The Effects of BPA and its Structural Analogues on Adipocyte Differentiation

Misha Singh

Thesis submitted to the University of Ottawa
in partial fulfillment of the requirements for the
MSc degree in Biochemistry

Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

ABSTRACT

Obesity and the metabolic complications associated with it are increasing globally. Sedentary lifestyles, poor diet and genetic predisposition contribute to obesity. In addition, environmental chemicals such as Bisphenol A (BPA) may play a significant role. Exposure to BPA has been correlated with an array of adverse health effects on the endocrine system and whole-body homeostasis. This has resulted in manufacturers replacing it with structural analogues such as Tetra Methyl Bisphenol F (TMBPF), Bisphenol F (BPF), Bisphenol AP (BPAP), and fluorine-9-bisphenol (BHPF). BPA is a suspected obesogen as it can induce adipogenesis in human and murine preadipocytes. The effects of the BPA analogues listed above on adipogenesis have yet to be evaluated. The aim of this project is to investigate their adipogenic effects. For this purpose, we used 3T3-L1 mouse embryonic fibroblasts. This cell model can be differentiated into mature adipocytes with appropriate inducers including 3-isobutyl-1-methylxanthine (IBMX), insulin and dexamethasone, a synthetic steroid. To assess the effects of BPA analogues, the cells were treated with varying concentrations of TMBPF, BPF, BHPF, BPA, or BPAP in place of dexamethasone. The expression levels of mature adipocyte markers were assessed at mRNA and protein levels to determine the adipogenic potential of the analogues. Lipid accumulation was evaluated by Nile Red staining. A time course was performed to assess the expression levels of known transcriptional regulators of adipogenesis. The results indicate that TMBPF, BPF and BPA increase 3T3-L1 adipogenesis. BHPF and BPAP did not affect adipogenesis in this model. BPF appears to be at least as good as BPA at inducing adipogenesis. TMBPF, on the other hand, can induce adipogenesis to a greater extent than the other chemicals, including BPA, as evidenced by increased expression of adipogenic markers and lipid accumulation. Finally, key transcription factors *C/ebpδ* and *C/ebpα*, part of the adipogenic transcriptional cascade, were up-regulated at two and six hours post-treatment by TMBPF. BPA also up-regulated *C/ebpδ* at two hours post-

treatment. Though the adipogenic effects have become apparent for some of these analogues, the mechanism by which they elicit their effects remains to be discovered. More research is required to deduce the mechanism of action and to provide consensus on what the effects of these replacement bisphenols actually are.

ACKNOWLEDGEMENTS

First, I would like to extend my sincerest appreciation towards my Supervisor Dr. Ella Atlas for having given me the opportunity to be a Master's Student under her supervision. These past years have been a whirlwind of events to say the least. Thank you for continuing to show me kindness, understanding and support while simultaneously challenging me to meet my goals and expand my knowledge. I have grown from your mentorship in ways I could never have imagined, and for that, I will remain grateful.

I would also like to thank my Co-Supervisor, Dr. Alexander Sorisky for his welcoming atmosphere, guidance and support over the last few years. I appreciate your insights and attention to detail, as it has been incredibly valuable for the progression of my project. I sincerely value the feedback and unique perspectives you have contributed.

Thank you to my committee members Dr. Céline Aguer and Dr. Mary-Ellen Harper. The encouragement, support and constructive critiques from you both have helped immensely throughout my degree.

I would like to thank my lab mates Marc Rigden, Jennifer Crosthwait, and Karen Leingarter for their kindness and continuous help. It has been a pleasure to work along side you all, and I am especially grateful for your enthusiasm and patience towards my limitless joy for asking questions.

To my friends, I am grateful to have had your support over these years. Your constant perspectives, honesty, and positive outlook on life has been a source of encouragement in pushing me towards my goals. Thank you to my brother Vishal Singh for never letting me forget the good times; for knowing how to make me laugh and smile when I most needed it. Thank you to my brother Davin Singh, an incredibly supportive and inspiring role model constantly reminding me that I can achieve my goals. Thank you to my father, Lajendra Singh for teaching me to never give up.

Finally, to the most important woman in my life, my mother Janet Ramdayal; thank you for your unconditional love, strength, and sacrifice. You have shown me what it is to persevere. Your unwavering belief in me is, and will continue to be, my source of strength and drive in my future endeavours.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AT adipose tissue

ATCC American type culture collection

ATDS Adipose tissue derived stem cells

ATP Adenosine triphosphate

BCA Bicinchoninic acid

BHPF Fluorine-9-bisphenol

BMI Body Mass Index

BPA Bisphenol A

BPAF Bisphenol AF

BPAP Bisphenol AP

BPB Bisphenol B

BPF Bisphenol F

BPP Bisphenol P

BPS Bisphenol S

BPZ Bisphenol Z

C/EBPα CCAAT-enhancer-binding protein alpha

C/EBPβ CCAAT-enhancer-binding protein beta

C/EBPδ CCAAT-enhancer-binding protein delta

cAMP Cyclic adenosine monophosphate

CHOP C/EBP homologous protein

CMV Cytomegalovirus

CREB cAMP response element-binding protein

DAPI 4’,6-Diamindino-2-phenylindole dihydrochloride

DES diethylstilbestrol

DEX Dexamethasone

DMEM Dulbeco’s modified eagle’s medium

DMSO Dimethylsulfoxide

ERα Estrogen receptor α

ERβ Estrogen receptor β

FABP4 Fatty acid binding protein 4

FBS Fetal bovine serum

FFA Free Fatty Acid

GLUT2 Glucose transporter 2

GLUT4 Glucose transporter 4

GR Glucocorticoid receptor

GRE Glucocorticoid response element

HDAC1 Histone deacetylase 1

HRP horseradish peroxidase

IBMX 3-isobutyl-1methylxanthine

IL-6 Interleukin 6

IR Insulin receptor

IRS-1 Insulin receptor substrate-1

KLF4 Kruppel-like factor 4

LPL lipoprotein lipase

MCE Mitotic Clonal Expansion

MEF Mouse embryonic fibroblasts

MMTV Mouse mammary tumour virus

NHAES National Health and Nutrition Examination Survey

NOAEL No observed adverse effect

PBS Phosphate-buffered saline

PI3K Phosphoinositide 3-kinase

PIP_2 Phosphatidylinositol (4,5) bisphosphate

PIP_3 Phosphatidylinositol (3,4,5) trisphosphate

PKA Protein Kinase A

PLIN- Perilipin

PPARγ Peroxisome proliferator-activated receptor γ

PVDF polyvinylidene fluoride

REDOX Reduction oxidation

RXR Retinoid X receptor

SD Shine dalagarno

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SS DNA Salmon Sperm DNA

SVF Stromal vascular fraction

TBBPA Tetrabromobisphenol A

TMBPF Tetramethyl bisphenol F

TNFα Tumour necrosis factor alpha

UCP1 Uncoupling protein 1

WHO World Health Organization

LIST OF FIGURES AND ILLUSTRATIONS

LIST OF TABLES

1. INTRODUCTION

1.1 Obesity

1.1.1 *Definition and measurement of obesity*

Obesity is characterized by the excessive accumulation of adipose tissue, often leading to adverse effects on human health (World Health Organization, 2021). Body mass index (BMI) is the most common method by which obesity is calculated and takes into account the height and weight of an individual. An adult's calculation involves dividing weight by height squared (kg/m^2) (Ofei, 2005). The calculation for children is slightly different as it incorporates age. BMI, because of its simplicity, remains the most useful way to measure and track overweight and obesity at a population level. According to the World Health Organization (WHO), a BMI above or equal to $25kg/m^2$ qualifies as being overweight, and a BMI above or equal to 30 kg/m^2 qualifies as being obese for adults (World Health Organization, 2021). Although BMI is the standard method to assess obesity, it is important to note that bone density and muscle mass vary among individuals and will affect BMI calculation.

1.1.2 *Prevalence and Etiology of obesity*

The prevalence of obesity has increased worldwide and is now considered an epidemic. The most recent WHO estimates indicated that in 2016, 1.9 billion adults were overweight and 650 million of them were obese (World Health Organization, 2021). In Canada alone, the percentage of overweight adults for 2018 was roughly 63% (17.2 million) (Statistics Canada, 2019).

As technology continues to advance, physical activity becomes progressively limited, leading to low energy expenditure. This, in conjunction with an increased calorie intake, leads to

an energy imbalance resulting in increased fat deposition. Obesity is also influenced by biological, behavioural and genetic factors (García-Mayor et al., 2012). Twin adoption studies have provided strong evidence supporting the heritability of a high BMI (Stunkard et al., 1986; Sorensen et al., 1989; Maes et al., 1997). Another study by Wardel *et al.* analyzed the BMI and waist circumference of 5092 British twin pairs (identical and non-identical) between the ages of 8-11 years. The study concluded that heritability had greatly influenced BMI. Results showed heritability of BMI to be of the order of approximately 80% (Wardle et al., 2008).

Mackay *et al.* suggested that the endocrine system may influence appetite and satiety through regulating energy homeostasis. This process is responsible for controlling neuroendocrine circuits; therefore, by varying hormone levels and activity, one's appetite may increase, ultimately contributing to the development of obesity (MacKay et al., 2013). Therefore, in addition to genetics, lack of exercise and overnutrition, environmental toxicity may have a prominent impact on the obesity epidemic. A theory called the "obesogen hypothesis" introduced by Bloomberg *et al.* suggests that exposure to environmental chemicals named obesogens, may also predispose individuals to obesity (Grün and Blumberg, 2006). This theory will be discussed in detail in the following sections.

1.1.3 *Complications of obesity*

Obesity has been strongly linked to the metabolic syndrome, a condition associated with type 2 diabetes, insulin resistance, stroke and cardiovascular disease (Hossain et al., 2007). Coronary or ischemic heart disease is the main cardiovascular disease typically associated with the metabolic syndrome. It is caused by the build-up of cholesterol, foam cell macrophages, calcium and other cellular matter in coronary arteries creating a plaque-like atheroma that gradually reduces the amount of oxygen-rich blood reaching the heart (Bentzon et al., 2014). In the event that the

plaques rupture and the artery becomes completely blocked, the onset of a myocardial infarction or even death is possible (Gargiulo et al., 2015).

Type 2 diabetes is strongly associated with obesity and is characterized by a relative lack of insulin production that is unable to compensate for a state of insulin resistance (Pulgaron and Delamater, 2014). Insulin is a hormone secreted by beta cells within the islets of Langerhans in the pancreas (Rorsman and Ashcroft, 2018). This occurs in response to elevated glucose levels in the blood. Glucose is transported into the beta cells via Glucose transporter 2 (GLUT2) and is converted to Adenosine triphosphate (ATP) through cellular respiration (Sansbury et al., 2012). The ATP consequently closes potassium channels embedded within the membrane of beta cells, thus depolarizing the membrane and opening calcium channels. This allows for the influx of Ca^{2+}, which triggers the exocytosis of insulin-containing granules from the beta cell. Insulin, once secreted, will circulate and then bind to the insulin receptors present within various tissues to stimulate glucose uptake via Glucose transporter 4 (GLUT4) (Fu et al., 2012). This process serves as an essential and major pathway for the production of ATP within the muscle and heart (Wilcox, 2005). Glucose levels need to be tightly regulated; too much glucose in the blood could lead to damage of organs, blood vessels and nerves. On the other hand, low blood glucose could result in seizures, fainting, coma and even death.

1.2 Adipose Tissue

1.2.1 *Origin and types of adipose tissue*

Many studies have investigated the function of adipose tissue, leading to its recognition as an essential organ for the regulation of various biological processes. These include coagulation, appetite regulation, immunity, glucose and lipid metabolism, reproduction, angiogenesis,

fibrinolysis, body weight homeostasis , and vascular tone control (Coelho et al., 2013). There are two types of adipose tissue: brown adipose tissue (AT) and white AT, each serving different roles.

Brown AT is highly vascularized compared to white AT. This tissue appears brown and is densely packed with mitochondria which facilitate non-shivering thermogenesis (Casteilla et al., 1989; Coelho et al., 2013). Brown AT requires the storage, synthesis and metabolism of free fatty acid (FFA) for thermogenesis (Divakaruni et al., 2012). More specifically, uncoupling protein 1 (UCP1), a protein highly expressed in brown AT, functions to disrupt the proton gradient through the ATPase within the mitochondria, ultimately running futile metabolic cycles to produce heat (Casteilla et al., 1987; Berry et al., 2013). This thermogenic process is primarily important during the neonatal stages of mammalian life and less so during the adult stage. Major depots of brown AT can be found between the scapulae, along great vessels and within parts of the abdominal cavity of rodents and human infants. However, they tend to diminish with increasing age in humans (Lee et al., 2013; Gonçalves et al., 2017). Residual brown AT has been suggested as a target for human therapeutics to combat obesity and type 2 diabetes (Nedergaard and Cannon, 2010). This idea stems from studies in rodents showing that augmentation or increased mass of brown AT may be metabolically beneficial (Lee et al., 2013). One study in particular by Stanford and colleagues showed that brown AT transplants improved glucose homeostasis and insulin sensitivity in mice with high-fat diet-induced insulin resistance (Stanford et al., 2013). A similar study in humans found that an increase in thermogenic fat resulted in improved metabolic effects (Chondronikola et al., 2016). These studies suggest there is future potential in increasing the mass of brown like AT as a method of increasing energy expenditure, ultimately combating obesity and diabetes.

White AT is the principal site for the storage of excess nutrients in the form of triglycerides within adipocyte lipid droplets. It is more widely distributed anatomically compared to brown AT. It spans almost the entire subcutaneous region, the viscera of the abdominal cavity and around several muscle groups, thus providing thermal insulation and mechanical protection (Berry et al., 2013). White AT possesses fewer mitochondria than brown AT and plays a crucial role in maintaining energy homeostasis through two processes; lipolysis and lipogenesis (Berry et al., 2013). Lipolysis occurs when the body is in a fasted or active state, causing stored fat to be hydrolyzed and the resulting FFA to be used as energy. This process can be initiated by several hormones (glucagon, epinephrine and norepinephrine) (Coelho et al., 2013). Lipogenesis, on the other hand, is a process that occurs post-prandially, with elevated blood glucose post-absorption causing insulin to be released from beta cells, which stimulates glucose uptake within adipocytes for it to be converted into triglycerides for storage in adipocytes (Coelho et al., 2013; Eckel, 2018). This expansion of white AT is governed by hyperplasia and hypertrophy – two responses that define obesity (Ruiz-Ojeda et al., 2016). Hyperplasia occurs when there is an increase in the number of adipocytes for storage of excess energy made possible by the proliferation and differentiation of adipose progenitors; this is known as metabolically healthy adipose tissue. Expansion of white AT via hyperplasia shows relatively preserved insulin sensitivity and low ectopic lipid disposition (Kim et al., 2007; Blüher, 2020). Hypertrophy, on the other hand, occurs through an increase in the size of existing mature adipocytes and, in the case of sustained energy overload, can lead to fibro-inflammation; this is known as metabolically unhealthy adipose tissue (Wang et al., 2014). Hypertrophy of adipocytes within adipose tissue is considered metabolically unhealthy because it can induce hypoxia, oxidative stress as well as inflammation due to the secretion of proinflammatory adipokines as existing adipocytes expand (Skurk et al., 2007a; Muir

et al., 2016). These alterations can often result in metabolically dysfunctional cells, leading to insulin resistance, ectopic lipid disposition, and organ dysfunction (including within the liver and muscle). These events are often associated with cardiovascular disease and type 2 diabetes (Carrière and Casteilla, 2019).

1.2.2 *Role as an endocrine organ*

Although the adipose tissue was initially thought to be an organ mainly for the storage and release of energy, the discovery of several biologically active factors termed adipokines, such as leptin, led to its recognition as an endocrine organ (Zhang et al., 1994; Waki and Tontonoz, 2007). Leptin is a peptide hormone responsible for suppressing hunger and promoting energy expenditure via activation of hypothalamic pathways (Bjørbaek and Kahn, 2004; Myers et al., 2008). Adiponectin is an anti-inflammatory protein that has been suggested to play a role in regulating insulin sensitivity. This was demonstrated in a study by Berg *et al.* where wild type and insulin-resistant mice were injected with recombinant adiponectin. A significant decrease in plasma glucose levels independent of plasma insulin levels was observed thus suggesting that adiponectin may have enhanced insulin action (Berg et al., 2001). Furthermore, a strong negative correlation between expression of adiponectin and obesity exists, whereas circulating leptin levels are elevated with increased adiposity (Maffei et al., 1995; Arita et al., 1999).

Thus far, adipose tissue is known to secrete a variety of adipokines such as tumour necrosis factor-alpha (TNFα), adipsin, and interleukin 6 (IL-6), among others (Kershaw and Flier, 2004). Adipose tissue is diverse in its composition consisting of adipocytes, stromovascular cells, immune cells, and nerve tissue; thus, not all adipokines are secreted by adipocytes (Frayn et al., 2003). Therefore, in addition to serving as an organ for the regulation of energy storage and release,

adipose tissue is also a complex organ involved in maintaining metabolic homeostasis through the synthesis and secretion of adipokines.

1.3 Adipogenesis

Adipogenesis is the process whereby mesenchymal stem cells commit to the preadipocyte lineage and eventually become mature adipocytes. Once committed, these cells lose their ability to differentiate towards any other lineage. The first phase of commitment is called determination (where the cell commits to becoming a preadipocyte), and the second phase, terminal differentiation (when the preadipocyte becomes a mature adipocyte) (Ruiz-Ojeda et al., 2016). This elaborate cellular process called adipogenesis is controlled via a series of transcription factors. Some of these transcription factors are more crucial for adipogenesis than others. They will be discussed in more detail within the following section.

1.3.1 *Transcriptional cascade governing adipogenesis*

Transcription factors bind to specific sequences within the DNA called response elements which then trigger a cascade of events leading to gene expression, and in this case, the development of the phenotype of a mature adipocyte. Two key transcription factors that are critical for this terminal differentiation process are peroxisome proliferator-activated receptor γ (PPARγ) and CCATT-enhancer-binding protein α (C/EBPα).

PPARγ was first identified while pursuing the binding factor responsible for the regulation of the fatty acid binding protein 4 (FABP4) gene (Tontonoz et al., 1994a). This ligand-dependent transcription factor belongs to the nuclear hormone receptor superfamily and binds to DNA as an obligate heterodimer with members of the retinoid X receptor (RXR) (Lefterova et al., 2014). There are two isoforms of PPARγ, one found in virtually all tissues (PPARγ1) and the other

expressed predominantly in adipose tissue (PPARγ2) (Tontonoz et al., 1994a; Braissant et al., 1996). The same gene encodes these protein isomers; however, alternative promoter usage and splicing variants lead to various transcripts for PPARγ1 and 2. PPARγ2 consists of 30 additional amino acids at its N terminus relative to PPARγ1 (Tontonoz et al., 1994a). Although both isoforms have been shown to induce adipogenesis, PPARγ2 has been shown to lead to a more robust response compared to PPARγ1 under low ligand concentrations (Mueller et al., 2002). Following the discovery of PPARγ, several groups sought to confirm its expression during adipogenesis and hypothesized that it might be important for adipose cell development and function (Chawla et al., 1994; Sears et al., 1996) Its regulatory role was further demonstrated in gain of function experiments where PPARγ was ectopically expressed in NIH-3T3 fibroblasts. Despite the lack of adipogenic potential under normal conditions, these cells were able to differentiate and express many endogenous genes characteristic of adipocytes and acquired the lipid-laden phenotype similar to that of a mature adipocyte (Tontonoz et al., 1994b). An even more robust response has been observed with the expression of both PPARγ and C/EBPα in fibroblasts and myoblasts (Tontonoz et al., 1994b; Hu et al., 1995).

