006 (Ullah et al., 2018). It is also found in canned tomatoes, canned beers, and canned soft drinks (Rosenmai et al., 2014). Studies conducted have detected BPB in 28% of the 52 Italian endometriotic women tested, indicating that it is also leaching into the environment and could pose a health risk (Rosenmai et al., 2014).

Figure 4

Structure of Bisphenol B (BPB)



Bisphenol F

The BPA replacement Bisphenol F (BPF, Figure 5) has been used in the manufacturing of epoxy resins and polycarbonate products (Zhao et al., 2018). It is commonly found in consumer items such as potable water pipes, industrial floors, electrical varnishes, lacquer, dental sealants, and food packaging (Zhao et al., 2018). BPF has been detected in food packages and in the drinking water that has been pumped through BPF-lined water pipes (Ullah et al., 2018). This chemical has also been found in meat products, vegetables, and beverages (Ullah et al., 2018). Organ samples were taken in some studies, and BPF has been observed in the reproductive organs and is known to cross the placental barrier to the developing fetus (Ullah et al., 2018).

Bisphenol S

Bisphenol S (BPS, Figure 6) is an analogue of BPA used in industrial applications as well as in consumer products, Figure 6. Its industrial applications include wash fastening agents in cleaning products, electroplating solvent, and as a constituent of phenolic resins (Herrero et al., 2018). BPS can also be found in canned soft drinks and food products and in thermal receipt paper (Herrero et al., 2018). Because BPS is considered to be more stable when exposed to heat

Figure 5

Structure of Bisphenol F (BPF)

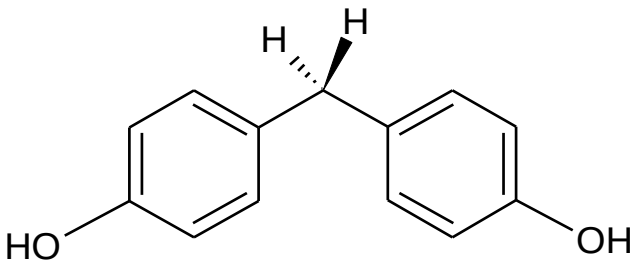
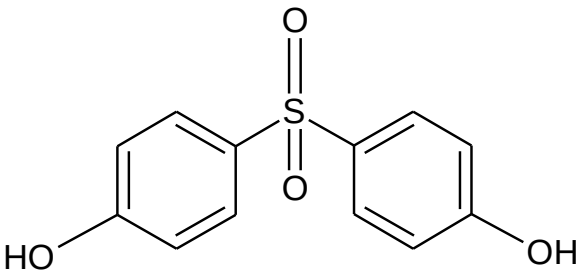


Figure 6

Structure of Bisphenol S (BPS)



and UV light, it is used in many products that are labelled BPA-free due to the assumption that it is a safer product (Herrero et al., 2018). There are no regulations regarding the use of BPS for industrial or consumer applications, which has led to a sharp increase in its production and use (Herrero et al., 2018). BPS has been detected in water, sediment, dust, and sewage sludge, as well as in human urine and blood samples (Herrero et al., 2018). The concentrations found are generally lower than that of BPA but are of the same order of magnitude (Herrero et al., 2018).

Health Concerns Regarding Usage of BPA and Its Analogues

As the usage of Bisphenol A increased, concerns about its safety grew. Studies regarding the estrogenic properties of BPA were reported as early as 1936 (Ullah et al., 2018). As more research studies were conducted, they began to link BPA and the induction of oxidative stress. These health concerns regarding BPA ultimately led to legislatures limiting its usage and resulting in the search for BPA replacements. Unfortunately, because the structure of BPA and its analogues are so alike, similar deleterious effects began to be discovered upon their usage as well (Rosenmai et al., 2014).

BPA and BPA Analogues and Endocrine Dysfunction

Endocrine dysfunction was one of the first side effects noticed with the usage of BPA. It was discovered that the structure of BPA—containing two hydroxyl (OH^-) groups and two benzene rings—allows it to fit into the binding pocket of the estrogen receptor (ER), mimicking the effects of estrogen (Ullah et al., 2018). Studies determined that BPA is a weak ER ligand, meaning that while it binds to ER, it binds with less affinity than estrogen (Ferguson et al., 2016). BPA also binds to the two isoforms of the nuclear ER—ERα and ERβ—with different affinities (Meli et al., 2020). BPA has also been shown to bind with other membrane and nuclear receptors, activating them at lower concentrations than what is required with the nuclear ERs (Meli et al., 2020). Two of these receptors to note are G protein-coupled estrogen receptor (GPER) and the estrogen-related receptor (ERR)γ (Meli et al., 2020). GPER is found in multiple cell types and is involved with cell proliferation and apoptosis (Meli et al., 2020). ERRγ is an orphan nuclear receptor that is highly expressed in fetal brain and placental tissue, indicating that BPA could have deleterious effects on infant development.

