IN VITRO AND *IN VIVO* EFFECTS OF BISPHENOL A ON CELL GROWTH, LIPID ACCUMULATION AND INFLAMMATION IN LIVER CELL LINE, RAT ADIPOSE AND COLORECTAL TISSUES

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by

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LIST OF SYMBOLS

g	Gram
mol	Mole
Kg	Kilogram
℃	Degree Celsius
ppm	Parts per million
%	Percent
ng	Nanogram
mL	Milliliter
µg	Microgram
bw	Body weight
mg	Milligram
L	Litre
µM	Micromolar
mM	Millimolar
min	Minute
v/v	Volume per volume
x g	Times gravity (relative centrifugal force)
nM	Nanomolar
M	Molarity
α	Alpha
γ	Gamma
β	Beta
δ	Delta

LIST OF ABBREVIATIONS

BPA	Bisphenol A
CYP	Cytochrome 450
C/EBP	CCAAT/enhancer-binding proteins
DLK	Protein delta homolog
IFG1	Insulin Like Growth Factor 1
PPARγ	Peroxisome proliferator-activated receptor gamma
LPL	Lipoprotein Lipase
THLE-2	Transformed Human Liver Epithelial-2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
UCP1	Uncoupling protein 1
BRS3	Bombesin receptor subtype 3
IL	Interleukin
CCL2	C-C Motif Chemokine Ligand 2
PPI	Protein-Protein Interaction
PPARα	Peroxisome-proliferator-activated receptor alfa
PPARβ/δ	Peroxisome-proliferator-activated receptor beta/delta
NHANES	National Health and Nutrition Examination Survey
CDC	Centre for Disease Control
TDI	Tolerated daily intake
WHO	World Health Organization
FDA	Food and Drug Administration
LOAEL	Lowest observable adverse effect level
EPA	Environmental Protection Agency
pH	Potential of hydrogen
UGTs	UDP-glycosyltransferase
SULTs	Sulfotransferases
DNA	Deoxyribonucleic acid
GPR30	G-protein coupled receptor 30
ERα	Estrogen Receptors Alpha
ERβ	Estrogen Receptors Beta
TNF	Tumor necrosis factor

CD36	Cluster of differentiation 36
FABP	Fatty acid-binding protein
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
TG	Triglyceride
LDL	Low-density lipoprotein
VLDL	Very low-density lipoprotein
TNF	Tumor necrosis factor
IFN	Interferons
HDL	High-density lipoprotein
TC	Total cholesterol
TBARS	Thiobarbituric acid reactive substances
NO	Nitric oxide
CAT	Catalase
GSH	Glutathione
SOD	Superoxide dismutase
ROS	Reactive oxygen species
mERs	Membrane estrogen receptors
IBDs	Inflammatory bowel diseases
UC	Ulcerative colitis
CD	Crohn's disease
CRC	Colorectal cancer
PPRE	Peroxisome proliferator-responsive element
RXR	Retinoid-X-receptor (RXR)
NCOR1	Nuclear receptor corepressor 1
LBD	Ligand binding domain
DBD	DNA binding domain
PND	Postnatal depression
PBS	Bovine pituitary extract
hEGF	Hydrocortisone, human epithelial growth factor
FBS	Fetal bovine serum
EGF	Epidermal growth factor
DMSO	Dimethyl sulfoxide
ATCC	American type culture collection

CO_2	Carbon dioxide
RNA	Ribonucleic acid
DF	Dilution factor
TBE	Tris-borate-ethylenediaminetetraacetic acid
EtBr	Ethidium bromide solution
PCR	Polymerase chain reaction
UV	Under ultraviolet
dNTP	Deoxyribonucleotide triphosphate
cDNA	Complementary DNA
BSA	Bovine serum albumin
HCl	Hydrochloric acid
BrdU	Bromodeoxyuridine/ 5'-bromo-2'-deoxyuridine
PI	Propidium Iodide
FITC	Fluorescein isothiocyanate
IPPT	Advanced Medical and Dental Institute
RIPA	Radioimmunoprecipitation assay
SDS	Sodium dodecyl sulfate
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
UPM	Universiti Putra Malaysia
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NASH	Nonalcoholic steatohepatitis
NAFLD	Non-alcoholic fatty liver disease
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
XRE	Xenobiotics response element
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
GO	Gene ontology
MCF-7	Michigan Cancer Foundation-7
HCT116	Human colorectal carcinoma-116
HeLa	Henrietta cells
GPER	G protein-coupled estrogen receptor
ERFR	The epidermal growth factor receptor
ERK1	Extracellular signal-regulated kinases

IGF-1R	Insulin Like Growth Factor 1 Receptor
VIM	Vimentin
CXCL12	C-X-C motif chemokine 12
TWIST	Twist-related protein
JAK/STAT	Janus kinase/signal transducers and activators of transcription
ZEB	Zinc Finger E-Box Binding Homeobox
GSK	Glycogen synthase kinase
MMP	Matrix metalloproteinase
PI3K/AKT	Hosphoinositide-3-kinase
SnoN	Ski novel
APLN	Apelin
MMPs	Matrix metalloproteinases
FOXA1	Forkhead in rhabdomyosarcoma
BCL2L11	BCL-2 Interacting Mediator of cell death
BAX	BCL2-associated X protein
PDCD5	Programmed cell death 5
MAPK/ERK	Mitogen-activated protein kinases /extracellular signal-regulated kinases
ND	Normal diet
HFD	High-fat diet
V	Vehicle

LIST OF APPENDICES

KESAN *IN VITRO* DAN *IN VIVO* BISFENOL A TERHADAP PERKEMBANGAN SEL, PENGUMPULAN LIPID DAN KERADANGAN DALAM TITISAN SEL HATI, TISU ADIPOS DAN KOLOREKTAL TIKUS

ABSTRAK

Bisfenol A (BPA) ialah pengganggu endokrin eksogen yang meniru hormon berkait rapat dengan komplikasi kesihatan, seperti perkembangan kanser. BPA juga dikaitkan dengan peningkatan kelaziman obesiti dan penyakit yang berkaitan obesiti kerana tindakan obesogeniknya. Kajian ini bertujuan untuk menilai kesan BPA pada sel hati manusia dan tisu haiwan. Daya tahan sel THLE-2 yang dirawati BPA mula-mula dinilai dengan menggunakan ujian MTT. Pengumpulan lipid sitoplasma kemudiannya diperiksakan dalam sel THLE-2 yang dirawati BPA dengan menggunakan pewarnaan *Oil Red* O. Ekspresi pelbagai gen adipogenesis pada metabolisme lipid, termasuk klusterin, PPARs, CYPs, UCP1, dan BRS3 dalam sel THLE-2 yang dirawati BPA, disiasatkan dengan menggunakan masa-nyata PCR. Selepas itu, sitometri aliran mengukurkan sintesis DNA dalam sel THLE-2 yang dirawati BPA dengan menggunakan pewarnaan BrdU/PI. Protein pro-radang IL6 dan CCL2 dalam media terkondisi sel THLE-2 yang dirawati BPA diperiksakan oleh ELISA. Setelah itu, pelbagai ekspresi gen adipogenesis pada metabolisme lipid, termasuk klusterin, PPARs, CYPs, UCP1, dan BRS3, dalam tisu adiposa dan kolorektal dalam tikus yang diberikan diet (normal atau berlemak tinggi) yang ditambah BPA dan tanpa BPA juga disiasatksn dengan menggunakan masa-nyata PCR. Akhirnya, rangkaian PPI gen yang dikajikan secara *in vitro* dan *in vivo* dianalisis dengan menggunakan pangkalan data STRING. Keputusan menunjukkan bahawa BPA mengurangkan daya maju sel dengan ketara dan meningkatkan pengumpulan