Interestingly, PPARγ alone can promote adipogenesis in mouse embryonic fibroblasts (MEFs), whereas C/EBPα cannot (Mueller et al., 2002). It is for these reasons that PPARγ is considered to be the master regulator of adipogenesis. Nevertheless, C/EBPα remains an important transcription factor in the adipogenic cascade as it increases the expression of *Pparγ*. Together these transcription factors work via a feedback loop where PPARγ increases expression of *C/ebpα,* which then further up-regulates *Pparγ* (Rosen et al., 2002). These two transcription factors work to maintain the phenotype of the mature adipocyte.

Other members of the C/EBP transcription factor family (C/EBPβ and C/EBPδ) undergo a transient and early increase in their mRNA and protein levels within preadipocytes, whereas expression of C/EBPα occurs slightly later in the differentiation process (Cao et al., 1991; Yeh et al., 1995; Rosen, 2005). The importance of these transcription factors was demonstrated in a study by McKnight and colleagues' in which C/EBPβ and C/EBPδ were ectopically expressed in both 3T3-L1 and NIH-3T3 cells. The experiments showed that ectopic expression of C/EBPβ and C/EBPδ, without hormonal stimulation, induced expression of C/EBPα at day 3 during the differentiation process in 3T3-L1. Furthermore, ectopic expression of C/EBPβ converted non-adipogenic NIH-3T3 cells into adipoblasts that were capable of differentiating into lipid-laden adipocytes upon hormonal stimulation (Yeh et al., 1995). This suggested that C/EBPβ may upregulate PPARγ. Analysis of *Pparγ* and *C/ebpα* promoters revealed functional C/EBP response elements within which C/EBPβ and C/EBPδ may bind (Christy et al., 1991; Clarke et al., 1997). These studies indicate an important role for C/EBPβ and C/EBPδ at inducing *Pparγ* and *C/ebpα* expression.

It is now established that there is a transcriptional cascade where C/EBPβ and δ are expressed early in adipogenesis. Although expressed, C/EBPβ remains inactive, as it is bound to C/EBP homologous protein (CHOP), a protein that renders the DNA binding domain inactive. However, upon stimulation, CHOP dissociates allowing for C/EBPβ to up-regulate C/EBPα which then upregulates PPARγ (Tang and Lane, 2000). Finally C/EBPα and PPARγ mutually maintain expression levels in a feedback loop that ultimately drives the terminal differentiation process towards a mature adipocyte (Wu et al., 1999).

1.3.2 *3T3-L1 cell model*

Much of what is known about the transcriptional cascade of adipogenesis was investigated in 3T3-L1 mouse embryonic fibroblasts derived from Swiss 3T3 mouse embryos (Green and Meuth, 1974). These cells display a fibroblast-like morphology and can differentiate into mature adipocytes when exposed to adipogenic stimuli. 3T3-L1 cells can be extensively maintained in culture and are easily manipulated. Although these cells are aneuploidy (Fu et al., 2005a), this model system has been extremely useful for identifying molecular markers and transcription factors required for adipogenesis. This model is ideal for screening chemicals for their adipogenic effects. There are many mature adipocyte markers that can be used to assess whether or not a chemical is adipogenic. For example, adipsin and adiponectin are adipokines (cell signalling proteins) that are secreted from adipocytes. Adipsin, also known as factor D, is a serine protease that plays a role in stimulating insulin secretion as well as maintaining β cell function (Lo et al., 2014; Song et al., 2016). Expression of this adipokine is low in obese and diabetic mice models. Low circulating levels of adiponectin have also been associated with the metabolic syndrome (Arita et al., 1999; Yamauchi et al., 2001). Adiponectin has been postulated to play a role in adipocyte differentiation, regulation of glucose metabolism and augmenting insulin sensitivity (Fu et al., 2005b). Other molecular markers include perilipin (PLIN), FABP4, and lipoprotein Lipase (LPL). PLIN is expressed on the surface of lipid droplets and allows for fats to be metabolized once it is phosphorylated (Sztalryd and Brasaemle, 2017a). FABPs are intracellular lipid chaperones that can bind and unbind long chain FAs. FABP4 is found in adipocytes and plays a role in fatty acid uptake and metabolism (Furuhashi et al., 2014). Finally, LPL is a central enzyme to lipid metabolism. It is synthesized and secreted by mature adipocytes and is responsible for the

hydrolysis of triglycerides from lipoproteins with subsequent fatty acid uptake (Walton et al., 2015).

Although it has been suggested that mitotic clonal expansion (MCE) is absolutely necessary for 3T3-L1 adipognesis, a recent study by Qiu *et al.* found that inhibition of MCE did not affect 3T3-L1 adipocyte differentiation (Qiu et al., 2001; Tang et al., 2003). MCE refers to growth-arrested cells synchronously re-entering the cell cycle to undergo two rounds of mitosis upon stimulation by adipogenic inducers. This differentiation cocktail has been well established and consists of insulin, dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (IMBX). DEX is a synthetic glucocorticoid and is responsible for activating the glucocorticoid receptor (GR) (Pantoja et al., 2008).

Upon activation, GR is able to rapidly induce expression of *C/ebpδ* by binding glucocorticoid response elements (GRE) on its promoter (Cao et al., 1991). GR simultaneously acts to deplete and thus dislodges histone deacetylase-1 (HDAC1) from the promoter of *C/ebpα,* allowing C/EBPβ to regain its DNA binding abilities (Abdou et al., 2011). This ultimately results in the up-regulation of C/EBPα and PPARγ (Hamm et al., 2001; Salma et al., 2006). As previously mentioned, PPARγ and C/EBPα are the two major transcription factors involved in the adipogenic process. Not only do they mutually induce expression of each other, but they also cooperate to induce and maintain the phenotype of the mature adipocyte (Farmer, 2006). IBMX is a phosphodiesterase inhibitor responsible for elevating cytosolic cyclic adenosine monophosphate (cAMP) levels leading to protein kinase A (PKA) activation. This enzyme activates cAMP response element-binding protein (CREB) and the Krüppel-like factor 4 (KLF4), the first wave transcription factors involved in the differentiation process of adipogenesis (Reusch et al., 2000). Finally, insulin binds and activates the insulin receptor (IR), resulting in the auto-phosphorylation

of tyrosine residues present within the β subunit of the IR (Van Obberghen et al., 2001). These phosphorylated residues are recognized by the insulin receptor substrate 1 (IRS-1), which ultimately leads to the phosphorylation of IRS-1. Once phosphorylated, IRS-1 attracts and docks the lipid kinase phosphoinositide 3-kinase (PI3K), which phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP_2) to phosphatidylinositol (3,4,5) trisphosphate (PIP_3) (Gagnon et al., 1999; Saltiel and Kahn, 2001). This insulin signalling pathway also further activates CREB and increases expression of C/EBPβ (Siersbaek and Mandrup, 2011).

1.3.3 *Human Primary Preadipocytes*

Though murine *in vitro* models are useful systems for the initial assessment and study of adipogenesis, primary human preadipocytes are more reflective of human physiology. These cells are derived from the stromal vascular fraction (SVF) after the isolation of human adipose tissue. The SVF isolate is composed of a variety of cells such as preadipocytes, adipose-tissue derived stem cells (ATDS), vascular smooth muscle cells, macrophages, neutrophils and lymphocytes (Esteve Ràfols, 2014; Ramakrishnan and Boyd, 2018). Their adipogenic capacity is linked to their depot of origin (intra-abdominal/visceral versus subcutaneous origin) and are also reflective of donor characteristics (Lessard et al., 2014; Michaud et al., 2014). This can be useful in studying obesity between individuals ranging in age, sex, and other biological factors. Despite the many advantages of using human primary preadipocytes, the restricted access to donor tissue and their limited renewal capacity in culture remain limiting factors (Skurk et al., 2007b).

Like 3T3-L1 preadipocytes, human primary preadipocytes are already committed to the adipocyte lineage and therefore will differentiate into mature adipocytes when treated with a series of inducers. The differentiation protocol required for human preadipocytes to mature takes 14

days and is induced with insulin, IBMX, a PPARγ agonist, and a glucocorticoid such as DEX (Boucher et al., 2014a). These inducers act in the same way as discussed in the 3T3-L1 cell model.

1.4 Endocrine-disrupting Chemicals and Obesogens

1.4.1 *Definition*

Endocrine disrupting chemicals are compounds capable of interfering with the endocrine system (Monneret, 2017). Exposure to such chemicals may stimulate or inhibit the important responses that hormones trigger, ultimately affecting the normal function and development of tissues and organs (National Institute of Environmental Health Sciences, 2010). There have been ongoing studies concerning contributors to the obesity epidemic. There is growing evidence that several environmental factors may be implicated. An "obesogen hypothesis" was proposed by Grun and Blumberg (2006), defining an obesogen as a xenobiotic that can promote significant lipid accumulation and adipogenesis (Grün and Blumberg, 2006; García-Mayor et al., 2012). Early exposure to such endocrine-disrupting chemicals would predispose individuals to obesity and metabolic complications under the influence of a high calorie diet (Grün and Blumberg, 2006; Janesick and Blumberg, 2011; García-Mayor et al., 2012; Chamorro-Garcia and Blumberg, 2017). The introduction of the obesogen hypothesis has since led to the discovery of several chemicals able to induce adipogenesis. Several *in vitro* and in *vivo* studies have implicated bisphenol A (BPA) as an obesogen. Analogues of BPA are now being investigated to assess their effects on adipogenesis as well.

1.4.2 *BPA*

A.P. Dianin originally synthesized BPA in 1891, while in pursuit of synthetic estrogens to prevent multiple pregnancy-related problems such as miscarriage and premature birth in women

(Rubin, 2007; Vogel, 2009). Although BPA was proven to be estrogenic, it was not as effective as diethylstilbestrol (DES) and therefore was not used (Dodds and Lawson, 1936). However, in the 1940s, BPA was taken up by the plastics industry, where it was used as a polymer within polycarbonate plastics such as baby bottles and other food containers (Shelby, 2008). BPA-containing epoxy resins were also used within dental sealants as well as for the lining of food and beverage containers, amongst others. The presence of BPA within the environment is quite significant. It has been estimated that over 6 billion pounds of BPA are produced annually (Welshons et al., 2006). Although polymerized, BPA can leach from these polycarbonate plastics by hydrolysis of BPA-linked ester bonds upon exposure to high temperature and to acidic or basic foods (Welshons et al., 2006; Vandenberg et al., 2007). This is problematic because food and beverages stored within polycarbonate plastics run the risk of absorbing BPA, resulting in routine ingestion of BPA.

A study conducted in 2003 by Calafat *et al.* measured the amount of BPA detected within urine samples of 2517 participants (6 years or older) of the cross-sectional NHANES (National Health and Nutrition Examination Survey) study (Calafat et al., 2008). The study revealed detectable levels of BPA in 92.6% of the participants. An average of 4.5 μg/L was found within children 6-11 years of age, 3.0 μg/L in adolescents (12-19 years) and 2.5 μg/L in adults (20 plus years). Higher concentrations of BPA were found in children compared to both adolescents and adults. This is an important finding because animal studies have shown increased vulnerability to BPA upon prenatal exposure, often affecting the development of the brain and reproductive systems amongst others (Calafat et al., 2008). BPA is not only detectable in urine samples. Studies have also determined that BPA is detectable in amniotic fluid, placental tissue, breast milk,

neonatal blood, serum, and umbilical cord (Ikezuki et al., 2002; Yamada et al., 2002; Vandenberg et al., 2007).

BPA is an endocrine disruptor and can mimic estrogen. Estrogen is responsible for a vast array of functions and is particularly important for reproduction and development in females, such as breast development and the menstrual cycle. In both males and females, estrogen is responsible for maintaining bone strength and is also known to affect the brain, heart, skin, as well as other tissues (The Endocrine Society, 2014).

Aside from its estrogenic effect, human BPA exposure has been correlated with increased metabolic disease, including diabetes, cardiovascular disease, liver dysfunction, recurrent miscarriages, and an increase in premature infants (Sugiura-Ogasawara et al., 2005; Lang et al., 2008; Melzer et al., 2010). As evidence accumulated showing the adverse health effects of BPA, governments and the public questioned its safety. The growing concern over the health and safety of BPA led to more restriction of its use in many countries across the globe. In 2010 Canada became the first jurisdiction to ban BPA from use in baby bottles (Mittelstaedt, 2010). Since then other countries have followed suit in prohibiting its use in some products, particularly baby bottles. As a result, manufacturers have been prompted to replace BPA with other structural analogues, one of which is bisphenol S (BPS), currently used in many industrial applications (Thoene et al., 2020). Studies have shown the presence of other analogues in plastics, including bisphenol F (BPF), bisphenol AF (BPAF), bisphenol B (BPB), bisphenol AP (BPAP) and bisphenol Z (BPZ) (Pivnenko et al., 2015; Mesnage et al., 2017). Many of these products are marketed as BPA-free, but the presence and effects of the analogues has not been fully evaluated.

1.4.3 *BPA analogues*

Once consumers became aware of the potential health effects of BPA, they started avoiding products containing BPA. This forced companies to replace it with other chemicals that could perform equally as well, however the biological effects of these chemicals were not assessed. These replacement bisphenols are structurally similar to BPA and may have similar effects. As such, the emergence and use of BPA analogues in plastics and other products triggered a number of studies looking to evaluate human exposure to the BPA analogues. One study by Wang *et al.* measured human exposure to BPA and its analogues by analyzing human urinary excretion data and wastewater-based measurements. The study found that humans are exposed to BPA and its analogues (µg/person/day) ranked from high to low exposure levels: BPA (2.53µg/p/day), BPF (0.68µg/p/day), BPS (0.60µg/p/day), bisphenol P (BPP) (0.41µg/p/day), BPAP (0.36 µg/p/day), BPB (0.29µg/p/day), BPZ (0.24µg/p/day), and BPAF (0.06µg/p/day) (Wang et al., 2020). Although BPA remains a chemical of concern, other analogues are being detected in urine samples across the globe. This is concerning because as BPA is being phased out by industry, it is being replaced with other analogues, and thus human exposure to these analogues may increase over time.

Much of the literature surrounding the effects of BPA analogues remains centred around estrogenic activity. A study by Mesnage *et al.* looked at the effects of 6 bisphenol analogues (BPF, BPAP, BPZ, BPS, BPB, and BPAF) and compared them to the estrogenic activity of BPA in human breast cancer cell lines. The study concluded that all analogues were estrogen receptor agonists in MCF-7 cells. In fact, of the bisphenols tested, three (BPAF, BPB, BPZ) turned out to be even more estrogenic (Mesnage et al., 2017). Other research has shown some bisphenol analogues to be anti-estrogenic. Xiao *et al.* evaluated the estrogenic and anti-estrogenic effects of

BPAP using *in vitro*, *in vivo*, and *in silico* tools. This research concluded that although BPAP exhibited weak estrogenicity, it was strongly anti-estrogenic (Xiao et al., 2018). Another recent study by Zhang *et al.* looked at the estrogenic activity of another BPA analogue, fluorine-9-bisphenol (BHPF), and found this compound to be anti-estrogenic *in vitro* and *in vivo* (Zhang et al., 2017). A recent study has suggested that one bisphenol analogue, tetramethyl bisphenol F (TMBPF), lacks estrogenic activity and thus may be a plausible replacement for BPA (Soto et al., 2017; Maffini and Canatsey, 2020). *In vivo* studies have shown that some of these bisphenols (BPAP, BHPF, and BPF) affect reproductive and sexual development in male and female mice or rats (Xiao et al., 2018; Jiao et al., 2019; Ullah et al., 2019). Human and environmental exposure to TMBPF has yet to be evaluated.

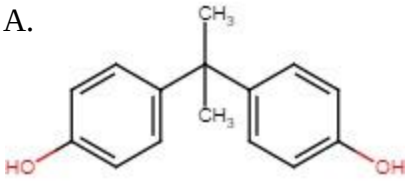
BPA has also been shown to have other effects besides reproductive effects. Of concern is BPA's ability to elicit adipogenic effects. Studies have shown that treatment of murine cells with estrogen inhibits adipogenesis (Jeong and Yoon, 2011; Lee et al., 2021), thus suggesting that BPA may mediate its adipogenic effects independent of its estrogenic activity. On the other hand, others claim estrogen can induce adipogenesis, however the experimental conditions used in this study were different than those used in other studies, including our own (Fatima et al., 2019). This remains an area for further research, nevertheless the potential obesogenic effects of the BPA analogues is worth investigating.

To date, there have not been any *in vitro* or *in vivo* studies investigating whether or not BPAP or BHPF may affect lipid accumulation. There are a few recent papers looking at the effects of TMBPF and BPF. A study by Tetzlaff *et al.* looked at the effects of BPF on adipogenesis in the 3T3-L1 preadipocyte cell model. They found BPF increased lipid accumulation similar to BPA (Ramskov Tetzlaff et al., 2019). However, Drobna and colleagues claim the opposite to be true

where BPF decreased the expression of adipogenic genes in differentiated 3T3-L1 cells (Drobna et al., 2019). With regards to TMBPF, adipogenesis has yet to be investigated using the 3T3-L1 cell model. However, its effects on human mesenchymal stem cells has been investigated by Cohen and colleagues. This research group found that TMBPF reduced adipogenesis (Cohen et al., 2021). These studies reveal a gap in the literature whereby the effects of BPA analogues on adipogenesis remain unclear and require further investigation.

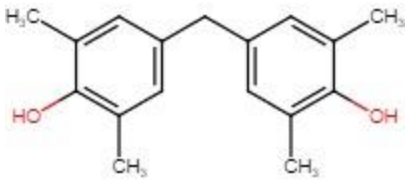
Our lab has previously used the 3T3-L1 cell model to investigate the effects of BPA on adipogenesis. The results showed that BPA was able to induce adipocyte differentiation markers and lipid accumulation in the absence of DEX (Atlas et al., 2014). These experiments were also replicated within human primary preadipocytes where BPA was again found to increase adipogenesis (Boucher et al., 2014a). Our lab then sought to investigate whether or not replacement analogues such as BPS, could elicit the same effects as BPA. Experiments were performed in both murine and humans cell models as previously shown with BPA. The results indicated that BPS induced greater expression of mature adipocyte markers and lipid accumulation compared to BPA in the murine cell model (Ahmed and Atlas, 2016). BPS was also found to induced lipid accumulation and increased expression of adipocyte genes within the primary human preadipocytes as well (Boucher et al., 2016a).

A.



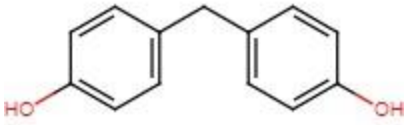
BPA

B.



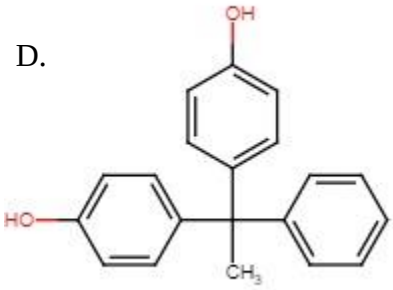
TMBPF

C.



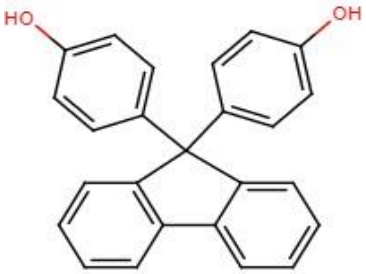
BPF

D.



BPAP

E.



BHPF

Figure 1: Chemical Structures of BPA, TMBPF, BPF, BPAP and BHPF.

RATIONALE

Although studies have shown that BPA and its analogues have adverse effects on human health, their effects on adipogenesis remain relatively unknown and require further investigation. Our lab has previously shown that BPA can accelerate fat cell formation *in vitro* for both murine and human cell models (Atlas et al., 2014; Boucher et al., 2014a). Given the structural similarities of the replacements (Figure 1), it is possible they might exhibit the same effects as BPA. Our lab showed that BPS increases adipogenesis in both 3T3-L1 and human primary preadipocytes (Ahmed and Atlas, 2016; Boucher et al., 2016a). This research area warrants further investigation to elucidate potential effects that the BPA analogues may have on adipogenesis and to determine a mechanism by which these chemicals are acting within the model system. **I hypothesized that BPA analogues may have an effect on adipogenesis similar to BPA.** To address this hypothesis, I proposed the following specific aims:

SPECIFIC AIMS:

Aim 1: Investigate the effects of the BPAAnalogues (TMBPF, BPAP, BPF, and BHPF) on adipogenesis.