The structural analogues of BPA have also undergone numerous studies to determine if they have estrogenic properties as well. While the exact estrogenic potential of these analogues depends on the type of estrogen receptor, all the bisphenol analogues mentioned in this paper exhibit weak estrogenic properties (Cao et al., 2017). Research has shown that BPAF and BPB are more estrogenic than BPA, BPF is about as estrogenic as BPA, and BPS is less estrogenic than BPA (Moreman et al., 2017).

The estrogenic nature of BPA and its analogues is a cause for concern, as they can function through the estrogen receptors and alter the expression of estrogen responsive genes.

Additionally, it is known that estrogen stimulates the production of RONS for cellular signaling (White et al., 2010). Therefore, BPA and its analogues have the potential to increase RONS levels within cells, leading to oxidative stress.

Other health risks linked to estrogenic chemicals include increased incidences of breast and testicular cancer, decreases in immune function, metabolic disease, and urogenital tract malformation (Moreman et al., 2017). Studies have indicated that postmenopausal women are at a greater risk for the deleterious effects of BPA and BPA analogue exposure due their low levels of endogenous estrogen (Meli et al., 2020).

BPA and BPA Analogues and Oxidative Stress

Studies have shown that Bisphenol A and its commonly used chemical replacements disturb the prooxidant/antioxidant balance within cells, inducing oxidative stress (Y. Wang et al., 2019). This occurs through both direct and indirect mechanisms, such as the increase of oxidative mediators and reduction of oxidative enzymes, alteration in cell signaling pathways, induction of apoptosis, and the determination of mitochondria dysfunction (Meli et al., 2020). BPA has also been shown to decrease the production of the antioxidant enzymes superoxide dismutase, glutathione reductase, glutathione peroxidase, and catalase, which in turn aids in the development of oxidative stress (Meli et al., 2020). Studies conducted show that the structural analogues used to replace BPA also induce the formation of ROS such as •OH, which is highly reactive (Mokra et al., 2018).

The hydroxyl radical, •OH, is considered to be the major ROS that can react with DNA bases, free nucleotides, and deoxyribose (Mokra et al., 2018). These radicals are responsible for the most oxidative DNA damage (Nelson & Cox, 2012, p. 295). The by-products of lipids and

proteins generated from oxidation due to the exposure to BPA and its analogues have also been implicated in DNA damage, including the formation of DNA adducts (Mokra et al., 2018). This oxidative DNA damage has been related the aging process as well as the development of cancer and atherosclerosis (Mokra et al., 2018).

To determine oxidative damage, one study looked at damage from BPA-induced oxidative stress on biomacromolecules (e.g., nucleic acids, proteins, and lipids) (Huang et al., 2020). The markers of oxidative damage, 8-OHdG for nucleic acids, protein carbonyls for proteins, and malondialdehyde (MDA) for lipids, were increased in the presence of BPA (Huang et al., 2020; Peluso et al., 2016). This biomacromolecular damage, especially with DNA damage resulting from BPA exposure, is one of the pathways that induces cell apoptosis (Huang et al., 2020). This study also showed that BPAF induced greater biomacromolecular damage in KGN cells than BPA, indicating that it might not be a suitable replacement for BPA (Huang et al., 2020).

Etiologies Associated with BPA and BPA Analogue Exposure and Oxidative Stress

Reproductive Dysfunction

Reproductive dysfunction is one of the etiologies associated with BPA and BPA analogue exposure and oxidative stress. BPA has been shown to interfere with ovarian follicular development, which can affect fertility (Huang et al., 2020). Apoptosis due to biomacromolecular damage is a primary mechanism in the development of follicular atresia and developmental disorder of the ovary (Huang et al, 2020). Additionally, maintenance of intracellular calcium homeostasis plays an important role in ovarian follicular development (Huang et al., 2020). Increases in intracellular calcium have been implicated in follicular

development disorders and overall ovarian dysfunction (Huang et al., 2020). While increased levels of intracellular calcium have been seen in the presence of BPA and its analogues, research must still be conducted on whether follicular disorders are induced as a direct result of increased intracellular calcium levels resulting from exposure to these compounds (Huang et al., 2020).