lipid dalam sel THLE-2. Fenomena ini menunjukkan bahawa sel THLE-2 mungkin mengalami pembezaan sel selepas rawatan BPA. Klusterin, PPARα, PPARγ, dan CYP2S1 adalah gen utama yang terlibat dalam perencatan pertumbuhan, pengumpulan lipid dan tindak balas keradangan sel THLE 2 selepas rawatan BPA. Perencatan ekspresi mRNA CYP1A1 dan CYP1B1 menunjukkan bahawa sel THLE-2 yang dirawati BPA mengurangkan kapasiti untuk metabolisme karsinogen, manakala ekspresi mRNA BRS3 yang meningkat dalam sel THLE-2 menunjukkan hubungan gen dengan gangguan metabolik manusia. Selain itu, BPA menghalang sintesis DNA dalam sel THLE-2 yang dirawati BPA. Di samping itu, kajian mendapati bahawa tahap ekspresi protein pro-radang, IL6 dan CCL2, meningkat dalam media terkondisi sel THLE-2 yang dirawati BPA. Untuk model haiwan, ekspresi mRNA klusterin, PPARα, PPARγ, PPARβ/δ, UCP1, CYP1A1, CYP1B1 dan CYP2S1 meningkat dalam tisu adiposa tikus yang diberikan diet berlemak tinggi yang ditambah BPA. Begitu juga keputusan menunjukkan bahawa ekspresi mRNA klusterin, PPARα, PPARγ, PPARβ/δ, CYP1A1, CYP1B1 CYP2S1, UCP1 dan BRS3 yang telah meningkat dalam tisu kolorektal tikus yang diberikan diet (normal atau berlemak tinggi) yang ditambah BPA. Sebaliknya, analisis bioinformatik mendedahkan bahawa interaksi langsung antara PPARs, CYPs, UCP1, IL6, dan CCL2 memainkan peranan penting dalam pembezaan sel dan keradangan yang membawa kepada gangguan metabolik, termasuk obesiti dan perkembangan kanser. Kesimpulannya, kajian ini menunjukkan bahawa BPA mempengaruhi pertumbuhan sel dengan menghalang sintesis DNA, mendorong pengumpulan lipid selular dan keradangan, serta mengekspresikan gen adipogenesis dalam sel dan model haiwan.

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ABSTRACT

Bisphenol A (BPA) is an exogenous endocrine disruptor that mimics hormones closely associated with health complications, such as cancer progression. BPA is also linked to an increase in the prevalence of obesity and obesity-related diseases due to its obesogenic action. The present study aimed to evaluate the effects of BPA on human liver cells and animal tissues. The BPA-treated THLE-2 cell viability was first evaluated using an MTT assay. Cytoplasmic lipid accumulation was then examined in the BPA-treated THLE-2 cells using Oil Red O staining. The expression of various adipogenesis genes in lipid metabolism, including clusterin, PPARs, CYPs, UCP1, and BRS3 in BPA-treated THLE-2 cells, was investigated using real-time PCR. Subsequently, flow cytometry measured the DNA synthesis in BPA-treated THLE-2 cells was performed using BrdU/PI staining. The pro-inflammatory proteins IL6 and CCL2 in the conditioned media and cytoplasmic proteins of the BPA-treated THLE-2 cells were examined by ELISA. After that, various adipogenesis genes' expression in lipid metabolism, including clusterin, PPARs, CYPs, UCP1 and BRS3, in adipose and colorectal tissues of the rats fed a diet (normal or high fat) supplemented with and without BPA was investigated using real-time PCR. Finally, the PPI networks of the genes studied *in vitro* and *in vivo* were analysed using the STRING database. The results revealed that BPA significantly reduced cell viability and increased lipid accumulation and inflammatory response in THLE-2 cells. The clusterin, PPARα, PPARγ and CYP2S1 are likely the primary genes involved in the growth inhibition,

lipid accumulation and inflammatory response of the THLE-2 cells following BPA treatment. Inhibition of CYP1A1 and CYP1B1 mRNA expression implied the decreased capacity of BPA-treated THLE-2 cells for carcinogen metabolism, while up-regulated BRS3 mRNA expression in THLE-2 cells showed a connection of the gene with human metabolic disorders. Moreover, BPA inhibited DNA synthesis in the BPA-treated THLE-2 cells. In addition, the study found that the expression level of pro-inflammatory proteins, IL6 and CCL2, were increased in the conditioned media and cytoplasmic proteins of the BPA-treated THLE-2 cells. For the animal model, the mRNA expressions of clusterin, PPARα, PPARγ, PPARβ/δ, CYP1A1, CYP1B1, CYP2S1 and UCP1 were up-regulated in the adipose tissue of the rats fed a high-fat diet supplemented with BPA. Similarly, the result shows that the mRNA expressions of clusterin, PPARα, PPARγ, PPARβ/δ, CYP1A1, CYP1B1 CYP2S1, UCP1 and BRS3 were increased in the colorectal tissue of the rats fed with a diet (normal or high-fat) supplemented with BPA. On the other hand, the bioinformatics analysis revealed that direct interaction between PPARs, CYPs, UCP1, IL6 and CCL2 plays an important role in cell differentiation and inflammation that leads to metabolic disorders, including obesity and cancer progression. In conclusion, the study demonstrates that BPA influences cell growth by inhibiting DNA synthesis, inducing cellular lipid accumulation and inflammation and expressing adipogenesis genes in the cells and the animal model.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Bisphenol A (BPA) or 4,4′-(Propane-2,2-diyl) diphenol is one of the most abundant environmental chemicals to which individuals are widely exposed worldwide (Quesnot et al., 2014). BPA is an exogenous endocrine disruptor associated with human health complications. BPA is a solid crystal, organic, colourless compound with a phenolic odour under ambient conditions, with chemical formula $C_{15}H_{16}O_2$ and a molecular weight of 228.29 g/mol. BPA is highly soluble in lipids and weakly soluble in water. BPA is primarily used to synthesise plastic, epoxy resin, thermal paper, dental sealant, flame retardant, baby bottles and lacquer coatings for metal products (Bertoli et al., 2015). Numerous studies have indicated that BPA easily contaminates food and drinks, such as heating, under certain conditions. During the fabrication, degradation and treatment of BPA-containing materials, BPA is released into the environment (Peyre et al., 2014; Eweda et al., 2019). It easily enters the human body through the respiratory and digestive tracts and is absorbed via the skin (Peyre et al., 2014). BPA release from food products is increased by heating, contact with acid and alkaline substances, exposure to microwaves and repeated use of plastic containers which contain BPA (Ohore & Songhe, 2019). According to the magnitude value analysis, daily human exposure to BPA ranges from 0.4 to 1.4 g/Kg body weight/day (Geens et al., 2012). BPA is also a xenobiotic endocrine disruptor chemical. In mammary glands, xenobiotic-metabolising enzymes metabolise BPA via two pathways: glucuronidation and sulfation (Jalal et al., 2018). Cytochrome P450 (CYP) enzymes are key enzymes in the liver that mediate the metabolism of BPA into BPA-o-quinone and BPA-semiquinone (Kang et al., 2006; Kourouma et al., 2015).