Aim 2: Investigate the mechanism by which these chemicals may increase adipogenesis.

2. MATERIALS AND METHODS

2.1 Cell Culture and Differentiation

2.1.1 *3T3-L1 Differentiation Protocol*

3T3-L1 mouse embryonic fibroblasts previously obtained from the American Type Culture Collection (ATCC) were stored in liquid nitrogen. One vial of cells was thawed in a 10cm dish containing 10mL of pre-warmed Dulbecco's modified Eagle's medium (DMEM) 5.6mM glucose media (Hyclone, Mississauga, ON, Canada) supplemented with 1% penicillin/streptomycin (P/S) (Life Technologies, Burlington, ON, Canada) and 10% bovine calf serum (ATCC, Manassas, VA, USA). Cells were replenished with fresh maintenance medium the following day to prevent any cell death that may occur due to the presence of 5% dimethylsulfoxide (DMSO) within the freezing medium. Cells were incubated at 37°C with 10% CO_2 and left to proliferate until appropriate confluence was achieved. Once 60-65% confluent, cells were split into multiple plates depending on the number of experiments and left to proliferate until approximately 80% confluent. Caution was taken to avoid letting the cells reach a higher confluence level, as this can reduce adipogenic potential. These cells were then seeded into 6 well plates in preparation for differentiation. Cells ready to be passaged were rinsed with 5mL of phosphate-buffered saline (PBS) solution (Wisent) before adding 1mL of Trypsin and incubating within a humidified 37°C incubator with 10% CO_2 for 1-2 min. The appropriate amount of maintenance medium was then dispensed on the now lifted cells, mixed and then distributed among sterile 10cm dishes containing fresh maintenance medium.

The same passaging process was followed when seeding cells within 6 well plates for differentiation. Cells were then left to reach 100% confluence (Day -2), after which point the cells undergo MCE. Two days post confluence (Day 0), cells were differentiated using DMEM medium

with 5.5mM glucose supplemented with 10% fetal bovine serum (FBS; Wisent, Montreal, QC, Canada) along with 1% P/S (Life Technologies, Burlington, ON, Canada).

Differentiation was induced with 0.5mM IBMX and 100nM insulin (MI) (Roche Diagnostics, Laval, QC, Canada) with either 250nM DEX or varying concentrations of the following bisphenols; bisphenol A (BPA) (0.01µM-20µM; Sigma), bisphenol F (BPF) (0.01µM-20µM; Sigma), tetra methyl bisphenol F (TMBPF) (0.01µM-20µM; Sigma), fluorine-9-bisphenol (BHPF) (0.01µM-20µM; Sigma), bisphenol AP (BPAP) (0.01µM-20µM; Sigma). Solvent controls for these experiments were 0.1% ethanol (for cells treated with DEX) and 0.1% DMSO (for cells treated with 0.01–20 µM BPA, BPF, TMBPF, BHPF, BPAP). On day 2 of the differentiation process medium was replaced with 100nM of insulin plus varying concentrations of the bisphenols listed above or solvent control. Medium was subsequently replenished every two days throughout the differentiation time period (until Day 8) and the indicated time points (Days 2,4,6).

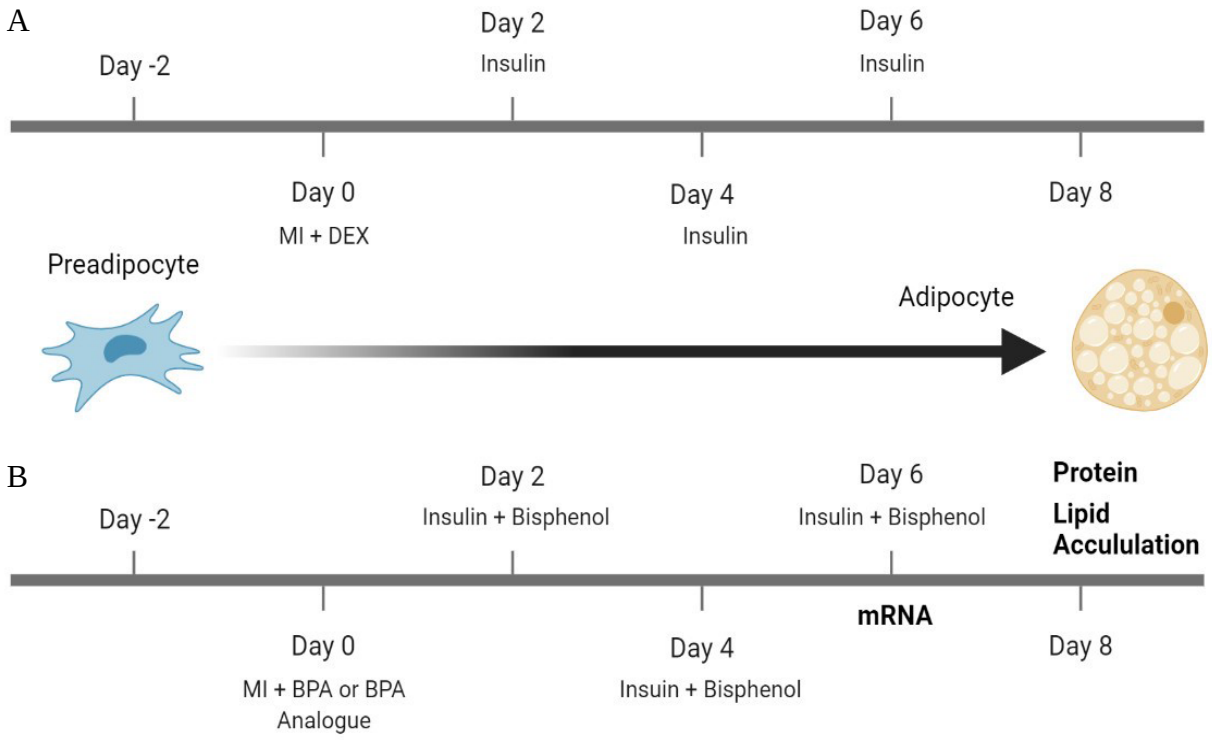


Figure 2: 3T3-L1 cell differentiation timeline. 3T3-L1 preadipocytes were left to reach confluence. Two days post-confluence (Day 0), cells were then treated as in (A) with positive control dexamethasone or as in (B) with varying concentration of BPA or BPA analogues (no dexamethasone). Expression of mature adipocyte makers was assessed on Day 6 for mRNA. On Day 8, protein expression and lipid accumulation were assessed. Figure created with BioRender.

2.2 Real-Time Reverse-Transcriptase PCR Reaction

2.2.1 *RNA extraction and purification*

3T3-L1 adipocytes were harvested on Day 6 of the differentiation process. Total RNA was extracted using reagents from the RNeasy Mini Kit (Qiagen). Genomic DNA was removed using the RNase-Free DNase Kit (Qiagen, Mississauga, ON, Canada) as per the manufacturer's instructions. Cells were lysed using 350µL/well of Buffer RLT in the 6 well plates. A rubber policeman was then used to scrape the wells' surface to ensure maximum lysate collection. Lysates were homogenized with a syringe and needle before being purified or transferred to a 1.5mL Eppendorf tubes for storage at -80^{o}C.

In order to induce the precipitation of nucleic acids out of solution, 350µL of 70% ethanol was added to each Eppendorf tube containing the homogenized lysate and thoroughly mixed. Up to 700µL of sample were then transferred on to a RNeasy spin column placed in a 2mL collection tube supplied by the Qiagen kit and centrifuged (8000 x g) for 20 seconds. Flow through was discarded and 350µL of Buffer RW1 added to the RNeasy spin column which was then centrifuged (8000 x g) again for 20 seconds. A subsequent DNase digestion was performed by the addition of 80 uL of DNase obtained from the RNase-Free DNase Kit to the spin column. Samples were left sitting at room temperature for 15 minutes before the addition of 350µL of Buffer RW1 followed by centrifugation (8000 x g) for 20 seconds. After discarding the flow through, 500µL of Buffer RPE was added to the spin column and centrifuged (8000 x g) for 20 seconds. This was done a second time before all samples were centrifuged for 1 min dry spin to ensure minimal ethanol carryover during the RNA elution. Spin columns were then transferred to new 1.5mL collection tubes and 30uL of RNase-free water added directly on to the spin column membrane. Samples

were again centrifuged (8000 x g) for 1 min to elute the RNA before being flash frozen and stored at -80°C in liquid nitrogen.

2.2.2 *RNA quantification and quality analysis*

To quantify and analyze the quality of RNA in preparation for cDNA synthesis and real-time qPCR, the DS-11 Spectrophotometer (DeNovix) was used. Before measuring the amount of RNA in each of the samples, 1µL of RNase-free water was used as a blank to ensure accurate readings. The amount of RNA in 1µL of each sample was then determined; 500ng of RNA was used for reverse transcriptase reactions.

2.2.3 *Reverse transcription and real-time PCR analysis*

Expression levels of *Lpl, Plin, Ap2, Adipsin, Adiponectin, Pparγ, C/ebpα, C/ebpβ, C/ebpδ* and *GR* were quantified using real-time quantitative PCR (qPCR) using *β-actin* as an internal control. Each reaction consisted of 5µL SYBR Green Master Mix (Applied Biosystems), 0.25µL of primers (forward and reverse), 0.5µL of RNase free water, and 4µL of cDNA. Samples were run in triplicate on 96 well plates (Applied Biosystems) using the CFX96 Real-Time System C1000 Thermal Cycler (BioRad) and quantified using the Bio-Rad CFX Software. All primers used are listed in appendix A.

2.3 Protein Extraction and Western analysis

2.3.1 *Whole-cell extracts*

Whole-cell extracts were prepared on day 8 of the differentiation process. 3T3-L1 cells were washed twice with ice-cold 1X PBS, scraped and collected into 1.5mL eppendorf tubes. The samples were then centrifuged at 4°C for 3 minutes at 4427 x g to facilitate a cell pellet formation

at the bottom of the tube. The supernatant (PBS) was aspirated and the pellet re-suspended in 100 µL of lysis buffer containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA, 0.5% nonidet P-40 and freshly added 1mM DTT and 1X complete protease inhibitor cocktail (Roche). Samples were incubated on ice for 10 minutes before being sonicated for 3-5 minutes at an amplitude of 45 Amperes in the sonicator (QSonica).

2.3.2 *SDS-PAGE and Western transfer*

The Pierce BCA (Bicinchoninic acid) assay (Thermofisher) was used to determine the protein amount in each sample. Based on the strength of the antibody and protein expression level, 10-20µg of protein were prepared using the Laemmli loading buffer (62.5mM Tris pH6.8, 10% Glycerol, 2% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue, 355mM 2-mercaptoeethanol). Samples were then boiled at 95^{o}C for 5 minutes, briefly centrifuged and loaded onto the appropriate gel for electrophoresis. The proteins of interest ranged from 10 - 70 kDa; therefore, 12% SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) gels were used; gels were run at 100V through the stacking gel and 140V through the resolving gel until desired migration was achieved. Gels were transferred at 4^{0}C onto a Polyvinylidene fluoride (PVDF) membrane (BioRad) overnight at 25V for 16 hours following standard transfer protocols.

2.3.3 *Immunoblotting*

Each of the membranes were removed from transfer apparatus and placed in 10mL 1 X TBST (TBS with 0.1% (v/v) of Tween-20 detergent) containing 5% (m/v) skim milk. Membranes were left to block for 1 hour at room temperature or overnight at 4^{o}C, after which membranes were incubated with the primary antibody. This was done in 5mL 1X TBST with 5% dissolved skim milk. Incubation with the primary antibody varied depending on its strength; weak antibodies were

incubated overnight at 4°C while stronger antibodies were incubated for 2 hours at room temperature (see appendix B for details). Before applying the secondary antibody, membranes were rinsed 3 times with approximately 20mL 1X TBST for 5 minutes. The membranes were then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature using a 1:10,000 dilution for anti-rabbit (Jackson ImmunoResearch Laboratories Inc, Baltimore Pike, West Grove, PA) and anti-goat (Jackson ImmunoResearch Laboratories Inc, Baltimore Pike, West Grove, PA) in 5 mL 1 X TBST with 5% skim milk. Membranes were rinsed three times again as previously described to wash off any excess unbound secondary antibody. Membranes were then placed between acetate sheets, and ECL developing reagent mix (enhanced chemiluminescence, Perkin Elmer) was pipetted onto the membranes. They were left at room temperature for 5 minutes before being exposed in the ChemiDoc XRS+ Imaging System (BioRad). After exposure, membranes were rinsed once again, stripped with 5mL re-blot solution (Millipore) for 15 minutes at room temperature and blocked once again as previously described.

2.4 Quantification of lipid accumulation using Nile Red/DAPI Staining

3T3-L1 cells were differentiated in 96- black well plate with clear bottoms (PerkinElmer, Wellesley, MA) for 8 days before being stained. Medium was aspirated from the wells, and cells were washed twice with 1X PBS. Cells were then fixed with 100µL/well of 10% formalin (Electron Microscopy Sciences, Hatfield, PA) for 30 minutes at room temperature. The fixing agent was then removed, and cells rinsed twice with 1X PBS before taking a blank reading of the plate with 100µL of 1X PBS. Two staining reagents were used for lipid quantification; the first was a Nile Red Bio Reagent (Sigma) and the second, 4’6-Diamindino-2-phenylindole dihydrochloride (DAPI) (Sigma). Once the PBS solution was removed, 100µL of the 1µg/mL Nile Red/DAPI solution (0.2% Triton w/v in PBS) was dispensed in each well and left to incubate for

15 minutes at room temperature. Wells were then rinsed three times with 1 X PBS before taking the fluorescence readings. Fluorescence readings were taken using the Synergy H4 Hybrid Reader (BioTek). Readings were taken at 355/360 (excitation/emission) for DAPI and 485/540 for Nile Red.

To visualize lipid accumulation within the cells post staining, a confocal microscope (Leica SP8) was used. Photos taken are a representation of the overall lipid accumulation present within the wells.

2.5 Dual Luciferase Assay

2.5.1 *COS-7 cell culture*

COS-7 (CV-1 in Origin SV40) cells previously obtained from the ATCC were maintained in DMEM 4.5g/L high glucose medium (Gibco, Gaithersburg, MD) supplemented with 10% FBS and 1% P/S. Cells were incubated at 37°C with 10% CO_2. Once cells reached approximately 80% confluence, they were seeded into 12 well plates using charcoal-stripped FBS and phenol red-free DMEM high glucose media. Approximately $3X10^5$ cells were seeded in each well and left to reach confluence before being transfected with the appropriate plasmids.

2.5.2 *Transfection and Luciferase Assay*

Close to 100% confluence, cells were transfected using FuGENE HD reagent (Promega) as recommended by the manufacturer. The ratio of FuGENE: DNA used for transfections was 3µL:1µg. Cells were transfected with 100ng of the mouse mammary tumor virus (MMTV) -promoter Firefly Luciferase reporter plasmid, 25ng of the pTL-GR construct, 10ng of PRL-CMV (Cytomegalovirus-driven Renilla-Luciferase reporter plasmid) as an internal control and 356ng of

Salmon Sperm DNA (SS DNA) to bring the total transfected DNA to 500ng per well. The appropriate volume of FuGENE HD reagent was added to a 5mL polystyrene tube followed by the addition of DNA. After a brief vortex, the tube was left to incubate for 15 min at room temperature. The appropriate amount of Serum Free and Phenol Red Free 4.5g/L DMEM medium was added, gently mixed, and then dispensed into each well in the 12 well plates (50µL/well). The next day, 16-24 hours after the transfection, cells were treated with either 0-20µM TMBPF or BPA, solvent control or 1µM DEX in charcoal-stripped FBS and Phenol Red Free high glucose medium. The following day (16-24h later), medium was removed from cells and rinsed with 1 X PBS before being lysed with 150µL/well of 1 X Passive Lysis Buffer obtained from the Dual-Luciferase Assay Kit (Promega). The 12 well plates were left at room temperature on a shaker for 10-15 min in order for the cells to lyse. Luciferase assay was conducted using an opaque 96 well plate; 20µL of lysate from each sample was dispensed per well. Using the substrates provided in the Dual-Luciferase Assay Kit (Promega), luciferase activity of each of the samples was read using the Glow Max 96 Microplate Luminometer (Promega) with the Glowmax software. Experiments were performed in triplicate and expressed in relative luciferase units.

2.6 AlamarBlue Assay

To assess the effects of these bisphenols on 3T3-L1 cell viability an alamarBlue assay was performed. AlamarBlue is an indicator also known as resazurin that is virtually non-fluorescent. This cell permeable, non-toxic reagent is used to determine cell viability by measuring reduction-oxidation (REDOX) reactions occurring in the cell, particularly in the mitochondria. Thus upon entering living cells, resazurin is reduced to resorufin, which in turn fluoresces. The intensity of this fluorescence can be quantified and is indicative of the number of metabolically healthy cells undergoing cellular respiration (Rampersad, 2012). 3T3-L1 cells were grown and maintained as

previously described. Once cells reached the appropriate confluence, cells were seeded in 96 black well with clear bottom plates (Costar) at 2500 cells/well. Cells were left in the incubator at 37°C with 10% CO_2 to adhere overnight. The following day, medium was aspirated and cells treated with 200µL of varying concentrations (0.005-100µM) of the bisphenol analogues (BPA, TMBPF, BPF, BHPF and BPAP). Cells were then left to incubate for 48 hours before removing 100µL of medium from each of the wells and adding 25µL of CellTiter-Blue (Promega) to each test well. The plate was then incubated for 1 hour before fluorescence readings were obtained using the BMG Flow star (Labtech) and Optima Software. Fluorescence readings were taken at 530/590 (excitation/emission).

2.7 Statistical Analysis

All P-values were calculated using the SigmaPlot 12.5 software using either a one-way or two-way ANOVA followed by Tukey's post-hoc analysis or a t-test. One-way and Two-way ANOVA followed by Tukey's post-hoc analysis were used to determine statistical significance between multiple means. A t-test was used to determine the statistical significance between two means.

3. RESULTS

3.1 Investigating the effects of BPA and its analogues (TMBPF, BPAP, BPF, BHPF) on 3T3-L1 cell viability at varying concentrations

To assess the effects of these bisphenols on 3T3-L1 cell viability an alamarBlue assay was performed. Cells were grown as previously described in the Material and Methods section. Cells were then seeded in 96 black well plates with clear bottoms until 60-70% confluent and treated with varying concentrations of each of the bisphenols the following day (0-100µM). After treatment with the redox dye, CellTiter-Blue, fluorescence readings were taken.

The results revealed that most bisphenols tested in this study were not highly cytotoxic with the exception of TMBPF which reduced cell viability by approximately 50% at 50µM and 100µM. BPAP and BHPF were also found to be cytotoxic only at 100µM. BPA and BPF did not affect cell viability at any of the tested concentrations (Figure 3).