Endogenous estrogen plays a vital role in sexual differentiation during the pubertal stage (Leonardi et al., 2017). However, the estrogen-like action of BPA and its analogues has been implicated in the pathogenesis of precocious puberty, menstrual irregularities, and polycystic ovary syndrome (Leonardi et al., 2017). These compounds can also affect the morphology and function of the male and female genital tract and mammary glands (Hassani et al., 2017). The mechanisms regarding the development of these pathogeneses are still largely unknown. However, it is thought that BPA and its analogues may trigger a positive feedback process for the activity of the gonadotropin-releasing hormone pulse generation, increasing the secretion of luteinizing and follicle-stimulating hormones, and inducing precocious puberty (Leonardi et al., 2017).

Diabetes

BPA has been documented in the interference of glucose metabolism (Song et al., 2014). While the exact mechanism is not known, the estrogenic and prooxidative properties of this compound are implicated. While much of this research was conducted in a rat model, higher urinary levels of BPA in human patients with type 2 diabetes have been shown (Song et al., 2014). BPA is also known to be estrogenic, increase insulin secretion from β-cells in the pancreas, as well as induce insulin resistance by interfering with insulin transduction of target cells (Song et al., 2014). Two indicators of oxidative stress are serum MDA, an end product of

lipid peroxidation and indicative of oxidative stress in cells and tissues, and superoxide dismutase (SOD), which is an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Song et al., 2014). BPA has also been shown to reduce adiponectin (ADP), which is another factor related to insulin resistance (Song et al., 2014).

Obesity

It is well known that obesity increases the risk of insulin resistance and oxidative stress, as well as its role as an underlying factor in the pathogenesis of several diseases (Marseglia et al., 2015). BPA has been indicated to increase the risk of obesity through its ability to change endocrine-metabolic pathways in adipose tissues (Kim et al., 2019). One study conducted by Song et al. (2014) showed that perinatal exposure to BPA induced significantly higher weights in male rat offspring through their lifespan in comparison to the control group. Other studies have shown that in both human and rat models that prenatal and early postnatal BPA exposure is associated with increased body weight (Song et al., 2014).

Proposed Experimental Design

Purpose

The purpose of this experiment was to determine whether Bisphenol A will induce oxidative stress in a HepG2-Luciferase (HepG2-Luc) cell culture. HepG2 is an immortalized cell line that consists of human liver carcinoma cells from the liver tissue of a 15 year old Caucasian male who had a well-differentiated hepatocellular carcinoma (“HepG2 in cell culture”, 2014). The cells were transformed with an antioxidant response element (ARE) promoter-driven luciferase reporter gene (BPS Bioscience, n.d.). The luciferase gene used was transfected from firefly luciferase (ExPASy Bioinformatics Resource Portal, 2017). In the presence of RONS, the

ARE will initiate the luciferase reporter gene expression (Xin et al., 2016b). Analysis of the quantitative luciferase activity can reflect the level of oxidative stress induced by the ligand (Xin et al., 2016b).

The ARE promoter is found in the Nrf2 (nuclear factor E2-related factor 2) antioxidant response pathway (Nguyen et al., 2009; BPS Bioscience, n.d.). This pathway is responsible for controlling the expression of genes that generate protein products that detoxify and eliminate reactive oxidants and electrophilic agents (Nguyen et al., 2009). Gene transcription through the action of the ARE is mediated primarily through Nrf2 (Nguyen et al., 2009). The activity of Nrf2 is largely regulated by the actin-associated Keap1 protein, through binding and tethering the transcription factor in the cytoplasm (Nguyen et al., 2009). However, in response to stress, Nrf2 is released from the Keap1 protein, allowing it to translocate to the nucleus for its transcriptional activity (Nguyen et al., 2009).

The HepG2 cell line was used in this experiment because of its integral role in gene regulation for protein products responsible for detoxifying and eliminating RONS and electrophiles (Nguyen et al., 2009). Additionally, all three forms of the estrogen receptor: ERα, ERβ, and the GPER are found in this cell line, which allows for better modeling of *in vitro* conditions (Meng & Zong, 2019). Because BPA is a weak estrogen receptor ligand, the binding of BPA to the estrogen receptor may induce oxidative stress, leading to the upregulation of the luciferase reporter (Meli et al., 2020).

The hypothesis tested in this experiment was that BPA induces oxidative stress in the HepG2-Luc cell line, quantified by a strong luciferase response. Estrogen was used as a control

for this assay. The following materials and methods were used with permission by Dr. Gregory Raner at Liberty University.

Materials

HepG2-Luc cells were used for the cell culture and were placed in BPS Bioscience Thaw Media in T25 cell culture flasks. 10% FBS DEM with 1× penicillin/streptomycin solution and 4.5 g/L glucose, L-glutamine, and sodium pyruvate from Corning Cellgro composed the cell culture medium. Trypsin and 50 mg/mL Gibco Geneticin were also used to supplement the cell culture medium.