Several studies have indicated that BPA also mimics hormones like estrogens and androgens. Epidemiological studies confirm positive correlations between BPA exposure and the occurrence of hepatotoxicity, cardiotoxicity, type 2 diabetes, obesity and cancer (Rochester, 2013; Chen et al., 2015). BPA is a lipophilic chemical that accumulates preferentially in adipose tissues (Szkudelska et al., 2021). Adipose tissue produces a variety of biologically active molecules, serving as an endocrine organ (Fasshauer & Blüher, 2015). BPA may promote the differentiation of adipose cells. It also changes the expression of adipogenic genes, including C/EBP, DLK, IFG1, PPARγ and LPL (Ohlstein et al., 2014; Ariemma et al., 2016; Akash et al., 2020). Mechanistically, BPA induces adipogenesis and inflammation of adipose tissue, which contribute to the pathophysiology of obesity (Legeay & Faure, 2017; Engin & Engin, 2021). In this regard, BPA exposure stimulates the proliferation and differentiation of adipocytes and their lipid storage capacity (Desai et al., 2018).

This study investigated the effects of BPA on human liver cells (THLE-2) and animal tissue. Initially, the viability of BPA-treated THLE-2 cells was assessed using the MTT assay. Subsequently, the expression of genes (clusterin, PPARs, CYPs, UCP1 and BRS3) in BPA-treated THLE-2 cells along with adipose and colorectal tissues of Sprague Dawley rats were measured by real-time PCR. In addition, cytoplasmic lipid accumulation in BPA-treated THLE-2 cells was determined. DNA synthesis in BPA-treated THLE-2 cells was also measured using flow cytometry. The enzyme-linked immunosorbent assay was used to observe the expression of IL6 and CCL2 proteins in BPA-treated THLE-2 cells. Finally, the PPI network and gene ontology on the above genes was performed. The present results are important to raise awareness among the public and policy-makers on general BPA use in the food and

beverage industry and to provide a foundation for developing strategies to curb the high prevalence of obesity and obesity-related disease.

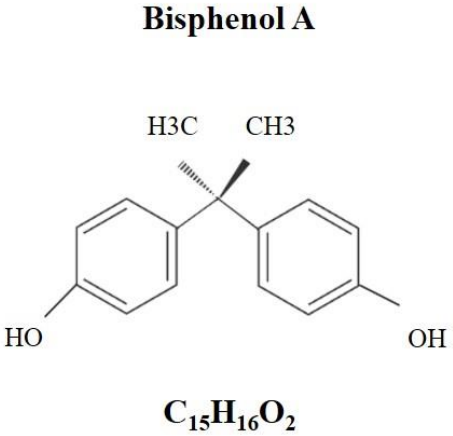


Figure 1.1 Chemical structure of bisphenol A. The structure was plotted with ChemDraw.

1.2 Research questions

BPA is ubiquitous in epoxy resins and polycarbonate plastic commonly used in manufacturing water bottles or food containers. BPA is found to have entered the human food chain from the BPA containers, which are placed in microwaves for extended periods or exposed to vegetable oil and/or sodium chloride solutions. The prevalence of obesity and obesity-related disease is increasing in Malaysia now. However, whether BPA-induced lipid accumulation or obesity is a risk factor for obesity-related diseases remains unclear. Other research questions to be answered in the present study include the following: -

- Will BPA also affect the obesity-related gene expression at mRNA and protein levels in liver cells?
- What has been altered in adipose and colorectal tissues after chronic exposure of rats to BPA?

- How does a high-fat diet in combination with BPA induce obesity and obesity-related diseases?

1.3 Problem statements

BPA is a common environmental chemical utilised in a wide range of products, including plastic bottles and components of life-saving medical devices like incubators and renal dialysis machines. Epoxy resins, known for their durability, are typically employed in coating food and beverage cans, which contribute to preserving the quality and security of our food supply (Bacle et al., 2019). It is concerning that BPA leaches into the contents of food and beverage containers manufactured with BPA under certain conditions, such as heating. Previous scientific evidence indicates that population-wide health risks associated with BPA exposure are negligible (Vom Saal & Vandenberg, 2021). Due to its adverse effects and potential health risks to humans and organisms, some nations have limited the use of BPA in certain applications. BPA-related health consequences are complex and controversial. BPA is known to be associated with a number of adverse health effects, including obesity, diabetes and cancers. In animal models, BPA has multi-system and multi-organ toxicity (Ma et al., 2019). Nonetheless, many questions regarding the consequences of BPA exposure on humans still need to be answered.

Investigations into the definitive processes by which BPA acts as an obesogen remain in their infancy. Although BPA has been shown to affect adipogenesis and adipocyte differentiation in animal models, its effect on these processes *in vitro* has only been shown in a small number of investigations. Recent research indicates that PPARγ, hormone interference and inflammation significantly influence obesogenic consequences (Griffin et al., 2020). However, only one study used the human liver

HL-7702 cells as an *in vitro* model to study the effect of BPA on PPARs (Li et al., 2021). It is essential to investigate the influence of BPA on adipogenesis-related gene expression to understand obesity better. The BPA effects on PPARα and PPARβ/δ genes related to obesity remain unexplored. In addition, the influence of BPA on metabolic enzymes (CYPs) has rarely been studied. *in vivo*, studies have shown that exposure to BPA increases the risk of obesity by disrupting the normal mechanism of the endocrine signalling pathway (also known as the metabolic pathway) in adipose tissue. Specifically, BPA can influence adipose tissue and increase fat cell numbers or sizes by regulating the expression of genes that play a pivotal role in metabolic homeostasis and obesity. Although BPA is an obesogen in animal models, its effects on clusterin, PPARs, CYPs, UCP1 and BRS3 in adipose and colorectal tissues have not been explored.