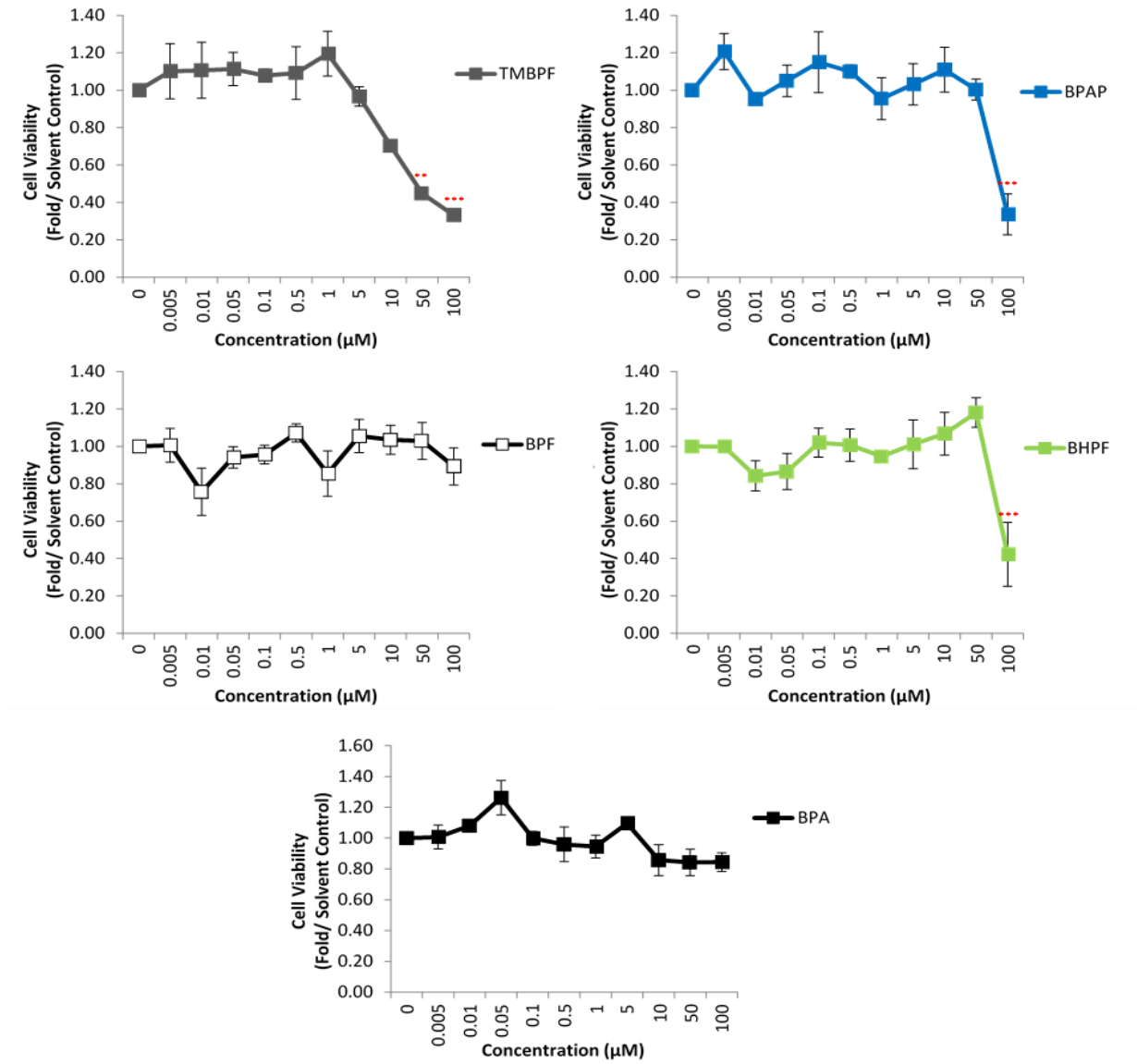


Figure 3: Effects of BPA and its analogues on 3T3-L1 cell viability. Murine 3T3-L1 preadipocytes were treated with varying concentrations of either solvent control (DMS0), BPA, TMBPF, BPAP, BPF or BHPF (0.005 – 100µM). Cell viability was assessed using CellTiter-Blue (Promega). Results were normalized to a blank well reading and expressed as fold over DMSO (MI) control. Results from 3 experiments are graphically represented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis. Experiments performed by Marc Rigden.

3.2 Effects of BPA analogues (TMBPF, BPAP, BPF, BHPF) on adipocyte differentiation

The model system being used for these experiments is the 3T3-L1 mouse embryonic preadipocyte cell line (Green and Meuth, 1974). It is well established in the literature that 3T3-L1 cells treated with insulin, IBMX and DEX achieve 90-100% differentiation. Previous experiments in the lab (unpublished data) showed that IBMX and insulin are required for BPA-mediated adipogenesis. Thus, in order to assess the effects of the BPA analogues (TMBPF, BPAP, BPF, and BHPF), we treated the cells with 0.01-20μM of the BPA analogue in the presence of IBMX and insulin. We then looked at the gene expression of mature adipocyte markers. Based on previous experiments in the lab, mRNA was extracted after 6 days of exposure to the adipogenic protocol (Atlas et al., 2014). As previously mentioned *Pparγ* and *C/ebpα* are the two most important transcription factors within the cascade governing adipogenesis. Upregulation of these two transcription factors leads to downstream expression of the mature adipocyte markers *Adipsin*, *Adiponectin*, *Perilipin* (*Plin*), *Fatty acid binding protein 4* (*Fabp4*) and *Lipoprotein lipase* (*Lpl*). Therefore, we first examined mRNA expression levels of *C/ebpα* and *Pparγ*.

3.2.1 *BPA, TMBPF and BHPF up-regulate the mRNA levels of Pparγ*

Our results for expression of *Pparγ* are depicted in Figure 4A. The data show that expression of *Pparγ* was increased by BHPF at 1 and 10μM to 1.94 and 1.86-fold over MI control respectively. BPA increased *Pparγ* mRNA expression levels by 2.2-fold at 20μM as compared to MI. TMBPF also increased expression of *Pparγ* 2.5-fold at 20μM (Figure 4A, left panel). Cells treated with our positive control (250nM DEX) showed an 8.9-fold increase over MI control in *Pparγ* expression levels (Figure 4A, right panel). BPAP and BPF did not have an effect on *Pparγ* expression in this model (Figure 4A, left panel).

3.2.2 *BPA, TMBPF and BPF up-regulate the mRNA levels of C/ebpα*

BPA, TMBPF and BPF also induced significant expression of *C/ebpα* (Figure 4B, left panel). BPA increased *C/ebpα* expression levels 3.47 and 4.26-fold at 10 and 20μM respectively. TMBPF induced elevated expression of *C/ebpα* as well at 10 and 20μM; mRNA levels reached 3.12-fold over MI control at 10μM and 3.47-fold over MI control at 20μM. At 10 and 20μM, BPF also increased mRNA expression levels of *C/ebpα* to 3.04 and 3.52-fold respectively. Cells treated with DEX showed a 7.48-fold increase in mRNA expression of *C/ebpα* (Figure 4B, right panel).

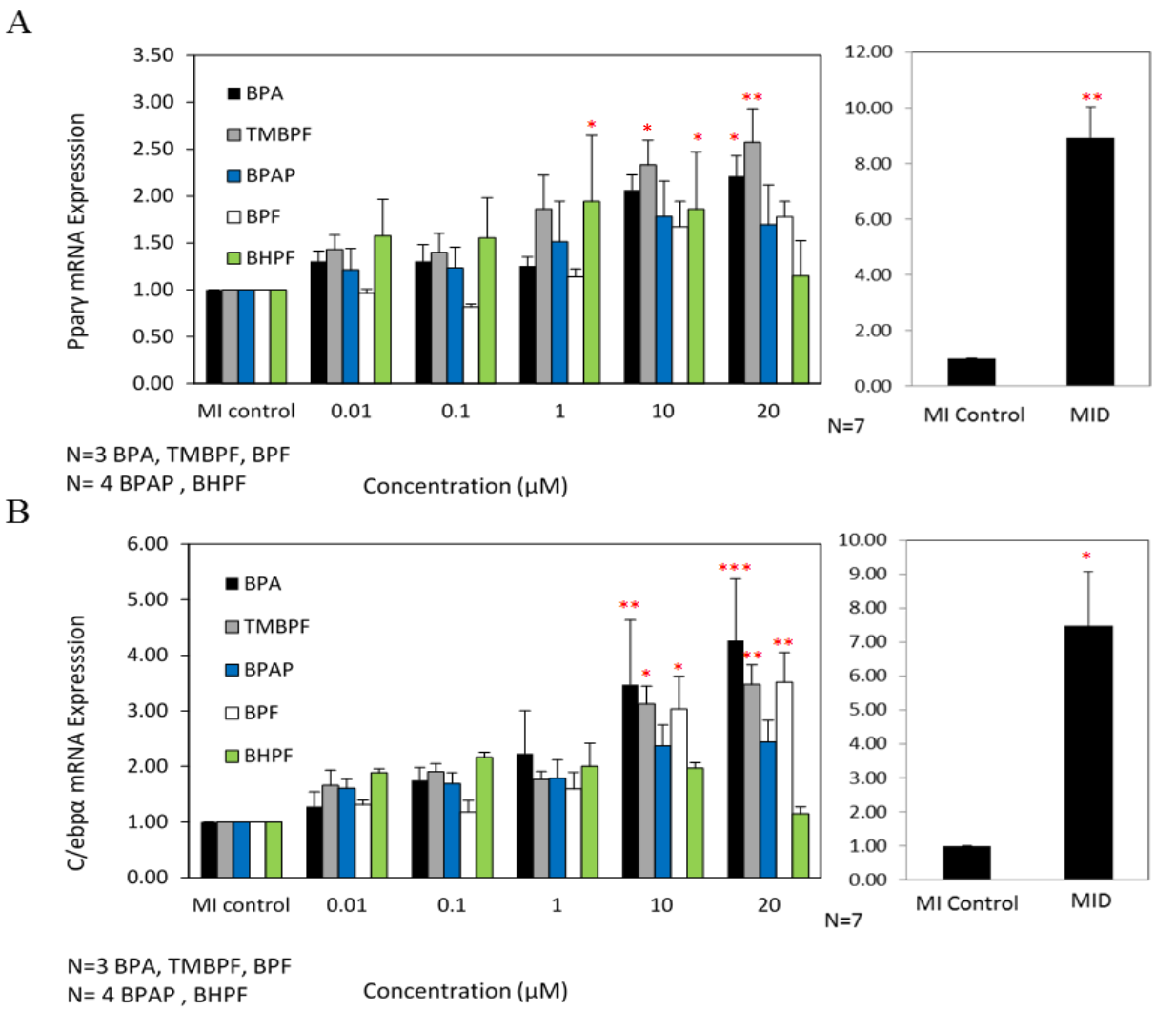


Figure 4: The effects of BPA and its analogues on mRNA expression levels of transcription factors *Pparγ* and *C/ebpα* in differentiation 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 6 Days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), BPA, TMBPF, BPAP, BPF, BHPF (0 – 20µM) or DEX as a positive control. At day 6 of differentiation RNA was isolated and the expression levels of transcription factors, *Pparγ* (A) and *C/ebpα* (B) were quantified by real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control, * P<0.05, ** P<0.01, *** P<0.001 relative to dose-matched BPA treatment using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.2.3 *BPA, TMBPF, and BPF up-regulate the mRNA levels of Adipsin*

Figure 5 depicts the gene expression of adipokines *Adipsin* (Figure 5A) and *Adiponectin* (Figure 5B). Increased expression of *Adipsin* is observed with BPA, TMBPF and BPF at 10μM (Figure 5A, left panel). BPA also increased expression of *Adipsin* at 20μM. BPA increased expression levels of *Adipsin* 2.78-fold over MI control at 10μM and 2.7-fold over MI control at 20μM. TMBPF increased expression levels of *Adipsin* 3.16-fold over MI control at 10μM. BPF increased mRNA expression levels of *Adipsin* 2.18-fold over MI control at 10μM. Treatment of cells with our positive control DEX increased *Adipsin* mRNA expression 7.48-fold over MI control (Figure 5A, right panel). The other bisphenols (BPAP and BHPF) did not have an effect on expression levels of *Adipsin* (Figure 5A, left panel).

3.2.4 *BPA, TMBPF, and BPF up-regulate the mRNA levels of Adiponectin*

Expression levels of *Adiponectin* were increased by BPA and BPF and TMBPF at 10 and 20μM, with TMBPF increasing *Adiponectin* expression at a concentration as low as 1μM (Figure 5B, left panel). BPA increased *Adiponectin* mRNA expression levels 2.36-fold over MI control at 10μM and 3.07-fold over MI control at 20uM. Elevated mRNA expression was also induced 2.19-fold over MI control at 10μM and 2.34-fold over MI control at 20μM in cells treated with BPF. TMBPF increased *Adiponectin* expression levels 2.09-fold over MI control at 1μM, 3.24-fold over MI control at 10μM and 2.48-fold over MI control at 20μM. Treatment of cells with DEX caused a 8.95-fold increase over MI control in mRNA expression levels of *Adiponectin* (Figure 5B, right panel). BPAP and BHPF did not affect *Adiponectin* mRNA expression levels in this model (Figure 5B, left panel).

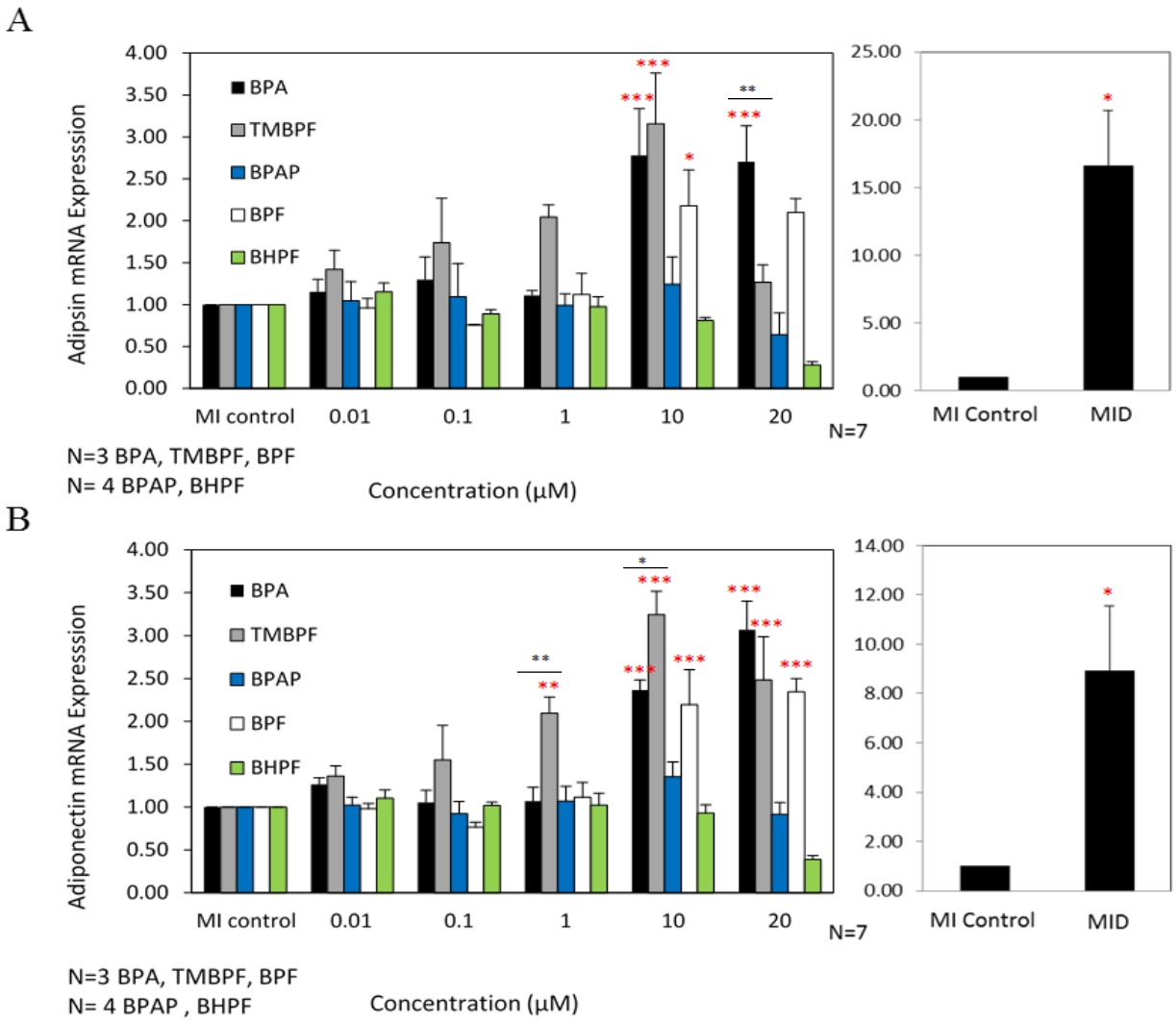


Figure 5: The effects of BPA and its analogues on mRNA expression levels of adipokines in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 6 Days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), BPA, TMBPF, BPAP, BPF, BHPF (0 – 20µM) or DEX as a positive control. At day 6 of differentiation RNA was isolated and the expression levels of adipokines , *Adipsin* (A) and *Adiponectin* (B) were quantified by real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control, * P<0.05, ** P<0.01, *** P<0.001 relative to dose-matched BPA treatment using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.2.5 *BPA, TMBPF and BPF up-regulate the mRNA levels of Fabp4*

Expression levels of mature adipocyte markers *Fabp4*, *Lpl* and *Plin* are shown in Figure 6. The results show that BPA and TMBPF increased mRNA levels of *Fabp4* at 10 and 20 μM. BPF increased *Fabp4* expression at 20 μM (Figure 6A, left panel). BPA increased mRNA expression levels of *Fabp4* 2.95-fold over MI control at 10μM and 3.46-fold over MI control at 20μM. TMBPF induced an increase in *Fabp4* mRNA expression to a greater extent than BPA at 10μM achieving a 4.43-fold increase over MI control. TMBPF also induced a 4.27-fold over MI control increase in *Fabp4* expression at 20μM. BPF increased *Fabp4* expression levels 2.6-fold over MI control at 20μM. Treatment of cells with DEX induced a 9.95-fold increase over MI control in *Fabp4* expression levels (Figure 6A, right panel). BPAP and BHPF did not affect *Fabp4* expression levels in this model (Figure 6A, left panel).

3.2.6 *BPA and TMBPF up-regulate the mRNA levels of Lpl*

Lpl mRNA expression was increased by BPA and TMBPF at 10 and 20 μM (Figure 6B, left panel). BPA increased mRNA expression of *Lpl* 1.63-fold over MI control at 10μM and 1.8-fold over MI control at 20μM. TMBPF increased mRNA expression of *Lpl* to a greater extent than BPA at both 10 and 20μM. At 10μM, TMBPF induced a 2.3-fold over MI control increase in *Lpl* expression levels. At 20μM, TMBPF induced a 2.72-fold over MI control increase. Treatment with the positive control DEX resulted in a 6.63-fold over MI control increase of *Lpl* mRNA expression levels (Figure 6B, right panel). BPAP, BHPF and BPF did not change *Lpl* expression levels in this model (Figure 6B, left panel).

3.2.7 *BPA, TMBPF and BPF up-regulate the mRNA levels of Plin*

BPA, TMBPF and BPF all increased expression of *Plin* (Figure 6C, left panel). BPA and BPF increased its expression at 20 μM while TMBPF increased mRNA levels at 10 and 20 μM. An increase of 3.46-fold was observed in cells treated with 20μM BPA compared to MI control alone. BPF induced a 3.06-fold over MI control increase in mRNA expression levels of *Plin* at 20μM. TMBPF elevated mRNA expression of *Plin* 2.73-fold over MI Control at 10μM and 2.84-fold over MI control at 20μM. Cells treated with the positive control, DEX, exhibited a 16.78-fold increase over MI control in *Plin* expression levels (Figure 6C, right panel). BPAP and BHPF did not increase *Plin* mRNA expression levels in these experiments (Figure 6C, left panel).