For this experiment, the cell cultures were placed on a Corning I 96-well assay plate (tissue culture treated). Estrogen and BPA stocks were provided from the lab of Dr. Cameron Sheeler at Liberty University.

Methods

Preparation of Cell Culture

All tools used in the preparation of the cell culture were sterilized using 70% ethanol, and all steps involving the use of cell culture were performed in a biosafety hood in order to prevent contamination. Incubation was performed at 37ºC unless otherwise noted.

Preparation of the cell culture began by the removal of the frozen HepG2-Luc cells from vapor phase nitrogen. The cells were then thawed using a combination of a water bath and friction. The thaw media was placed in a 37ºC water bath to warm, and then 1 mL of thaw media was added to the thawed HepG2-Luc cells. 8 mL of the thaw media was then added to the HepG2-Luc cells in a 15 mL control tube. The now-suspended cells were plated in a T25 flask, labelled, and placed in the incubator.

24 hours after the primary plating took place, the thaw media was removed and replaced with 10 mL of fresh 10% FBS DMEM that was warmed to 37ºC, and the flask was placed back into the incubator. Additionally, the cells were analyzed under a dissecting microscope to determine their confluence. 48 hours after initial plating, the old media in the T25 flask was removed, replaced with 6 mL of 37ºC 10% FBS DMEM, and then returned to the incubator. 72 hours after the initial plating, trypsin and 10% FBS DMEM media was warmed to 37ºC in a water bath. The old media was removed from the original T25 flaks, and 1 mL of trypsin was added to the flask and allowed to incubate for 2 minutes. In a separate 15 mL conical tube, 11 mL of 10% FBS DMEM was combined with 140 µL of 50 mg/mL of Geneticin, diluting the solution to 0.6 mg/mL. The flask containing the original cell culture and trypsin was hit to dislodge the cells, and then the trypsin/cell culture mixture was added to the 11 mL mixture of DMEM and trypsin. The new cell suspension was mixed by inversion, and 6 mL of the cell suspension was transferred into each of two new T25 flasks, labelled, and then placed back in the incubator.

Estrogen and BPA Treatment

Estrogen and BPA stocks were prepared in the lab of Dr. Cameron Sheeler at Liberty University. All stocks of BPA and estrogen were prepared in DMSO, and in order to promote cell integrity, the stocks were added to the cell media in a 1:50 ratio. For this experiment, 2 µL of estrogen or BPA stock will be added to 100 µL of media and incubated for 24 hours. The final concentrations of estrogen used were in between $1x10^{-6}$ M and $3x10^{-10}$ M. The final concentrations of BPA used were between $1x10^{-4}$ M and $3x10^{-8}$ M.

Luciferase Assay

Media was removed from the 96-well plate, and 40 µL/well of media without FBS was added. In a separate tube, 40 µL of luciferin substrate was mixed with 4 mL of reaction buffer. 40 µL of substrate/buffer was then added to each well containing cells in the 96-well plate. The plate was shaken for 5-15 minutes at room temperature and then analyzed with the plate reader.

Results

One possible outcome of the experiment would show that BPA induces oxidative stress in a manner that exceeds that of estrogen. This would indicate that BPA does indeed induce oxidative stress via this pathway, and this could implicate its role in the harmful production of ROS. Another result could show that BPA induces the same amount of oxidative stress when compared to estrogen, or that it induces less than estrogen. This would indicate that BPA does not produce ROS in a manner that would be harmful for the cells. The results of these experiments did show that BPA did not induce an oxidative stress response greater than that of estrogen. In fact, neither BPA nor estrogen produced an oxidative stress response using this assay. However, more experimentation should be performed in order to support the results obtained by this experiment.

In the future, this experiment would have to be performed multiple times and produce similar results each time to determine its efficacy. The concentrations of estrogen and BPA used may also have not been in the correct range to elicit oxidative stress. To determine the correct concentrations to be used, a broad dose-response assay should be performed initially. Other common BPA structural analogues should also be tested using this method to ensure that a comprehensive analysis of the oxidative stress produced using this pathway is completed.

Additionally, if the results of this assay do not point to the overproduction of ROS and induction of oxidative stress, it does not necessarily mean that BPA and its analogues do not induce oxidative stress. An overproduction of ROS may not occur from this pathway, but there are other mechanisms available for the induction of oxidative stress.

Conclusion

Ultimately, the usage of BPA and its analogues must be more carefully analyzed in order to determine their health risks. The contribution BPA exposure and increased RONS and their implications in cell and tissue damage as well as the formation of many etiologies should not be ignored. This analysis is only a small window into the complex mechanisms of RONS and oxidative stress, and their roles need to be more clearly elucidated.

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