Obesity is a major worldwide health issue that has not only been associated with diabetes mellitus and cardiovascular disease, but it is also a major risk factor for developing cancers. Research studies showed that excess body fat increases the risk of several cancers, including colorectal, breast, kidney and pancreatic cancers (Pati et al., 2023). Despite intensive efforts, the detailed understanding of BPA- and obesity-related cancers is still under investigation.

1.4 Objectives

The general objective of this study is to determine the *in vitro* and *in vivo* effects of BPA on human liver (THLE-2) cells and animal tissue. The specific objectives of this study are:

- To analyse the lipid accumulation induced by BPA in THLE-2 cells.
- To decipher the effects of BPA on clusterin, PPARs, CYPs, UCP1 and BRS3 gene expression at mRNA level in BPA-treated liver cells.
- To detect the BrdU incorporation into newly synthesised DNA in BPA-treated liver cells.
- To determine the effects of BPA on the levels of secreted and cytoplasmic proinflammatory proteins (IL6 and CCL2) in BPA-treated THLE-2 cells.
- To decipher the effects of BPA on clusterin, PPARs, CYPs, UCP1 and BRS3 gene expression at mRNA level in adipose and colorectal tissues of the rats fed with a high-fat diet containing BPA.
- To study the PPI network and gene ontology on the above genes.

CHAPTER 2

LITERATURE REVIEW

2.1 Bisphenol A

Bisphenol A (BPA) is one of the most widely produced chemicals in the world. BPA is manufactured industrially through the condensation reaction of phenol and acetone (Figure 2.1; Chruściel et al., 2019), It is a colourless solid chemical that is soluble in organic solvents, but poorly soluble in water (Table 2.1; Ohore & Songhe, 2019). BPA plays a vital role in our daily lives, including as one of the raw materials used to manufacture polycarbonate plastics and epoxy resins (Wang et al., 2020). BPA was first synthesised in 1891 by a Russian chemist, A. P. Dianin, and its potential commercial use was investigated in the 1930s during the search for synthetic estrogens (Jalal et al., 2018). In the 1940s and 1950s, the plastics industry identified more BPA uses. BPA is a polycarbonate plastic component commonly used in food and beverage containers, such as baby bottles and as an additive in other plastics (Wang et al. 2020). BPA is also a component of some dental materials, including dental sealants, the lining of food and beverage containers and numerous other products (Fleisch et al., 2010). Additional uses for BPA include coating CDs, DVDs, electrical and electronic equipment, automobiles and sports safety equipment. The items come into contact daily at home and in the workplace (Lim et al., 2022). Polymerised BPA molecules are connected by ester bonds that are susceptible to hydrolysis when exposed to high temperatures, acids, or bases (Siddique et al., 2021). Studies have demonstrated that BPA can leach from polycarbonate plastics, epoxy resins and other products that come into contact with food and drink. Consequently, it is assumed that humans consume BPA routinely (Thoene et al., 2017).

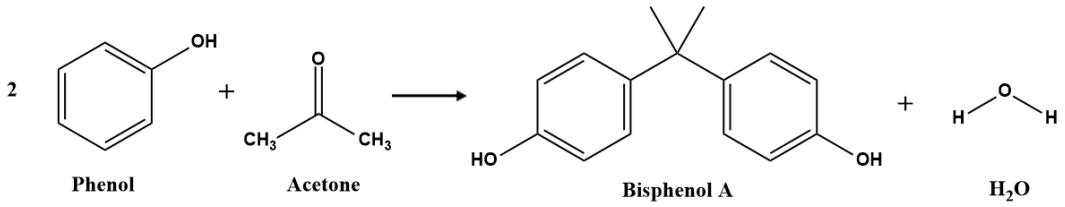
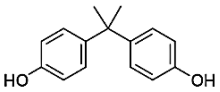


Figure 2.1 Synthesis of bisphenol A from phenol and acetone. The structure was plotted with ChemDraw (Chruściel et al., 2019).

Table 2.1 Physicochemical characteristics of bisphenol A (Ohore & Songhe, 2019).

Properties	**Values**
Chemical structure	
IUPAC name	4,4′-(Propane-2,2-diyl) diphenol
Molecular formula	$C_{15}H_{16}O_2$
Appearance	White solid
Odour	Mild phenolic
Molecular weight	228.29 g/mol
Melting point	158-159℃
Boiling point	220℃
Solubility (15℃)	120-300 ppm

Given the prevalence of BPA in our environment, it is unsurprising that the majority of individuals examined to date have detectable levels. BPA was found in humans' urine, blood, placenta, breast milk and umbilical cord serum (Lee et al., 2018). The National Health and Nutrition Examination Survey (NHANES) and Centre for Disease Control (CDC), revealed detectable levels of BPA in the urine samples of 92.6% of more than 2,500 participants. The reported adjusted mean BPA levels were between 2.5-4.5 ng/ml in children (6-11 years old), adolescents (12-19 years old) and adults (over 20 years of age) (Rubin, 2011; Lee et al., 2018).

Some countries have restricted the use of BPA in the application due to its possible health dangers to humans and other organisms (Meng et al., 2018; Usman et al., 2019). In France, the use of BPA in any food or beverage packaging has been forbidden since January 2015 (Inadera, 2015). BPA has been banned in all food contact materials in Europe since 2011-2015 (Hoepner et al., 2015; Legeay & Faure, 2017). In 2012 and 2014, the Food and Drug Administration (FDA) in the United States banned BPA in items intended for newborns and toddlers (Ma et al., 2019). In order to avoid potential dangers, Canada was the first nation to declare BPA a toxic substance and mandate its removal from all infant formula bottles in 2010 (Inadera, 2015; Manzoor et al., 2022). Despite an official ban on baby bottles in Malaysia in 2002, BPA is still present in other food containers and beverage packaging (Mahamuni & Shrinithivihahshini, 2017). Health organisations, such as the World Health Organisation (WHO), the FDA, the United States Department of Health and Human Services and the Centres for Disease Control and Prevention have expressed concern about its use (Michałowicz, 2014; Ohore & Songhe, 2019).

In the 1980s, 50 mg/kg body weight per day was established as BPA's lowest observable adverse effect level (LOAEL). The US Environmental Protection Agency (EPA) identified a "reference dosage" or the safe dose of 50 µg BPA/kg body weight per day (Rubin & Soto, 2009). Since then, numerous animal studies have demonstrated severe consequences of exposure to doses of BPA below the predicted safe values, especially in response to foetal, perinatal and neonatal exposure. Recent research, however, has revealed that even this 'safe' level of BPA can have profound impacts. Despite the European Food Safety Authority reducing the tolerable daily intake of BPA to 0.4-4 µg/kg body weight per day after thoroughly considering the US (Geens et al., 2012). Nonetheless, the "safe" level of BPA remains controversial. Given that most

humans are exposed to low doses of BPA daily, it is crucial to evaluate the long-term health implications of exposure to a 'safe' dosage of BPA, the so-called Tolerable Daily Intake (TDI), which is currently set at 50 µg/kg/day (Chen et al., 2017; Vom Saal & Vandenberg, 2021).