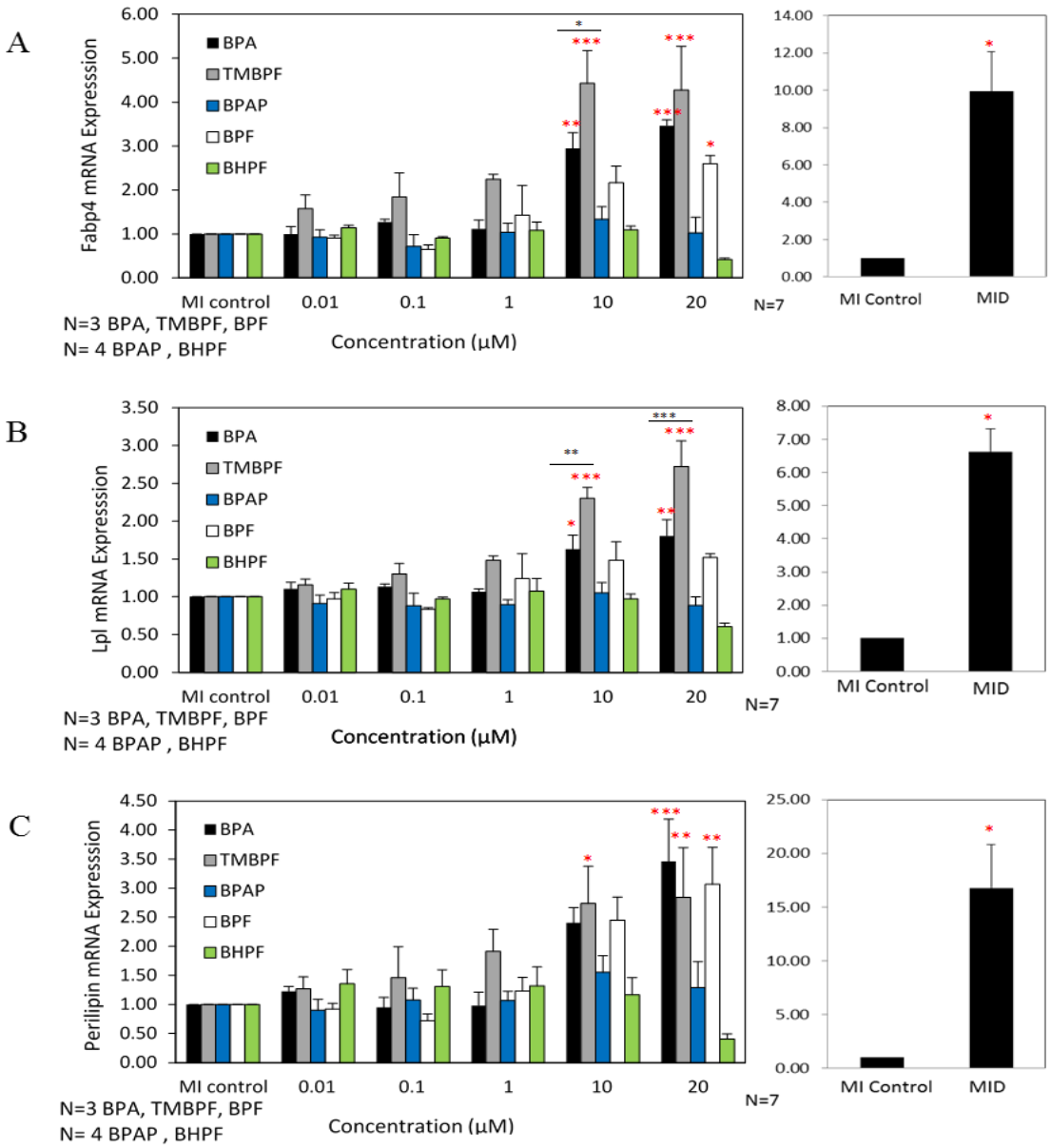


Figure 6: The effects of BPA and its analogues on mRNA expression levels of mature adipocytes markers in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 6 Days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), BPA, TMBPF, BPAP, BPF, BHPF (0 – 20µM) or DEX as a positive control. At day 6 of differentiation RNA was isolated and the expression levels of mature adipocyte markers, *Fabp4* (A), *Lpl* (B) and *Perilipin* (C) were quantified by real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control, * P<0.05, ** P<0.01, *** P<0.001 relative to dose-matched BPA treatment using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.2.8 *BPA and BPF up-regulate the protein expression levels of FABP4*

In addition to assessing mRNA expression levels at day 6, cells were also induced to differentiate for 8 days with induction medium in order to develop into fully mature, lipid-laden adipocytes. Protein expression levels of mature adipocyte markers (Figures 7-9) and lipid accumulation (Figure 8A) were assessed at day 8. Cells were also stained with Nile Red (Figure 8B) on day 8 of induction to visualize and quantify lipid accumulation within these cells.

Protein expression levels of FABP4 were increased by BPA (Figure 7A) and BPF (Figure 7D) at 10 and 20μM. BPA increased FABP4 protein expression 2.75-fold over MI control at 10μM and 3.37-fold at 20μM compared to MI control. BPF increased FABP4 expression 2.96-fold over MI control at 10μM and 3.83-fold at 20μM relative to MI control. Treatment of cells with DEX resulted in a 11.62-fold increase in FABP4 protein expression (Figure 7F). TMBPF (Figure 7B), BPAP (Figure 7C) and BHPF (Figure 7E) did not significantly affect protein expression of FABP4 in this cell model.

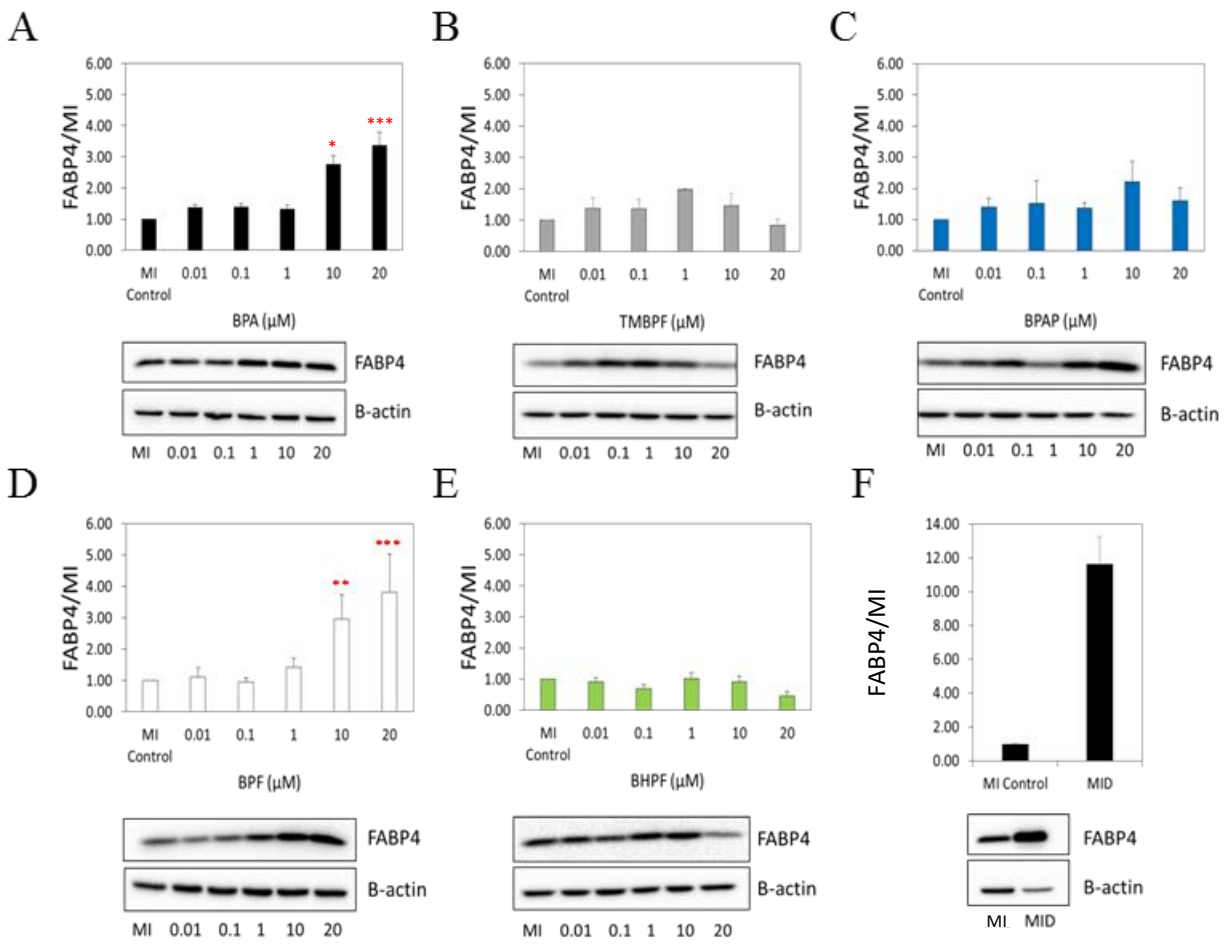


Figure 7: The effects of BPA and its analogues on protein expression levels of FABP4 in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 8 days in the presence of 500μM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF, (E) BHPF (0 – 20μM) or (F) DEX as a positive control. At day 8 of differentiation equal amounts of solubilized cellular protein were separated by SDS-PAGE and immunoblotted with the indicated antibodies. β-actin serves as a loading control. Levels were normalized to endogenous β-actin and expressed as fold over DMSO (MI) control. Results from 3 experiments are graphically represented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a one-way ANOVA followed by Tukey's post-hoc analysis.

3.2.9 *TMBPF and BPF up-regulate the protein expression of LPL*

An increase in LPL protein expression was observed with TMBPF at 1, 10 and 20μM (Figure 9B). BPF also increased expression levels of LPL at 10 and 20μM (Figure 9D). TMBPF increased LPL protein expression 2.27-fold at 1μM, 3.54-fold at 10μM and 3.44-fold at 20μM compared to MI control. Cells treated with DEX exhibited a 28.62-fold increase over MI control in LPL protein expression levels (Figure 9F). BPA (Figure 9A), BPAP (Figure 9C), and BHPF (Figure 9E) did not significantly induce increased expression of LPL in this cell model.

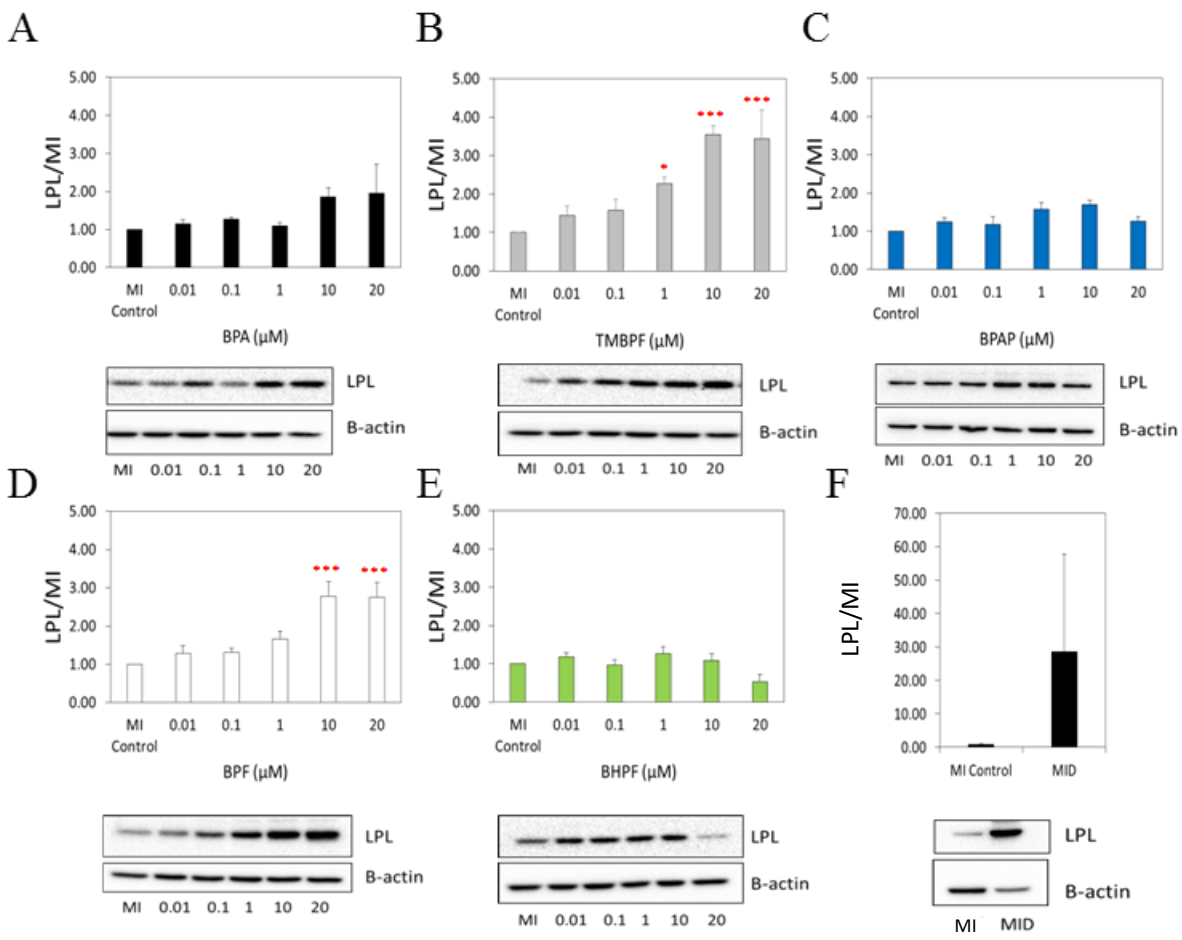


Figure 8: The effects of BPA and its analogues on protein expression levels of LPL in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 8 days in the presence of 500μM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF, (E) BHPF (0 – 20μM) or (F) DEX as a positive control. At day 8 of differentiation equal amounts of solubilized cellular protein were separated by SDS-PAGE and immunoblotted with the indicated antibodies. β-actin serves as a loading control. Levels were normalized to endogenous β-actin and expressed as fold over DMSO (MI) control. Results from 3 experiments are graphically represented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a one-way ANOVA followed by Tukey's post-hoc analysis.

3.2.10 *BPA, TMBPF, BPAP and BPF up-regulate the protein expression of PLIN*

PLIN protein expression was increased by BPA (Figure 8A) and BPF (Figure 8D) at 10 and 20µM where as TMBPF (Figure 8B) and BPAP (Figure 8C) increased PLIN expression at 10µM as compared to MI control. A 6.33-fold over MI control increase was induced by BPA at 10µM and a 8.89-fold over MI control increase at 20µM. BPF increased PLIN protein expression 8.53-fold over MI control at 10µM and 7.70-fold at 20µM compared to MI control. An increase in PLIN protein expression was induced 7.62-fold over MI control at 10µM with TMBPF. BPAP increased PLIN protein levels 6.05-fold over MI control at 10µM as well. Treatment of cells with the positive control DEX (Figure 8F) resulted in a 34.44-fold increase over MI control in PLIN protein expression. BHPF (Figure 8E) showed no response on PLIN protein expression in this model.

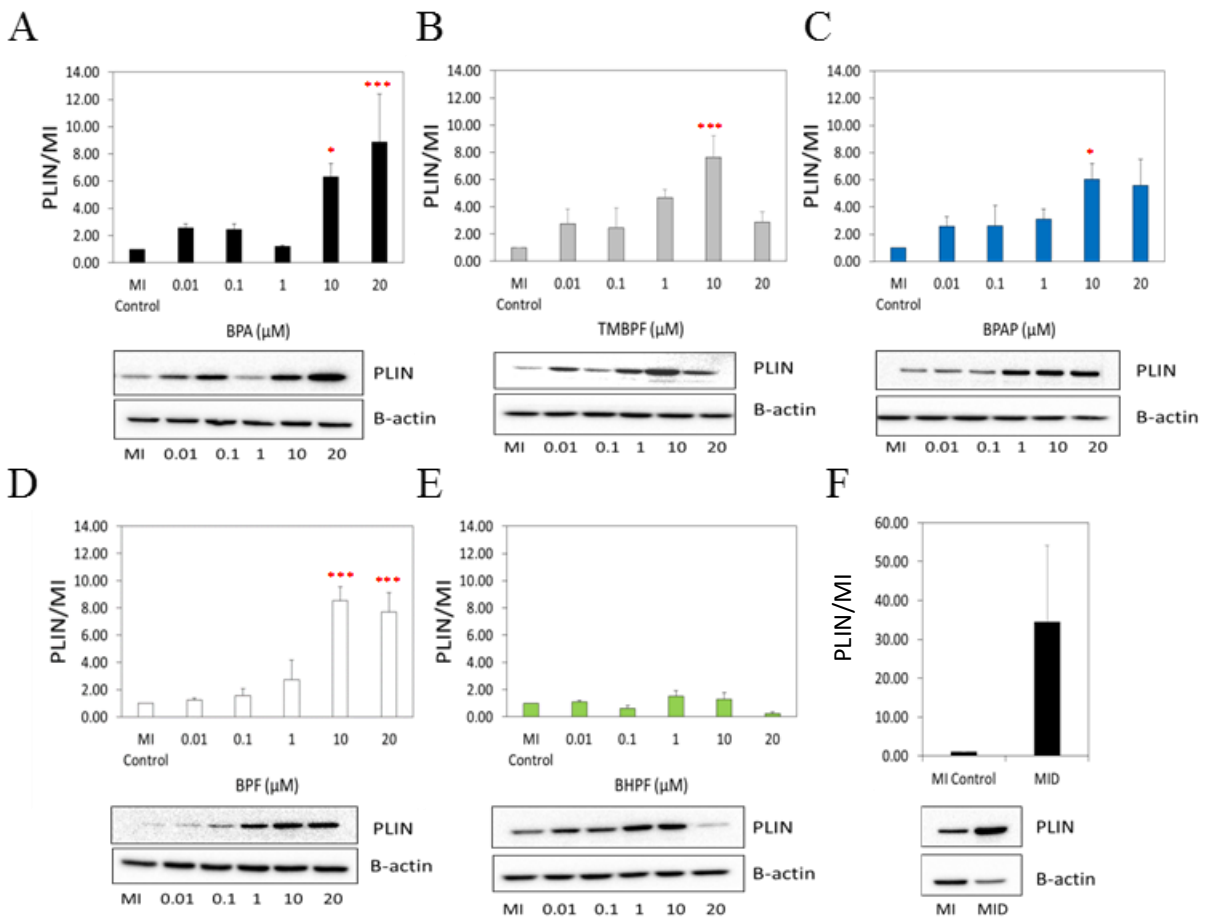


Figure 9: The effects of BPA and its analogues on protein expression levels of PLIN in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 8 days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF, (E) BHPF (0 – 20µM) or (F) DEX as a positive control. At day 8 of differentiation equal amounts of solubilized cellular protein were separated by SDS-PAGE and immunoblotted with the indicated antibodies. β-actin serves as a loading control. Levels were normalized to endogenous β-actin and expressed as fold over DMSO (MI) control. Results from 3 experiments are graphically represented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a one-way ANOVA followed by Tukey's post-hoc analysis.

3.2.11 *BPA and TMBPF induce lipid accumulation within 3T3-L1 preadipocytes*

As described in the materials and methods, cells were induced to differentiate for 8 days before being stained with DAPI and Nile Red. DAPI stains the nucleus of the cells while Nile Red stains the neutral lipid within the cells. Figure 10B depicts the staining of the cells treated with all the tested bisphenols at the varying concentrations. DAPI staining of the nucleus is seen as blue while Nile Red staining of the lipid is seen in green. After measuring the fluorescence of these dyes, analysis of the data revealed increased lipid accumulation with BPA at 10μM and TMBPF at 10 and 20μM (Figure 10A, left panel). BPA induced a 1.57-fold increase in lipid accumulation at 10μM compared to MI control. Cells treated with TMBPF showed a 2.72-fold increase at 10μM and 2.48-fold increase in lipid accumulation at 20μM. TMBPF increased lipid accumulation to a greater extent than BPA and appears to be almost as good as our positive control, DEX. Cells treated with DEX induced a 2.33-fold increase in lipid accumulation compared to MI control (Figure 10A, right panel). BPAP, BHPF and BPF did not significantly increase lipid accumulation in this cell model (Figure 10A, left panel).

Together these results indicate that BPA, BPF and TMBPF but not BPAP and BHPF were adipogenic. TMBPF was more potent than BPA and the other analogues at inducing mRNA expression of *Fabp4*, *Lpl* and *Adiponectin*. TMBPF, BPA and BPF were found to increase expression of Fabp4, Lpl and Plin for both protein and mRNA. Finally, TMBPF induced significantly more lipid accumulation compared to BPA, BPAP, BPF, and BHPF. Furthermore, BPAP, BHPF did not show a response. BPF did increase lipid accumulation, however this was not statistically significant.