The endocrine disruptor BPA has been linked to various harmful effects in animal studies due to its ability to imitate estrogen. Bisphenol A can particularly bind to and activate the same estrogen receptor as the natural hormone estradiol because its structure and function closely mimic estradiol. BPA's estrogenic action was found by accident. A study has reported that yeast produces estrogen (Ribeiro et al., 2017). The study discovered that the conditioned media's estrogenic material had leached from the polycarbonate flasks during autoclaving. It turned out that estrogen was leaching from the plastic rather than being produced by yeast. The team then experimented without yeast in the flask and discovered that this estrogenic molecule was still present in the medium, which they later identified as BPA. They discovered that it originated from the plastic flask and was absent when the experiment was conducted with glass flasks (Ribeiro et al., 2017). Nevertheless, BPA was 3 to 4 orders of magnitude less potent than estradiol (Lemmen et al., 2004). Importantly, BPA has numerous harmful consequences, including interaction with the endocrine system. BPA in low quantities over long periods can induce liver disease, diabetes, obesity, some types of cancer and various other illnesses (Kharrazian, 2014; Cimmino et al., 2020).

2.1.1 Human exposure and metabolism of BPA

There are numerous routes of human exposure to BPA, including ingestion, inhalation and dermal absorption (Konieczna et al., 2015). Due to the widespread availability of BPA in the environment, all pathways of exposure must be investigated to identify the substance sources (Legeay & Faure, 2017). Human exposure to BPA

occurs primarily through diet, contaminated water and air (Figure 2.2; Tarafdar et al., 2022).

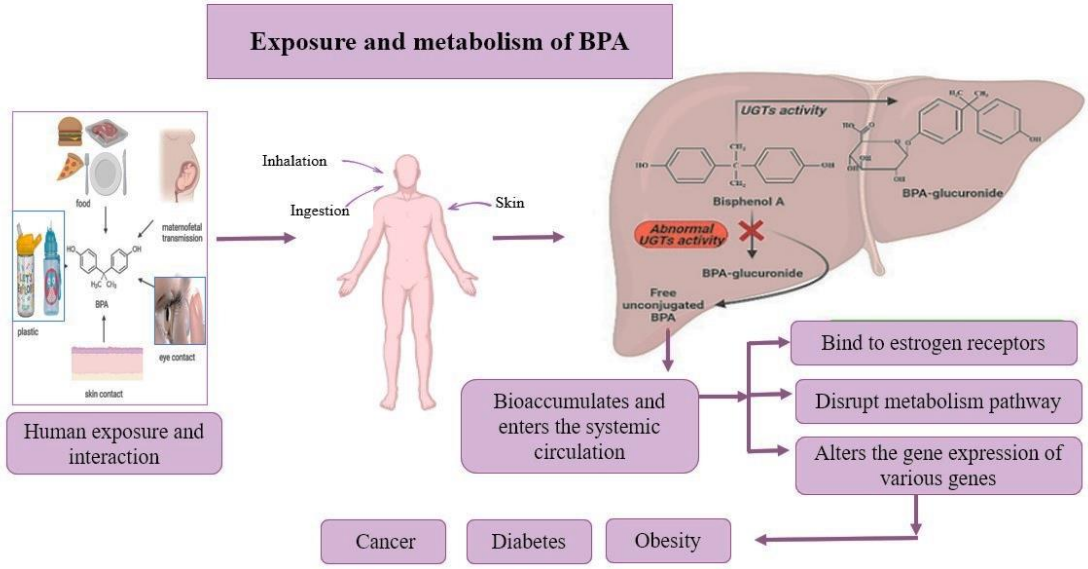


Figure 2.2 Bisphenol A exposure to humans and its metabolism (Mukhopadhyay et al., 2022).

Through the everyday usage of tin cans, infant bottles, reusable plastic water bottles, and polycarbonate plastic containers, BPA can leach into food and beverages. The rate of BPA leaching increases when polycarbonate is scratched or discolored (Ginter-Kramarczyk et al., 2022). Heat and non-neutral pH conditions (either acidic or basic) are two factors that influence BPA release, as hydrolysis of the ester bond connecting BPA monomers occurs with changes in temperature and pH, such as those that occur when BPA-containing plastics are cleaned with harsh detergents or contain acidic or high-temperature liquids (Gao et al., 2015).

BPA is absorbed by the gastrointestinal tract and metabolised by glucuronidation or sulfonation in the liver. The three major kinds of metabolising enzymes are known as phase 1 (oxidation), phase 2 (conjugation) and phase 3 (transport) enzymes (Figure 2.3; Quesnot et al., 2014). *in vivo* and *in vitro* systems have been used to investigate BPA metabolism. Ingested BPA undergoes first-pass processing in the liver and/or intestine, severely limiting its systemic bioavailability (Nachman et al., 2014; Gayrard et al., 2019). The liver enzyme known as UDP-glucuronosyltransferases 2B15 (UGTs) is responsible for the glucuronidation of BPA, which is then followed by its excretion from the body in the form of BPA glucuronide either through bile or urine (Khan et al., 2021). The abnormalities in the UGT enzyme cause an increase in the concentration of unconjugated BPA in the body. Numerous studies have documented the presence of unconjugated forms of BPA in human body fluids, including breast milk, maternal urine, amniotic and placental fluids, as well as in neonates, corresponding with numerous hormonal abnormalities (Inadera, 2015).

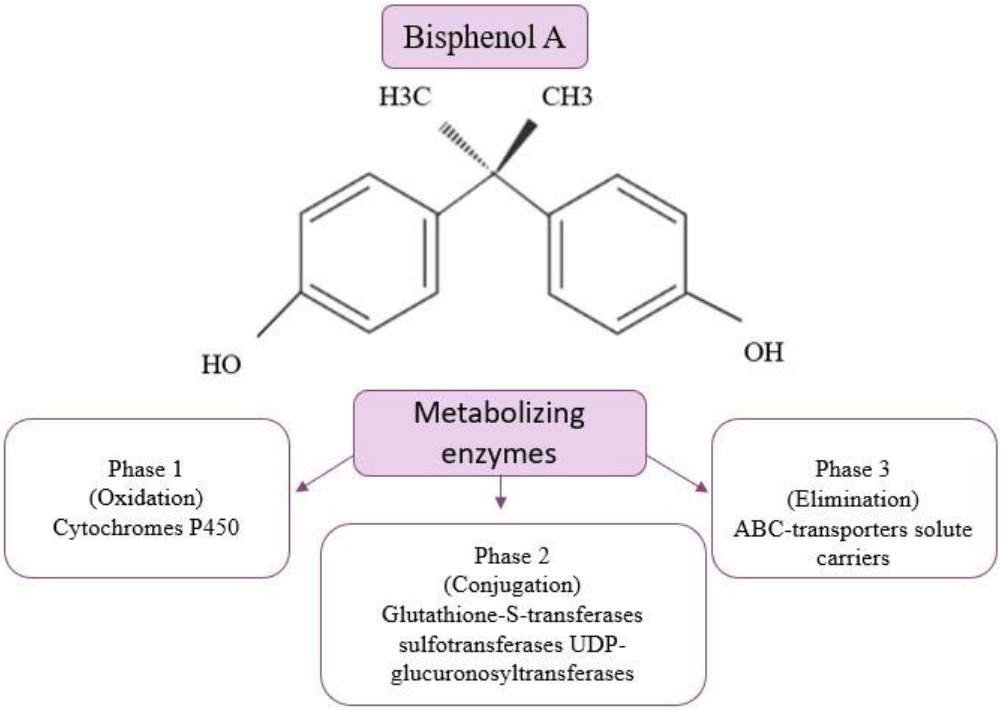


Figure 2.3 Categories of metabolising enzymes (Quesnot et al., 2014).