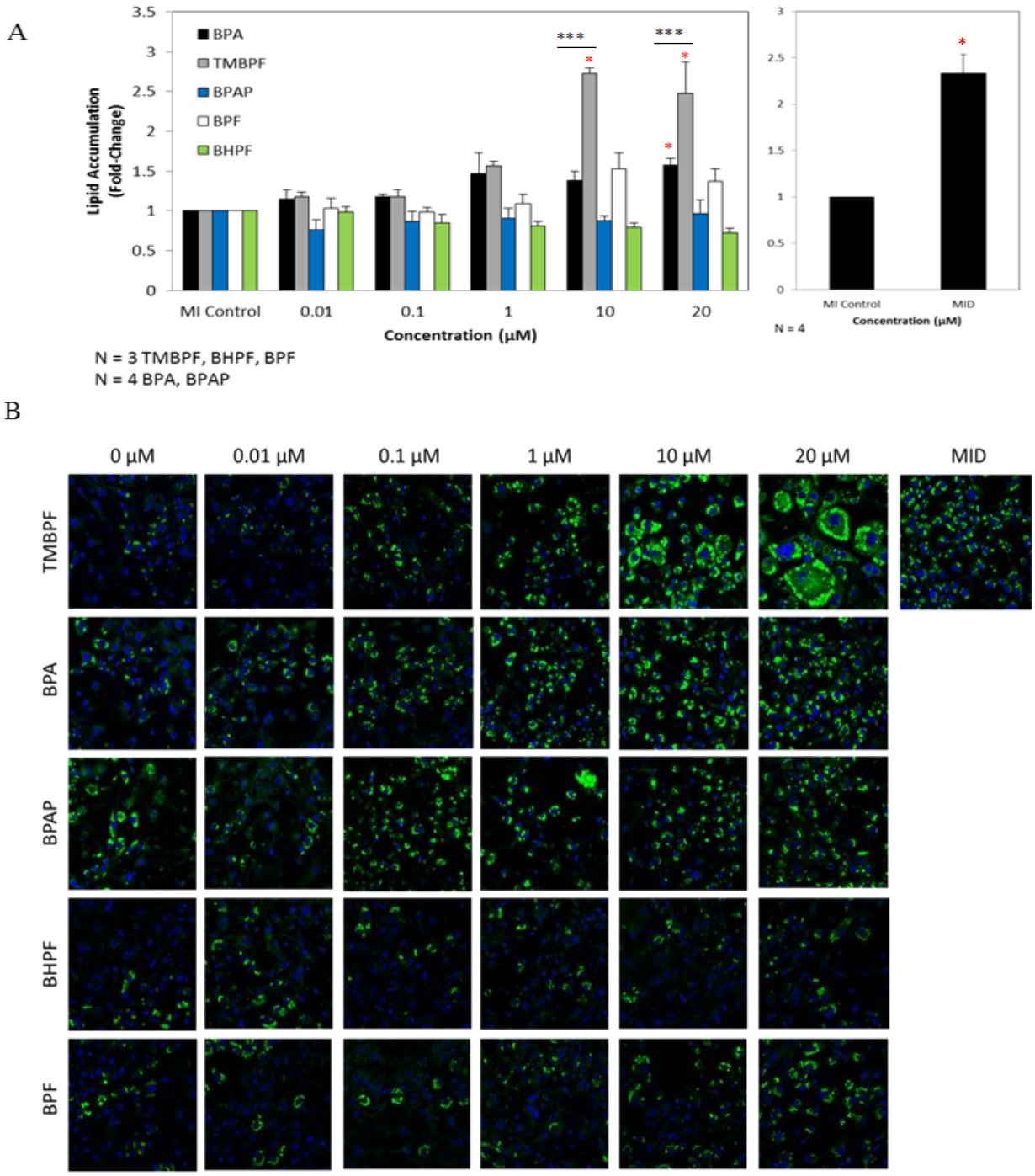


Figure 10: The effects of BPA and its analogues on lipid accumulation in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 8 days in the presence of 500μM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), TMBPF, BPAP, BHPF, BPF, BPA (0 – 20μM), or DEX as a positive control. At day 8 of differentiation (B) lipid accumulation was visualized using Nile Red staining and then quantified (A). Lipid accumulation was normalized to DAPI staining and expressed as fold over DMSO (MI) control.

Results from 3 experiments are graphically represented for TMBPF, BHPF and BPF; 4 experiments for BPA, BPAP and DEX performed in triplicate. Data represent mean ± S.E.M where * $P<0.05$ was determined relative to vehicle control (MI), *** $P<0.001$ relative to concentration matched treatment using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.3 Investigating expression of early and late transcription factors within the adipogenic cascade to explore potential mechanisms of action for BPA and its analogues

In order to determine the mode of action of BPA, TMBPF, BPAP and BPF, we investigated the temporal expression of known transcription factors within the adipogenic cascade. Two-day post-confluent 3T3-L1 preadipoytes were treated with IBMX, insulin and only 10μM of bisphenol (TMBPF, BPAP, BPF, BPA), a concentration which was found to elicit adipogenic effects in mRNA expression, protein expression as well as lipid accumulation. Though BPAP was not found to be adipogenic, it did increase mRNA expression levels of *Pparγ* and *C/ebpα*, thus we were curious to see the temporal expression of these two transcription factors as well. Cells were then harvested at several time points (0, 2, 6, 24, 48, and 96 hours), and mRNA expression levels of the transcription factors were assessed (Figures 11-15).

3.3.1 *TMBPF and DEX up-regulate C/ebpα early on in adipogenesis*

Our results show that TMBPF (Figure 11B) and DEX (Figure 11E) up-regulate *C/ebpα*. TMBPF appears to up-regulate *C/ebpα* 2.61-fold compared to MI control at 6 hours. DEX begins to up-regulate *C/ebpα* 2-fold at 24 hours, but an even greater induction of 7.29-fold over MI control is observed at 48 hours. BPA (Figure 11A), BPF (Figure 11D) and BPAP (Figure 11C) do not show much of a response on *C/ebpα* up-regulation, at these early time points.

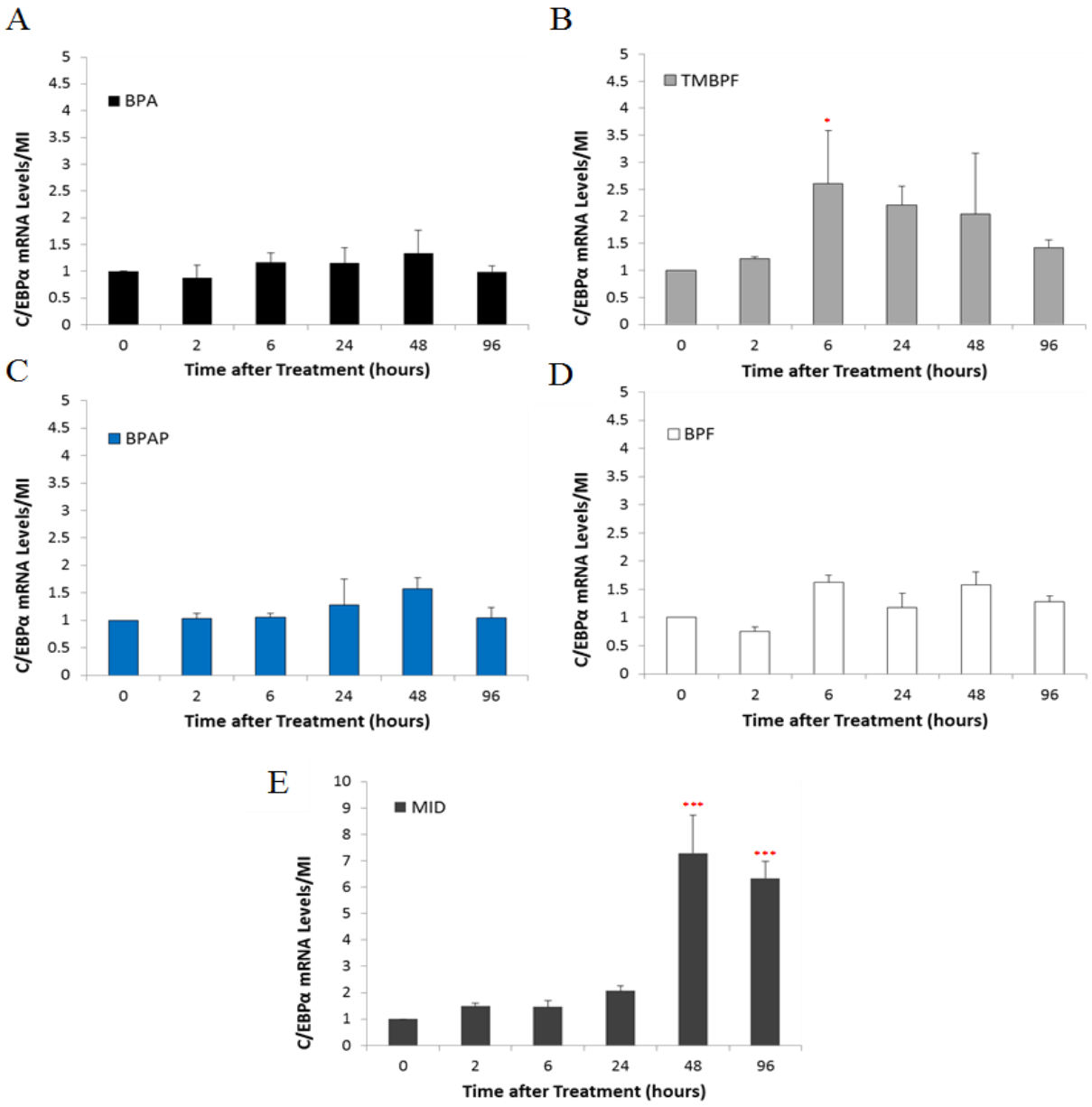


Figure 11: The effects of BPA, TMBPF, BPAP and BPF on temporal expression of *C/ebpα* in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 4 days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF (10µM) (E) or DEX as a positive control. Cells were harvested at the indicated time points (0, 2, 6, 24, 48, and 96 hours). RNA was isolated and the expression levels of *C/ebpα* were quantified using real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control for each time point. Results from 3 experiments are graphically presented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.3.2 *BPF and DEX up-regulate Pparγ early on in adipogenesis*

We also assessed the temporal expression of *Pparγ* during treatment with BPA (Figure 12A), TMBPF (Figure 12B), BPAP (Figure 12C) and BPF (Figure 12D). BPF up-regulated *Pparγ* expression roughly 2-fold over MI control at 2 hours. DEX began to up-regulate *Pparγ* at 2 hours and continued to increase its expression as peak levels were achieved at 48 hours. Increased expression of *Pparγ* is induced to 6.42-fold over MI control at 48 hours and 8.63-fold over MI control at 96 hours in cells treated with DEX (Figure 12E).

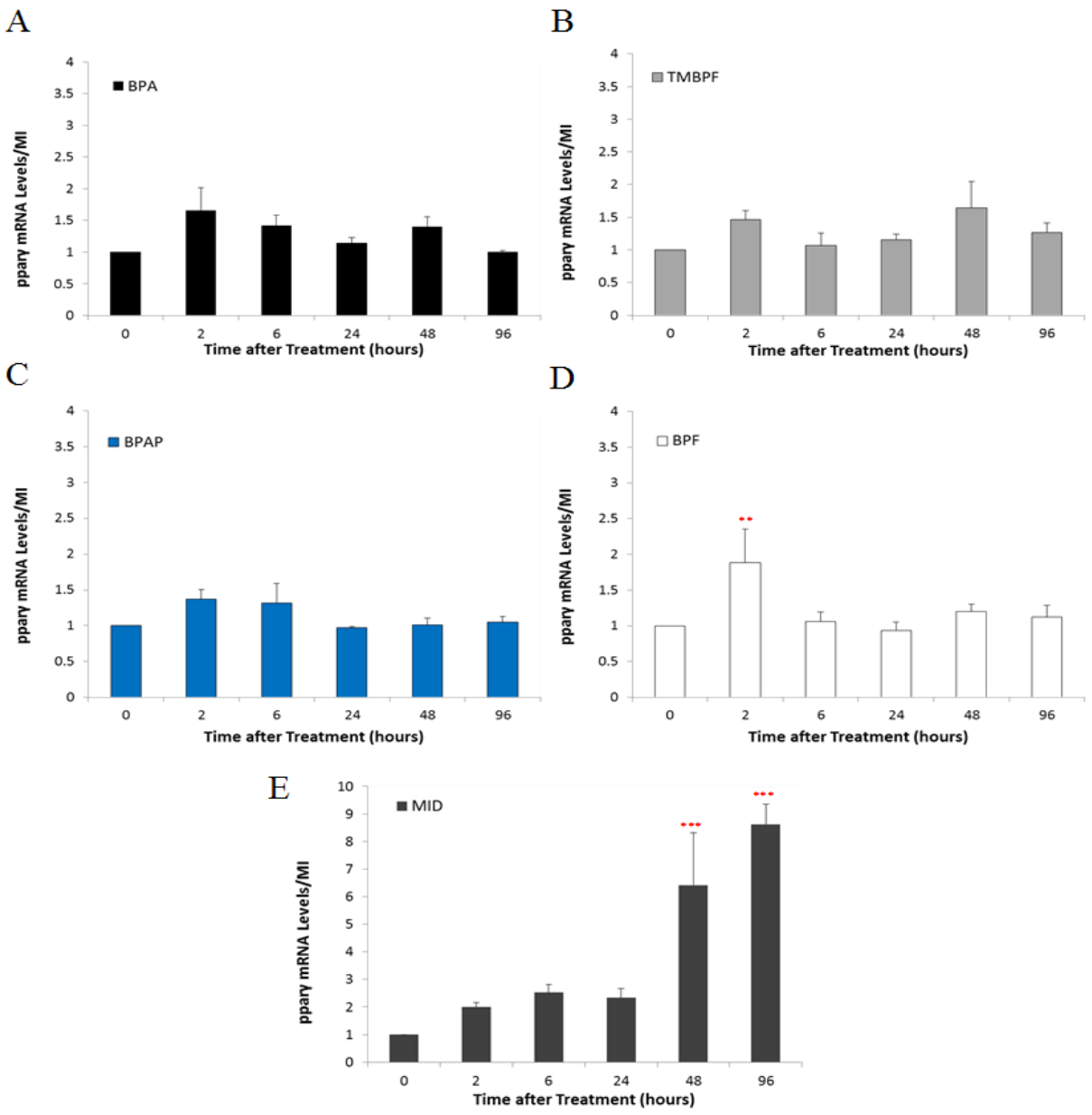


Figure 12: The effects of BPA, TMBPF, BPAP and BPF on temporal expression of *Pparγ* in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 4 Days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF (10µM) (E) or DEX as a positive control. Cells were harvested at the indicated time points (0, 2, 6, 24, 48, and 96 hours). RNA was isolated and the expression levels of *Pparγ* were quantified using real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control for each time point. Results from 3 experiments are graphically presented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.3.3 *BPA, TMBPF and DEX up-regulate one of the early transcription factors, C/ebpδ, within the adipogenic cascade*

BPA, TMBPF and DEX up-regulate *C/ebpδ* early on in apdipogenesis. Up-regulation of *C/ebpδ* is seen as early as 2 hours in cells treated with BPA where a 1.56-fold increase over MI control in expression is achieved (Figure 13A). TMBPF also up-regulated *C/ebpδ* at the 2-hour time point and the expression maintained until 6 hours before levelling back down. A 1.76-fold increase over MI control was observed at 2 hours and a 1.67-fold increase over MI control at 6 hours in cells treated with TMBPF (Figure 13B). Our positive control, DEX, which is known to bind and activate the glucocorticoid receptor (GR) showed a 4.39-fold up-regulation over MI control of *C/ebpδ* at the 2-hour time point (Figure 13E). Within the adipogenic cascade, activation of GR causes the receptor to translocate from the cell's cytoplasm to the nucleus where it can bind GREs within the promoter of *C/ebpδ*. Once up-regulated, C/EBPδ can transcriptionally up-regulate *Pparγ* (Hamm et al., 2001; Salma et al., 2006), thus inducing adipogenesis. BPAP (Figure 13C) and BPF (Figure 13D) did not induce up-regulation of *C/ebpδ* at any of the experimental time points.

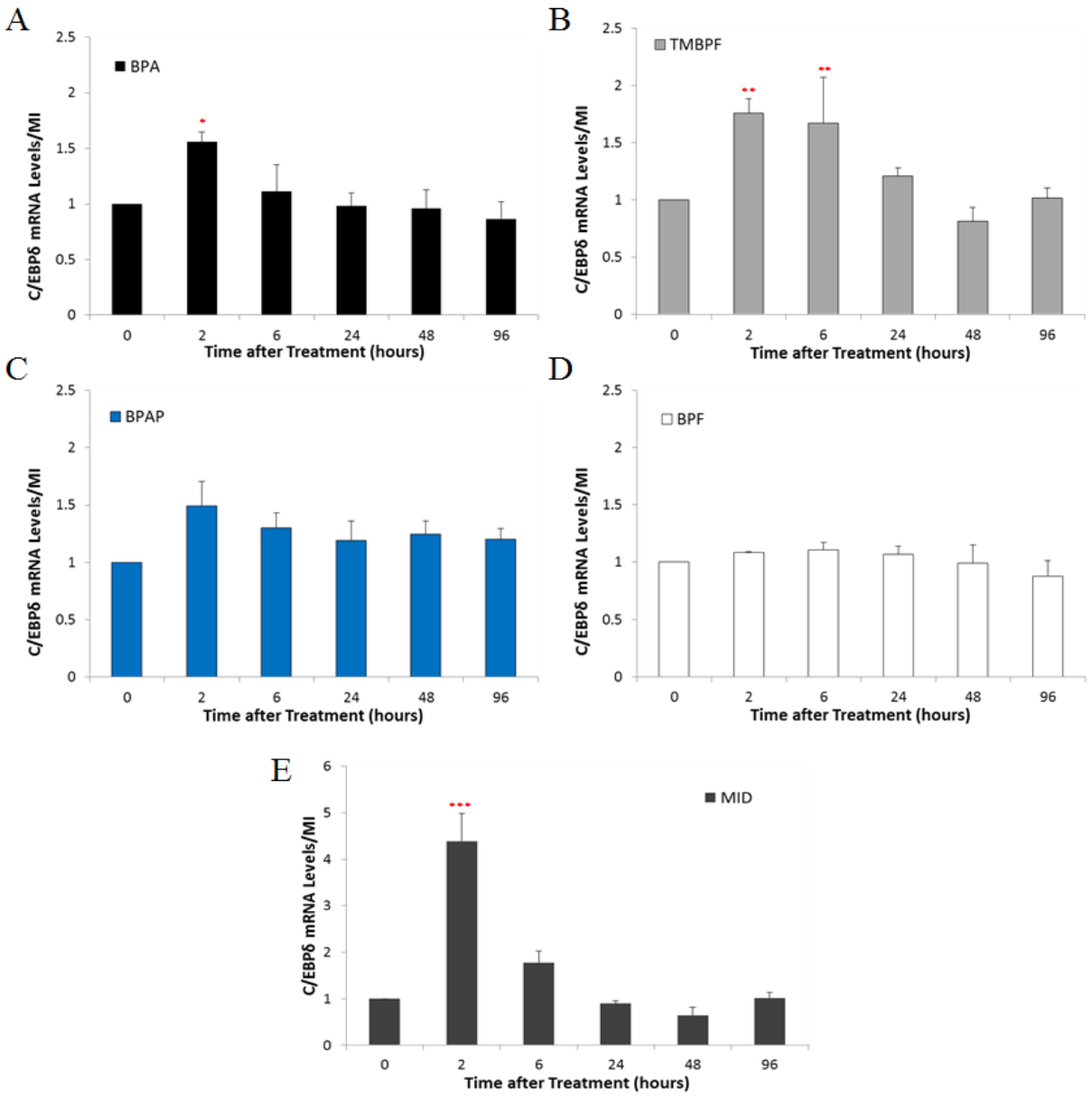


Figure 13: The effects of BPA, TMBPF, BPAP and BPF on temporal expression of *C/ebpδ* in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 4 days in the presence of 500 µM IBMX, 100 nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF (10 µM) (E) or DEX as a positive control. Cells were harvested at the indicated time points (0, 2, 6, 24, 48, and 96 hours). RNA was isolated and the expression levels of *C/ebpδ* were quantified using real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control for each time point. Results from 3 experiments are graphically presented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.3.4 *TMBPF, BPAP and MID increase early protein expression of C/EBPδ*

Protein expression levels of C/EBPδ were also assessed in addition to looking at mRNA expression levels (Figure 14E). Our results showed an early increase of roughly 1.5-fold over MI control in protein expression of C/EBPδ when treated with 10μM BPAP at 6 hours (Figure 14C). DEX increased protein expression levels of C/EBPδ approximately 2-fold at 2,4 and 6 hours (Figure 14D). BPA (Figure 14A) and TMBPF (Figure 14B) did not significantly affect protein expression of C/EBPδ. We did not assess protein expression levels when treated with BPF as mRNA expression was not affected by this bisphenol.

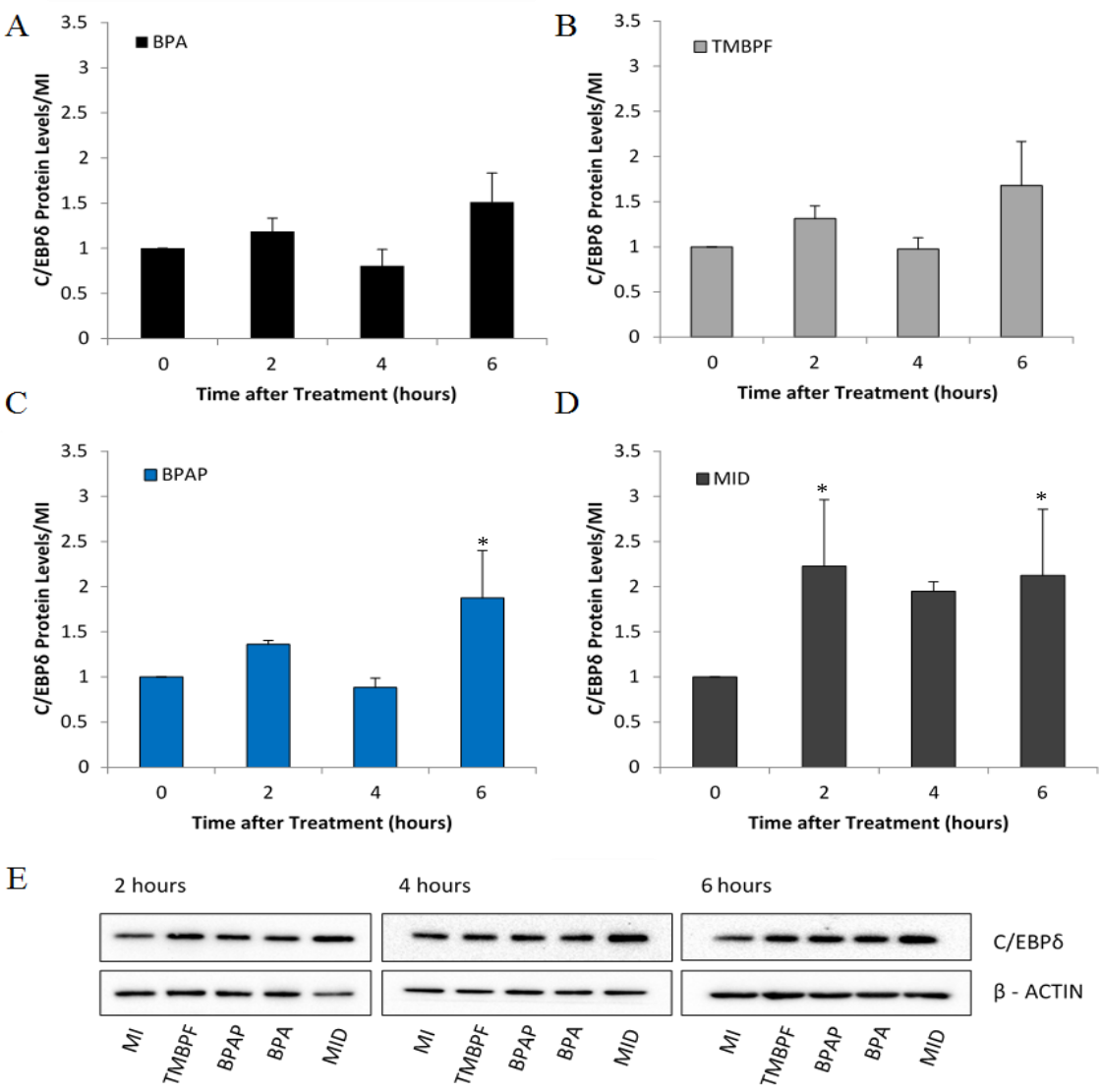


Figure 14: The effects of BPA, TMBPF, BPAP and BPF on temporal protein expression of C/EBPδ in differentiation 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 6 hours in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP (10µM) or (D) DEX. (E) Cells were harvested at the indicated time points (0, 2, 4, and 6 hours). Equal amounts of solubilized cellular protein were separated by SDS-PAGE and immunoblotted with the indicated antibodies. β-ACTIN serves as a loading control. Levels were normalized to endogenous β-ACTIN and expressed as fold over DMSO (MI) control. Results from 3 experiments are graphically represented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.3.5 *BPA, TMBPF, BPF and BPAP do not affect temporal expression of C/ebpβ or GR*

Treatment of cells with 10μM BPA, TMBPF, BPF or BPAP did not have an effect on the temporal expression of *C/ebpβ* (Figure 15A-D). DEX on the other hand showed increased expression levels at 96 hours and induced a 2.74-fold increase over MI control of *C/ebpβ* (Figure 11E). Temporal expression of *GR* also remained stagnant up treatment with BPA and its analogues. Figure 16 (A-D) depicts the temporal expression of *GR* upon treatment with 10μM BPA, TMBPF, BPF or BPAP. Treatment with DEX induced a 1.97-fold increase over MI control in *GR* expression as expected. This occurs as early as 2 hours (Figure 16E).

In summary, temporal expression for 3 early (*C/ebpβ*, *C/ebpδ* and *GR*) and 2 later transcription factors (*C/ebpα*, *Pparγ*) was assessed. Of the early transcription factors, BPA and TMBPF up-regulated mRNA expression of *C/ebpδ* as early as 2 hours. However, no effect was observed for protein expression of C/EBPδ at the protein level for TMBPF. Expression of the late transcription factors *C/ebpa* and *Pparγ* were increased by TMBPF and BPF respectively. TMBPF increased expression of *C/ebpa* at 6 hours while BPF increased expression of *Pparγ* at 2 hours.

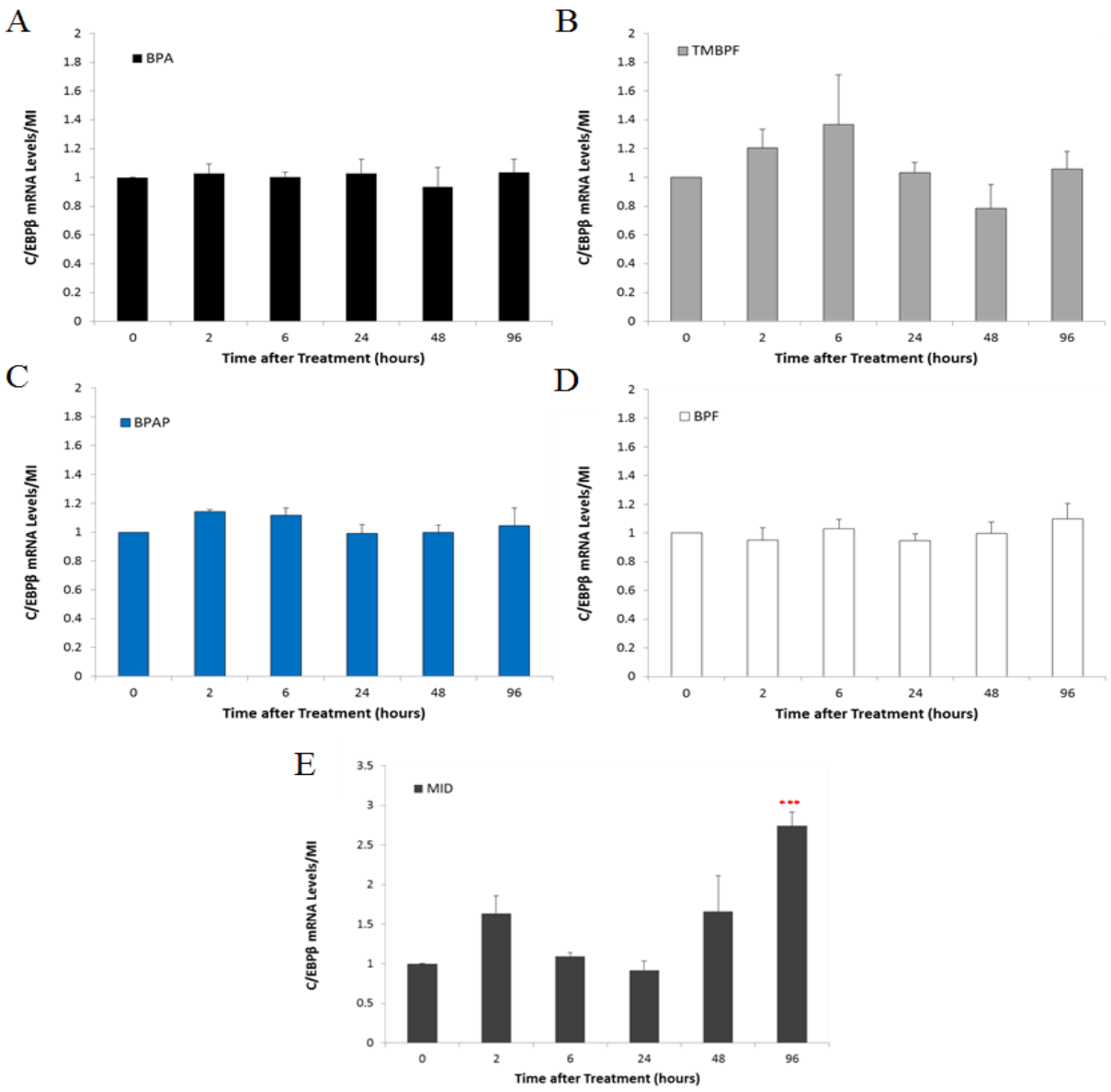


Figure 15: The effects of BPA, TMBPF, BPAP and BPF on temporal expression of *C/ebpβ* in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 4 Days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF (10µM) (E) or DEX as a positive control. Cells were harvested at the indicated time points (0, 2, 6, 24, 48, and 96 hours). RNA was isolated and the expression levels of *C/ebpβ* were quantified using real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control for each time point. Results from 3 experiments are graphically presented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis.

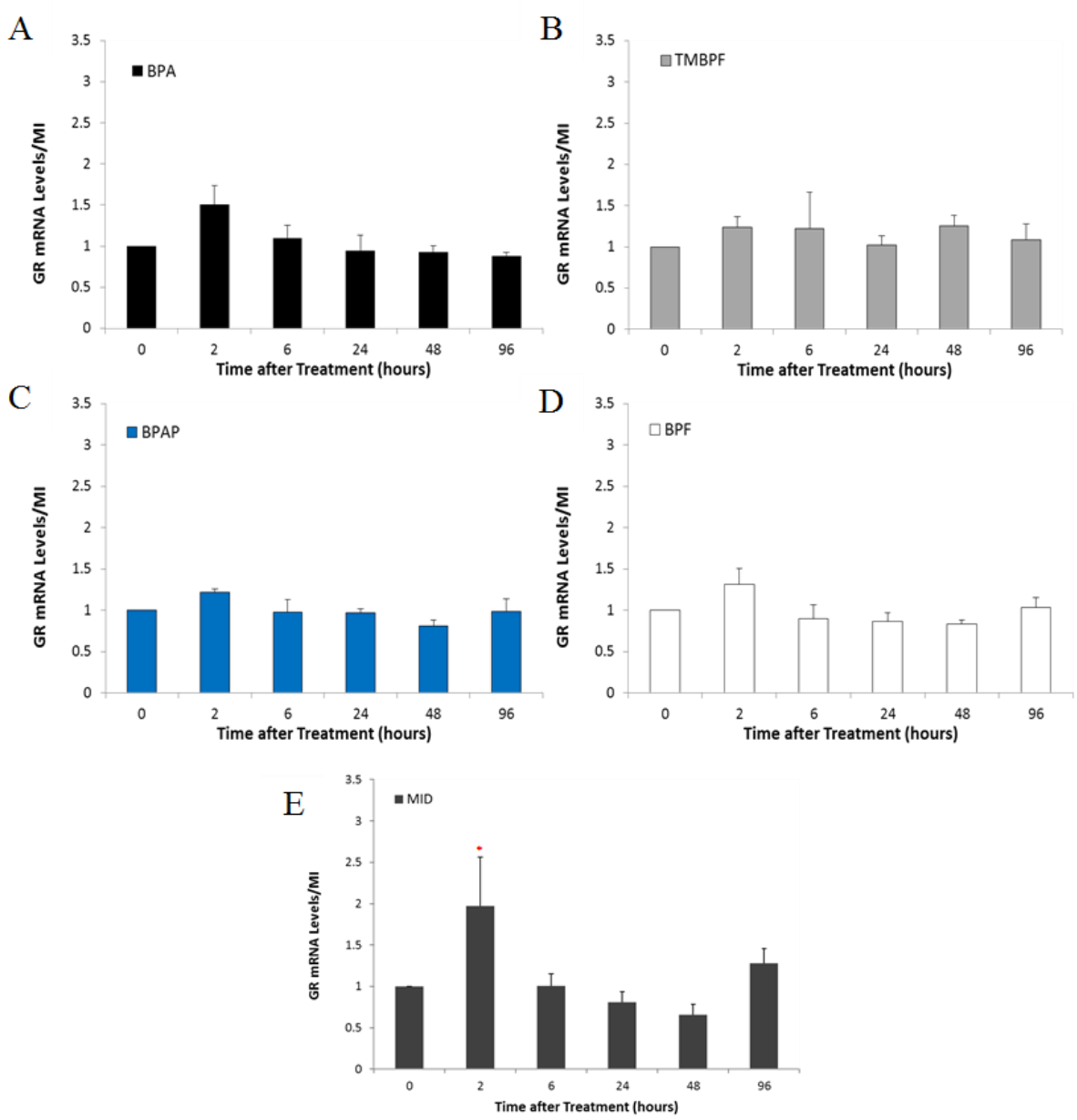


Figure 16: The effects of BPA, TMBPF, BPAP and BPF on temporal expression of *GR* in differentiating 3T3-L1 cells. Murine 3T3-L1 preadipocytes were induced to differentiate for 4 Days in the presence of 500µM IBMX, 100nM insulin, and supplemented with either solvent control (DMS0), (A) BPA, (B) TMBPF, (C) BPAP, (D) BPF (10µM) (E) or DEX as a positive control. Cells were harvested at the indicated time points (0, 2, 6, 24, 48, and 96 hours). RNA was isolated and the expression levels of *GR* were quantified using real-time qPCR. Levels were normalized to endogenous *β-actin* levels, and expressed as fold over the solvent control for each time point. Results from 3 experiments are graphically presented. Data represent the mean ± S.E.M where * P<0.05, ** P<0.01, *** P<0.001 relative to vehicle control using a two-way ANOVA followed by Tukey's post-hoc analysis.

3.3.6 *BPA and TMBPF do not transcriptionally transactivate GR on the MMTV promoter*

Based on the fact that *C/ebpδ* is a direct transcriptional target of GR, we hypothesized that TMBPF may activate this receptor, thus launching the adipogenic cascade. In order to assess this possibility, we performed a luciferase assay by transfecting COS-7 cells with a plasmid containing the promoter for the MMTV, a promoter known to be highly responsive to glucocorticoids (Atlas et al., 2014). The cells were also transfected with the expression vector, pTL-GR, and reporter plasmid pRL-CMV as an internal control. Following transfection, cells were treated with varying concentrations of BPA, TMBPF (0-20μM) or DEX being a positive control. After 24 hours of treatment, luciferase activity was assessed (Figure 17). The results confirm that DEX activates GR leading to nearly 200-fold increase in the luciferase activity of the MMTV promoter linked with the luciferase gene. TMBPF, on the other hand, did not result in transcriptional transactivation of GR on the MMTV promoter.

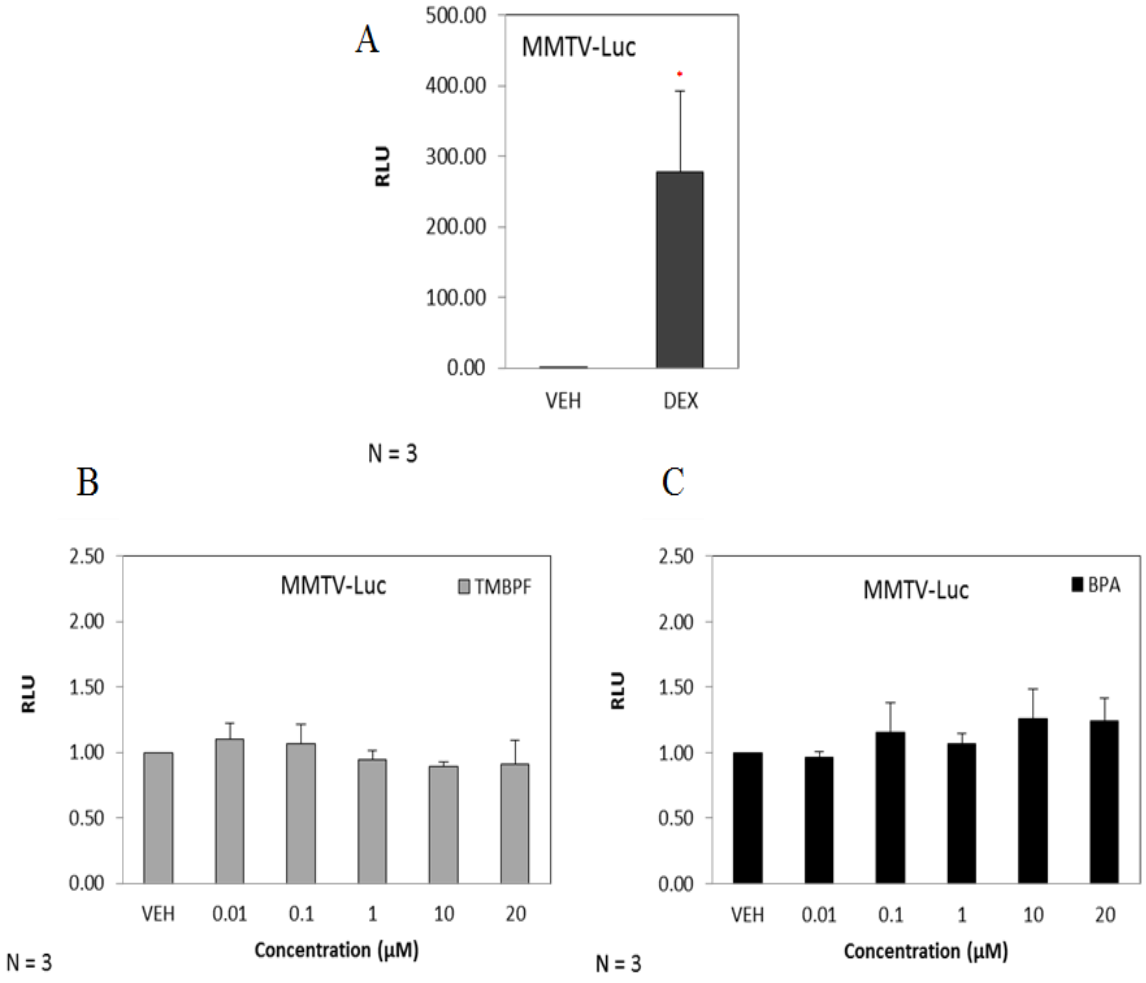


Figure 17: Effects of TMBPF, BPA and DEX on transcriptional transactivation of the glucocorticoid receptor on the MMTV promoter. Non-GR expressing COS-7 cells were transfected with 100ng MMTV-Luc, 25ng PTL-GR and 10ng pRL-CMV as an internal control. Following transfection cells were treated with (A) 1µM DEX or the indicated concentrations of (B) TMBPF, or (C) BPA. Luciferase activity is normalized to pRL-CMV of pTL-GR on the MMTV promoter, 16-24 h after treatment. Graphs show average relative luciferase units (RLU) (n = 3). Data represent the mean ± S.E.M where * $P<0.05$ using a t-test.