On the other hand, the metabolism of xenobiotics also involves phase I metabolism, which is carried out in the liver by cytochrome P450 (Quesnot et al., 2014; Kourouma et al., 2015; Hoque et al., 2021). CYP enzymes comprise a superfamily of heme-containing enzymes in phase I of biotransformation (Ghose, 2012). The majority of CYP enzymes in the human body are predominantly expressed in the liver. However, the enzymes are also found in the small intestine, heart, lungs, placenta, kidneys and other organs (Zanger & Schwab, 2013). There are around sixty CYP enzymes in the human genome. CYP enzymes are crucial for detoxifying foreign substances (xenobiotics), including carcinogens, steroids and the metabolism of numerous drugs (Zanger & Schwab, 2013). Human CYP enzymes are involved in the biotransformation of the majority of xenobiotics, including almost two-thirds of all clinically used drugs. BPA is metabolised to DNA-reactive bisphenol-o-quinon via 5-hydroxybisphenol and

bisphenol semiquinone. CYP inhibitors significantly reduce the formation of DNA adducts in a microsomal activation system, indicating that CYPs are closely associated with the metabolism and toxicity of BPA (Kourouma et al., 2015; Tomankova et al., 2017).

2.1.2 Mechanism and action of BPA

The potential mechanisms at the root of BPA's effects are another topic of considerable controversy in the study of BPA's activity. BPA is responsible for activating several different action mechanisms, some of which include the interference with the activity of nuclear receptors, noncanonical steroid hormone receptors and orphan receptors (Acconcia et al., 2015). In addition, enzymatic pathways that are involved in steroid production and/or metabolism, as well as a multitude of other processes that converge upon endocrine and reproductive systems, have been postulated as explanations for the activities of BPA (Yoon et al., 2014). It is unknown whether all of these mechanisms or just a few of the mechanisms concur with the nonmonotonic curves of BPA's propensity to disrupt hormones. Based on the relative binding affinity of BPA for the classical nuclear receptors estrogen receptors alpha (ERα) and beta (ERβ), which was estimated to be 1,000-10,000 times lower than that of estradiol, it was first believed that BPA was a weak environmental estrogen (Rubin, 2011). However, more recent research has shown that BPA can induce some biological responses at extremely low doses. Sometimes, BPA has the same efficacy as estradiol (Acconcia et al., 2015). Some activities are related to BPA's ability to bind to classical and non-classical membrane estrogen receptors and the G-protein coupled receptor 30 (GPR30) and to act via non-genomic pathways (Rubin, 2011). Several cellular sites have been proposed as action targets for BPA. Nevertheless, some BPA metabolites may be more potent estrogens than the parent substance (Rubin & Soto, 2009). BPA

has been shown to interact differently with the ligand binding domain of classical estrogen receptors than estradiol. Changes have also been observed in the transcriptional co-regulator recruitment, indicating that BPA is an estrogen mimic (Figure 2.4; Rubin, 2011; Gao et al., 2015).

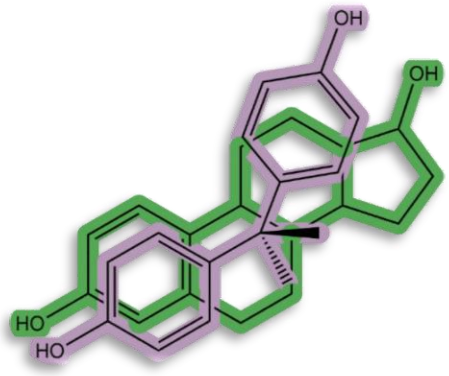


Figure 2.4 Estradiol, the predominant female sex hormone in humans, estradiol (green), and bisphenol A (purple). This diagram displays the structure–activity link that permits BPA to imitate the effects of estradiol and other estrogens.

2.1.3 Health complications of BPA

2.1.3(a) BPA and obesity

Obesity is one of the emerging health conditions throughout the world. The etiology of obesity involves interactions between genes and the environment because it is a multifactorial and complex endocrine disease. Poor nutrition and lack of exercise are significant contributors to the escalating obesity epidemic, but the built environment, stress and air pollution also play significant roles (Heindel et al., 2015). A significant amount of data points to the possibility that a class of endocrine-disrupting chemicals, which interfere with endocrine signalling, impair hormonally regulated metabolic processes (Heindel et al., 2015). These so-called "obesogens" may predispose

some people to gain weight despite their efforts to limit caloric intake and increase physical activity (Gupta et al., 2020). The endocrine-disrupting properties of BPA and its abundance in the environment have led to its classification as a possible environmental obesogen. Additionally, the potential for BPA to increase adiposity was accidentally discovered in 2001. The study discovered that rats treated with a high dose (1.2 mg/kg body weight/day) or a low dose (0.1 mg/kg body weight/day) of BPA produced offspring that were heavier than controls and that this phenotype persisted into adulthood (Rubin et al., 2001). This BPA raises the risk of obesity by disrupting the normal endocrine pathway (Table 2.2). BPA is a mimic estrogenic chemical that hinders adiponectin release by binding with estrogen receptors, specifically ERα and ERβ (Hugo et al., 2008; Choi et al., 2021). BPA reduces adiponectin and increases pro-inflammatory cytokines by acting on adipocytes and macrophages (Naomi et al., 2022). In this context, inflammatory cytokines, such as IL6 and TNFα, promote inflammation in adipose tissue, thereby inhibiting lipolysis (Ahmed et al., 2020). In this instance, the lipid overflow towards oxidative tissues, such as skeletal muscles and the liver, will promote ectopic fat distribution, resulting in abdominal obesity (Trouwborst et al., 2018).

Studies have shown that BPA activates a gene related to human lipid metabolism by inhibiting the peroxisome proliferator-activated receptors alpha (PPARα) signalling pathway (Lozada & Keri, 2011; Naomi et al., 2022). This phenomenon could intensify the induction of CD36 and FABP4 in adipose tissue. The expression of genes involved in the oxidation of fatty acids in mitochondria and peroxisomes is a crucial step in the catabolism of fatty acids, which involves PPARα (Naomi et al., 2022). Inhibition of PPARα causes lipids to accumulate in the hepatocytes, exacerbating obesity (Régnier et al., 2020). Furthermore, activation of another nuclear hormone receptor PPARγ by

environmental chemicals has been explored extensively as a mechanism underlying obesogen activity. The receptor modifies fat cell commitment, differentiation and function. PPARγ is called the 'master regulator' of adipogenesis and hence a reasonable candidate to explain the obesogen activity of substances mechanistically (Figure 2.5; Shao et al., 2016; Mahapatra et al., 2021). Alternately, BPA could induce dysfunction in adipocytes by acting directly on PPARγ. The PPARγ is generally recognised for its prime modulation activity on adipogenesis, particularly the isoform PPARγ2 abundant in adipose tissue (Lefterova et al., 2014). PPARγ will further activate its downstream signaling molecules following stimulation, including CD36, aquaporin 7, phosphoenolpyruvate carboxykinase and lipoprotein lipase. This phenomenon will cause excessive adipocyte differentiation and interfere with lipid metabolism (Naomi et al., 2022).

Table 2.2 Summary of *in vivo* studies on BPA and obesity.

Subject	Dosage	Finding	Reference
Male waster rat (6 weeks)	30 mg/kg	- Increased levels of ALT, AST and bilirubin in liver. - Increased level of TG, total cholesterol, LDL and VLDL. - Lipid droplet was seen in the liver tissue.	(Eweda et al., 2019)
Female mice (12-27 weeks)	50 µg/kg	- Gradual increase in offspring's body mass. - Increased fat levels in the offspring's gonadal, mesenteric and subcutaneous tissue.	(Neier et al., 2019)
Mice (8 weeks)	50 µg/kg/day	- Increased body mass in offspring. - Enhanced glucose tolerance among the offspring. - Increased liver levels of IL-17, IL-22, TNF and IFN.	(Malaisé et al., 2017)
Sprague-Dawley rat (15 weeks)	50 µg/kg/day	- Increased level of abdominal lipid. - Decreased level of HDL. - Increased levels of TG, TC, LDL and leptin.	(Shih et al., 2021)
Waster rat (3-4 weeks)	10 ppm and 10 mg/L/day	- The weight gradually increases. - Increased plasma levels of TG, lipid peroxidation and cholesterol in male offspring.	(Dabeer et al., 2020)
Female CD1 Mice (9-18 days)	5 or 500 µg /kg.bw/day	- Prenatal mice had increased body weight and gonadal fat - DNA demethylation at the transcription start site.	(Taylor et al., 2018)

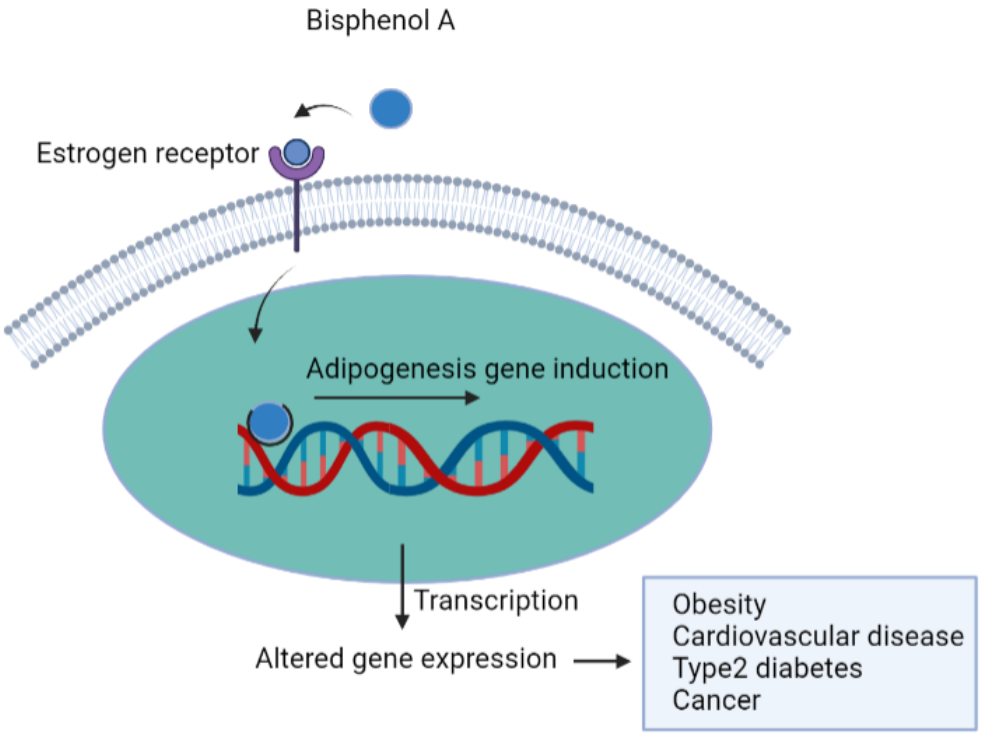


Figure 2.5 Illustration of the effects of BPA on adipogenesis in cells. The diagram shows how BPA gets into the cells, interacts with the ERs and then moves to the nucleus, which causes the transcription of key adipogenic genes.

2.1.3(b) BPA associated with liver disease

The impact of BPA exposure on liver function is another area that receives scant attention from researchers. The liver is the primary organ involved in detoxifying many medications and xenobiotics. The organ plays a key role in regulating several physiological processes in the body. As a result, it is essential in detoxifying BPA from the body. It was previously assumed that when unconjugated BPA (the active form of BPA) is ingested, it is rapidly conjugated in the liver and then removed in the bile or urine. Nonetheless, BPA can be deconjugated by the β-glucuronidase enzyme, which is found in many tissues and could lead to bioaccumulation of BPA (Ginsberg & Rice, 2009). Studies on development point to the rapid ontogeny of β-glucuronidase because the enzyme can be found prenatally in the liver, kidney, and lung of a wide variety of

laboratory species, with the activity being particularly high in the placenta (Linillos-Pradillo et al., 2023). The human placenta contains a lot of the enzyme β-glucuronidase, which plays a crucial role in embryonic growth and development (Nishikawa et al., 2010). A genetic deficiency causes hydrops fetal, a birth defect caused by improper foetal mucopolysaccharide breakdown and water accumulation. Because glucuronidation capacity is immature in early life, the net balance in animal models and tissues studied tends toward deconjugation. Deconjugation of BPA is a potentially significant pharmacokinetic factor during the perinatal period (Ginsberg & Rice, 2009). In fact, according to a recent study, the majority of plasma BPA is bound to serum protein and the accumulation of BPA in fat is approximately 3 times larger than in other tissues (Csanády et al., 2002). Evidence from preclinical studies has also demonstrated that the treatment of BPA in animals was associated with the modification of blood lipid profile and interference with the oxidant/antioxidant system in the liver (Korkmaz et al., 2010; Ke et al., 2016), which may eventually result in liver damage.

Accumulating evidence suggests that BPA exposure causes liver damage via oxidative stress. BPA induces severe hepatocellular damage regardless of the presence of an antioxidant system in the liver (Hassan et al., 2012). The study also revealed that BPA at a dose of 50 mg/kg once daily orally for 4 weeks dramatically triggered liver damage by altering the oxidant: antioxidant balance, where it raised oxidants, Thiobarbituric acid reactive substances (TBARS) and nitric oxide (NO) levels while decreasing antioxidants, catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD) levels (Hassan et al., 2012). This phenomenon is consistent with the findings of a study, which revealed that BPA caused the generation of reactive oxygen species (ROS) and lipid peroxidation, which led to cell damage or death (Kourouma et al., 2015). Furthermore, the oxidative damage to DNA caused by the generated ROS can

be detected with the TUNNEL assay (Kourouma et al., 2015). DNA damage can have long-lasting consequences, including teratogenesis, cancer and mutagenesis if repair attempts are unsuccessful or delayed (Rencüzoğulları & Aydın, 2019). The possible effect of BPA in interfering with redox signaling means that an imbalance between oxidants and antioxidants in the liver is inevitable in the presence of BPA.

Furthermore, BPA has been linked to the induction of inflammation through its role in the upregulation of genes encoding transcription factors implicated in inflammation and genes encoding cytokines engaged in this process. Research conducted by a study showed that BPA-induced liver damage was linked to the release of inflammatory cytokines in the liver (Moon et al., 2012). Animals injected with 1.2 mg/kg body weight per day BPA showed the hepatic expression levels of IL6 and TNFα were higher in the BPA-treated group compared to the control group. The result from another study shows that the BPA group had significantly elevated serum levels of IL1, IL6, IL8 and TNFα compared to the control group (Wang et al., 2019). Collectively, BPA can upregulate pro-inflammatory cytokines while simultaneously downregulating anti-inflammatory cytokines, resulting in an inflammatory response in the liver and subsequently causing liver inflammation and damage (Abdulhameed et al., 2022).

2.1.3(c) BPA association with cancer progression

Cancer development is a multistep process, beginning with cell genetic alterations and continuing with uncontrolled cell division and growth over time. This phenomenon means many cancers diagnosed today may result from exposures that caused genetic changes in cells many years ago (Cooper & Hausman, 2007). Endocrine disrupter chemicals exposure, especially BPA, has been linked to increased cancer risk (Maffini et al., 2006). BPA exposure affects multiple signalling pathways, including interference with a nuclear, steroid hormone and orphan receptor function. Multiple

studies have shown that BPA targets estrogen receptors, androgen receptors, estrogen-related receptors, thyroid hormone receptors, peroxisome proliferator-activated receptors (PPARs), pregnane X receptors and aryl hydrocarbon receptors, as well as their downstream signalling (Lee et al., 2012). BPA also targets numerous enzymatic pathways associated with steroid biosynthesis and metabolism in the endocrine and/or reproductive systems (Khan et al., 2021). The modulation of these pathways has been associated with the development of cancers. For instance, abnormal expression of estrogen receptors plays a critical role in the development of breast, ovarian and liver carcinoma (Gao et al., 2015). The primary mechanism of carcinogenesis induced by BPA may be its estrogenic activity. BPA binds to membrane estrogen receptors (mERs), (ERs) and receptor GPR30, modifies genomic and non-genomic signalling pathways differently in different cell types and disrupts normal biological functions, resulting in carcinogenesis (Wang et al., 2010). Taken together, BPA acts through both estrogen-dependent and estrogen-independent pathways in cancers.

Several previously published studies utilising *in vitro* and *in vivo* models demonstrated the carcinogenic potential of BPA (Gao et al., 2015). Numerous studies have shown that exposure to BPA, even at deficient concentrations, can increase the risk of cancers (Khan et al., 2021). *In vitro* and *in vivo* studies have demonstrated that BPA promotes cancer development by modifying cells' biological behaviour and inducing pro-carcinogenic signaling pathways (Khan et al., 2021). BPA induces proliferation, growth, migration and invasion in different cell types, including HCT116 (Qu et al., 2018), MCF7 (Kim et al., 2017), MDA-MB-231 and BT-549 (Zhang et al., 2016), prostate LNCaP (Bilancio et al., 2017), cervical SiHa (Wang et al., 2015), HeLa (Bolli et al., 2008), human trophoblasts HTR-8/ SVneo (Lan et al., 2017), lung A549 (Zhang et al., 2014), mesenchymal stem cells hUM-MSCs (Wang et al., 2013), ovarian

OVCAR-3 (Ptak et al., 2014), SkBr3 and colorectal SW480 (Pupo et al., 2012) through activation of signaling pathways. Several studies have used *in vitro* systems to show that BPA has genotoxic effects in addition to its role as a carcinogen (Khan et al., 2021). BPA affects genome-wide epigenetic changes that alter expression by modulating the epigenetic enzymes. The epigenetic changes induced by BPA exposure are thought to play a critical role in the pathophysiology of diseases, including cancers (Wang et al., 2019). Therefore, BPA is an attractive endocrine disrupter chemical that increases cancer progression. A summary of the *in vitro* and *in vivo* relationship between BPA and various cancer types is in Table 2.3 and Table 2.4.

Table 2.3 Summary of *in vitro* association of BPA to human cancer progression.

Cancer	Targets	Hallmarks	References
Breast	- Activation of GPER, ERFR, ERK1/2 and MMPs. - Downregulation of FOXA1, Forkhead Family Transcription Factor, P53, BIM and BAX, BCL2L11and PDCD5.	- Increase *in vitro* invasion, migration and proliferation. - Decrease the effectiveness of certain chemotherapy drugs.	(Zhang et al., 2014; Wang et al., 2015)
Ovarian	- Activates MAPK/ERK, JAK/STAT and PI3K/AKT - Upregulates mRNA levels of PPARγ, ERα, SnoN, APLN, IGF-1R, VIM and CXCL12.	- Increases cellular migration, growth, proliferation and invasion. - Raises intracellular ATP, pyruvic acid and lactate levels.	(Ptak et al., 2014; Kim et al., 2015)
Prostate	- Transcriptional activity stimulates AR-T877A. - Increases androgen receptor (AR) expression in ventral prostate and aromatase (CYP19A) activity.	- Increases cell migration and proliferation. - Changes cell morphology and cell cycle arrest. - Modifies tumour suppressor gene methylation.	(Derouiche et al., 2013; Bilancio et al., 2017)
Colorectal	- Increases expression of TWIST, SNAIL, VIM, ZEB and p38 phosphorylation. - Phosphorylates AKT and GSK-3β. - MMP becomes depolarised, which damages mitochondrial integrity. - Reduces ERβ extranuclear activities that E2 causes.	- Toxicity in human colon cancer cells induced at higher concentrations. - Increases invasion and migration. Oxidative damage and increased mitochondrial and intracellular reactive oxygen species.	(Chen et al., 2015; Qu et al., 2018)