4. DISCUSSION

Early exposure to BPA has been linked to the development of obesity and the metabolic syndrome. A study by Bhandari *et al.* found that urinary BPA was associated with obesity in children ranging from 6-18 years of age (Bhandari et al., 2013). This is in accordance with many studies published about the effects of BPA on adipogenesis. Our lab showed that BPA enhanced the adipogenic potential of murine 3T3-L1 cells at concentrations as low as 0.1nM (Atlas et al., 2014). In primary human preadipocytes, BPA was also found to show effects at 25-30µM (Boucher et al., 2014a). Furthermore, *in vivo* studies showed that perinatal exposure to BPA increased body weight in weaning rodents (Somm et al., 2009; Ryan et al., 2010). Given that BPA is replaced with other structural analogues, the question of whether or not they exhibit the same effects remains. Therefore, in this study, we investigated the effects of the bisphenol analogues (TMBPF, BPAP, BPF and BHPF) on adipogenesis by measuring mRNA, protein and lipid accumulation using the 3T3-L1 cell model.

First, we aimed to assess the cytotoxicity of the replacement bisphenols by performing an Alamar blue assay. Cytotoxicity was assessed using an extensive concentration range (0.005-100µM) to be better able to calculate the EC_{50}. We found cell viability to be affected at the highest concentration of 100uM for BPAP, BHPF and TMBPF. To date, there remains no study assessing the environmental risk exposure of humans to TMBPF however; recent studies have assessed some of its toxicological effects. The most recent study by Cohen *et al.* evaluated cytotoxicity of TMBPF (0.01 and 0.1 µM) on human female adipose-derived stem cells and found it to be extremely toxic at these concentrations (Cohen et al., 2021). Harnett *et al*. discovered similar results in experiments where female rat adipose-derived stem cells and human mesenchymal stem cells were treated with 0.01-50 µM TMBPF. Results showed that TMBPF was toxic to stem cells and induced apoptosis

in a biphasic manner (Harnett et al., 2021). On the other hand, a 90-day dietary systemic toxicity study in rats reported the no observed adverse effect level (NOAEL) to be 750 mg/kg-bw/d for female rats and 1000 mg/kg-bw/d for the males. However, dose-dependent increases in thymus cell proliferation and ovarian follicular cysts were observed; these effects subsided after a 28-day recovery period. Furthermore, increased kidney and liver weights persisted in males with 1000 mg/kg-bw/d even after the 28-day recovery period (Maffini and Canatsey, 2020).

One of the major concerns regarding BPA is its known interactions with the ER. As such, it is possible for these substitute chemicals to exhibit similar endocrine disrupting capabilities based on their structural similarities (Figure 1). Most of the research on BPA analogues has been focused on the estrogenic activity of these compounds. For example, TMBPF was shown to lack estrogenic activity thus potentially avoiding the endocrine disrupting effects and making it a plausible alternative to BPA (Soto et al., 2017; Maffini and Canatsey, 2020). Other analogues have been found to present less promising results. A study by Xiao *et al.* strove to evaluate the estrogenic and anti-estrogenic capabilities of BPAP *in vivo*. Their results showed BPAP to exhibit weak estrogenicity, but strong anti-estrogenicity and significantly decreased uterine weight of CD-1 mice (Xiao et al., 2018). Similarly, female mice given water containing BHPF released from plastic water bottles also displayed decreased uterine weights and deceased expression of estrogen-responsive genes. This study concluded that BHPF exhibits anti-estrogenic properties as well (Zhang et al., 2017). BPF, on the other hand, has been found to exhibit estrogenic properties. In an experiment with zebrafish, BPF was found to activate three zebrafish ERs and induce aromatase gene expression within the brain. Furthermore, BPF was able to induce vitellogenin synthesis in adult male zebrafish (Le Fol et al., 2017).

One of the initial objectives of the project was aimed at assessing whether or not the estrogen receptor played an important role in BPA-mediated adipogenesis as well as with other the other analogues. This avenue of research stemmed from previous research in our lab where BPA-mediated adipogenesis was inhibited by a specific estrogen receptor antagonist ICI, in human primary preadipocytes (Boucher et al., 2014a). Estrogen receptor α (ERα) and estrogen receptor β (ERβ) have been shown to be expressed in 3T3-L1 adipocytes by other labs (Yi et al., 2008), however we needed to confirm these receptors were expressed in the preadipocyte and thus may play a role in mediating adipogenesis. Kassotis and colleagues were able to detect the expression of ERα and ERβ within 3T3-L1 preadipocytes obtained from the ATCC, thus we sought to confirm its expression. We were able to detect very low expression of ERα and β (data not shown) in the 3T3-L1 preadipocytes. Such low expression of the receptor presented an unlikely path through which BPA and its analogues could mediate their adipogenic effects. In addition, our experimental conditions contained estrogenic compounds such as phenol red and estrogen in the serum, thus reinforcing our conclusion.

We proceeded to evaluate the mRNA and protein expression profiles of cells that had been differentiated into mature adipocytes upon treatment with BPA and its analogues. We chose to measure the mRNA expression of genes (Figures 4-6, Table 1) known to be markers of mature adipocytes as explained within the introduction. Expression of 3 mature adipocyte markers was also assessed at the protein level (Figures 7-9, Table 2). Our data suggests that similar to BPA, TMBPF and BPF increase adipogenesis in 3T3-L1 preadipocytes; BPAP and BHPF on the other hand, do not.

Table 1: Summary of mRNA gene expression upon treatment with BPA analogues.

	µM	Pparγ	C/ebpα	Adipsin	Adiponectin	Fabp4	Lpl	Plin
BPA	0.01	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	10	N.S	↑	↑	↑	↑	↑	N.S.
	20	↑	↑	↑	↑	↑	↑	↑
TMBPF	0.01	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.	↑	N.S.	N.S.	N.S.
	10	↑	↑	↑	↑	↑	↑	↑
	20	↑	↑	↑	↑	↑	↑	↑
BPF	0.01	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	10	N.S	↑	↑	↑	N.S.	N.S.	N.S.
	20	N.S	↑	N.S.	↑	↑	N.S.	↑
BPAP	0.01	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	10	N.S	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	20	N.S	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
BHPF	0.01	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	1	↑	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	10	↑	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	20	↑	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Dex	250nM	↑	↑	↑	↑	↑	↑	↑

Table 2: Summary of protein expression upon treatment with BPA analogues.

	µM	FABP4	LPL	PLIN
BPA	0.01	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.
	10	↑	N.S.	↑
	20	↑	N.S.	↑
TMBPF	0.01	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.
	1	N.S.	↑	N.S.
	10	N.S.	↑	↑
	20	N.S.	↑	N.S.
BPF	0.01	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.
	10	↑	↑	↑
	20	↑	↑	↑
BPAP	0.01	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.
	10	N.S.	N.S.	↑
	20	N.S.	N.S.	N.S.
BHPF	0.01	N.S.	N.S.	N.S.
	0.1	N.S.	N.S.	N.S.
	1	N.S.	N.S.	N.S.
	10	N.S.	N.S.	N.S.
	20	N.S.	N.S.	N.S.
Dex	250nM	↑	↑	↑

BPAP did not increase any of the mature adipocyte gene markers suggesting that it does not affect adipogenesis. However, at the protein level, increased expression of PLIN was observed at 10µM. This is the first study within the literature to investigate its adipogenic properties as most of the literature addresses the potential estrogenicity of BPAP (Cano-Nicolau et al., 2016; Xiao et al., 2018).

Similar to BPAP, BHPF also did not increase any of the mature adipocyte gene makers except for *Pparγ*, by roughly 2 fold over MI control. Expression of mature adipocyte makers remained unchanged at the protein levels as well. This is an interesting result given that *Pparγ* triggers the downstream expression of many of the mature adipocyte markers. Even though BHPF up-regulated the mRNA it is possible the protein was not up-regulated and thus expression of downstream mature adipocyte markers was not observed. It is also possible PPARγ may have been present, yet not active. This presents an avenue for further investigation.

In the case of TMBPF and BPF, several mature adipocyte markers were up-regulated suggesting that these two BPA analogues are adipogenic. Treatment of cells with TMBPF resulted in a dose-dependent increase in gene expression of all mature adipocyte markers. Furthermore, TMBPF up-regulated *Adiponectin*, *Fabp4* and *Lpl* to a greater extent than BPA. This suggests that TMBPF is more potent than BPA at 10µM. Gene expression profiles also revealed an increase in the expression levels of *Adipsin* at 10 µM followed by a decrease in the expression at 20µM. *Adipsin* was roughly 3-fold higher as compared to MI control at 10 µM and dropped down to control levels at 20µM.

Adipsin is a serine protease homolog which is a protein responsible for cleaving peptide bonds (Ramskov Tetzlaff et al., 2019). Not much is known about the regulation of its expression;

however, *in vivo* mouse studies have shown a correlation between reduced mRNA expression of adipsin in obese mice while expression remains elevated in lean mice. One study in particular sought to discover whether endoplasmic reticulum stress responses may be responsible for this observed decrease of adipsin expression within obese mice. The study found decreased levels of adipsin in adipose tissue and serum while expression of endoplasmic recticulum stress markers was elevated compared to lean mice (Ryu et al., 2020). Another study by Lowell *et al.* showed that insulin and insulin-like growth factor-1 up-regulated adipsin gene expression throughout differentiation in 3T3-F442A cells (Lowell and Flier, 1990). Our cytotoxicity results indicated that at 20uM cell viability was decreased, however cells displayed no signs of cell death when observed under the microscope at this concentration. Furthermore, gene expression profiles only show decreased expression of adipsin while other markers of mature adipocytes remained elevated. An alamar blue assay was used to assess cell viability and is a measure REDOX reactions occurring within the mitochondria. Thus, this decreased expression in *adipsin* may not be due to cytotoxic effects, but a result of inhibitory effects within the cells metabolic pathways and requires further investigation. Notably, Cohen *et al.* analyzed the effects of TMBPF on adipogenesis in human female adipose-derived stem cells and found this analogue reduced adipogenesis (Cohen et al., 2021). However, our study is the first study to assess the effects of TMBPF on the gene expression profile of the mature adipocyte within the commonly known 3T3-L1 screening model system. At the protein level, peak expression levels of FABP4 were observed at 1µM with TMBPF. Expression of PLIN and LPL were also elevated. Interestingly, expression of PLIN show an abrupt decrease at 20µM. This may seem counterintuitive as lipid accumulation at 10 and 20µM appear to be relatively the same. However, there are other perilipins that can coat the outside of lipid

droplets and maintain their integrity (Sztalryd and Brasaemle, 2017b). Thus investigating the expression of other perilipins may be another avenue for further research.

Treatment of cells with BPF resulted in increased expression of all except two of the mature adipocyte markers in a dose-dependent manner. Though not significant, BPF appears to have elevated levels of *Pparγ* and *Lpl* with increasing concentration. Increased expression of adipogenic markers was also observed at the protein level (Figures 7D, 8D and 9D). Unlike TMBPF, the effects of BPF on adipogenesis have elicited more attention. Similar to our experiments, Tetzlaff *et al.* also used the 3T3-L1 cell model to assess the adipogenic effects of several analogues of BPA, one of which was BPF (Ramskov Tetzlaff et al., 2019). In this study, expression of three markers of mature adipocytes (*Pparγ*, *Lpl* and *Fabp4*) was assessed upon treatment with 0-10μM of BPF. None of these gene were found to be significantly increased throughout adipogenesis. Another paper by Drobna *et al.* also looked at the effect of BPF (0.01 and 1μM) on 3T3-L1 preadipocyte differentiation (Drobna et al., 2019). This study found that BPF decreased expression of several genes. In both studies cells were differentiated in the presence of DEX which is known to induce close to 100% differentiation. If this is the case any difference in gene expression with BPF treatment in combination with DEX treatment would be difficult to see. Our study is the first to investigate the adipogenic potential of BPF in the absence of DEX and within the 3T3-L1 cell model. A recent paper by Reina-Pérez *et al.* differentiated human adipose-derived stem cells with varying doses of BPF (0.01-25 uM) for 7 or 14 days after which gene expression profiles were analyzed. Though this study differs in that human cells require DEX to differentiate, the study found that BPF increased expression of *PPARγ*, *C/EBPα*, *LPL* and *FABP4* at 10 and 25μM compared to control after 7 days of induction (Reina-Pérez et al., 2021). These results are similar to the findings of our research.

Though mRNA and protein expression showed strong correlation for most genes, cells treated with TMBPF and BPAP showed poor mRNA and protein correlation for PLIN expression. Cells treated with 20μM TMBPF displayed elevated mRNA expression of *Plin*, however this was not seen at the protein level. Cells treated with 10μM BPAP displayed the opposite where mRNA expression was down yet protein expression was up. It can be difficult to explain why this may occur, however, the disconnect between mRNA and protein expression has previously been addressed. There are several reasons for why this may occur. The first of which may be due to post-transcriptional or translational parameters which may influence the efficiency of protein translation and expression. For example, translation efficiency is dependent on the Shine Dalgarno (SD) sequence present within the mRNA. A strong SD sequence is one that is mostly complementary to the 16S rRNA present within the ribosomal subunit, thus a strong SD sequence leads to faster initiation and thus elevated translational efficiency (Maier et al., 2009). Another factor that may influence translational efficiency is the mRNA conformation. These conformations can either expose or sequester the ribosomal binging sites. Expression of proteins can also vary due to differences in their half-lives thus, some proteins may degrade faster than others (Doherty et al., 2008). With regards to mRNA expression, it is assessed at a snapshot in time, thus it is possible for the mRNA to have already degraded at that time point. To mitigate this, a time course looking at *Plin* expression for chemicals showing poor mRNA/protein correlation could be done in future studies. Finally, it is possible that noise and experimental error could contribute to poor mRNA and protein correlation (Greenbaum et al., 2003).

Our results have generated a clearer picture of which BPA analogues are adipogenic and those that are not. In order to further confirm our results, lipid accumulation was visualized on day 8 of differentiation. Cells treated with TMBPF showed significant lipid accumulation at 10 and

20µM compared to MI control. Although BPA also significantly up-regulated lipid accumulation at 20µM, TMBPF was able to increase lipid accumulation to a greater extent than BPA at both 10 and 20µM. Furthermore, an increase in lipid accumulation was also observed with BPF at 10 and 20µM, however this increase was not sufficient to obtain significance. Our data also confirmed once again that BPAP and BHPF are not adipogenic as no change in lipid accumulation was observed.

Not only did TMBPF increase lipid accumulation to a greater extent than BPA, but these adipoyctes also displayed an interesting morphological difference. Within humans there are two ways in which lipid can accumulate within adipocytes. Hyperplasia is the process through which cell numbers increase in order to accommodate the storage of triglycerides, where as hypertrophy involves the enlargement of existing adipocytes (Sun et al., 2011). In humans, hypertrophic obesity has been correlated with reduced insulin sensitivity, and a change in the secretion of adipokines that contribute to the development of the metabolic syndrome (Hammarstedt et al., 2012). Given that cells treated with TMBPF embody the hypertrophic phenotype, it is possible that the function of the adipocyte may have been altered as well. Future studies could thus focus on evaluating changes in lipolysis, insulin sensitivity, glucose uptake and the overall function of the adipocyte.

This is the first study to analyze the effects of BPAP and BHPF on lipid accumulation within 3T3-L1 preadipocytes. While the effects of BPA on adipogenesis have been extensively covered in the literature, the effects of other bisphenols are starting to gain more momentum (Atlas et al., 2014; Boucher et al., 2014a, 2014b, 2016b). The effects of BPF on lipid accumulation have been studied in both human and murine cell models and the findings suggest that this analogue does in fact increase adipogenesis thus supporting the results from our study (Ramskov Tetzlaff et al., 2019; Reina-Pérez et al., 2021). There aren't any studies that investigate the effects of TMBPF

in 3T3-L1 preadipocytes, however Chappell *et al.* investigated the effects of Tetrabromobisphenol A (TBBPA), a chemical similar in structure, in 3T3-L1 preadipocytes. Upon treatment with TBBPA cells were found to have increased lipid accumulation (Chappell et al., 2018). Cohen *et al.* looked at lipid accumulation in human mesenchymal stem cells and found that TMBPF has the opposite effect (Cohen et al., 2021). We found TMBPF to be extremely adipogenic. This is the first study to investigate the effects of TMBPF in 3T3-L1 cells and the first to study the effects of BPAP and BHPF within this cell model as well. More research is warranted in order to deliver consensus on their adipogenic effects.

After having determined that TMBPF and BPF were adipogenic, we sought to uncover the mechanism by which these effects were mediated. To do this, a time course was performed to assess early mRNA expression of important transcription factors within the adipogenic cascade. We assessed mRNA expression of *C/ebpα* (Figure 11), *Pparγ* (Figure 12), *C/ebpβ* (Figure 15), *C/ebpδ* (Figure 13), and *GR* (Figure 16). BPAP was also included in the time course analysis because it increased mRNA expression of *Pparγ* and *C/ebpα*. Within the adipogenic cascade, activation of GR causes the receptor to translocate from the cytoplasm of the cell to the nucleus where it is able to bind GREs within the promoter of *C/ebpδ*. Once up-regulated, C/EBPδ along with C/EBPβ can transcriptionally up regulate *C/ebpα* and *Pparγ*, thus inducing adipogenesis. Upregulation of the transcription factors *C/ebpβ*, *GR*, and *Pparγ* was not observed at early time points for any of the bisphenols except for BPF. However, *C/ebpδ* expression was increased by TMBPF treatment. Based on the fact that *C/ebpδ* is a direct transcriptional target of GR, we hypothesized that TMBPF may activate this receptor based on its ability to increase mRNA expression of *C/ebpδ* after 2 hours.

Our results indicated that neither BPA nor TMBPF transcriptionally transactivate GR at the MMTV promoter. This is contradictory to what Sargis *et al.* found which is that BPA activates GR (Sargis et al., 2010), but consistent with previous results published from our lab [122]. There is a lack of literature pertaining to upstream regulators of *C/ebpδ* expression within 3T3-L1 preadipocytes. We therefore decided to take a closer look at the proximal promoter of *C/ebpδ*. Although putative, this analysis revealed binding sites for GR, RXR, C/EBPα, GATA-1 and Sp1 to name a few. It may be possible that TMBPF is acting via one of those or perhaps even a series of factors. Furthermore, several chemicals have been shown to activate PPARγ via transcriptional transactivation assays. One study in particular showed that BPS was able to activate PPARγ using a PPARγ-response element reporter plasmid (Ahmed and Atlas, 2016). This could be another mechanism by which the adipogenic bisphenols induce their adipogenic effects.

LIMITATIONS

Although this body of work contributes to the gap in knowledge about the effects of the BPA analogues, more research is necessary to confirm the effects of these bisphenols in other cell lines. More specifically, the use of the 3T3-L1 fibroblasts is a good starting point for screening adipogenic potential of these chemicals. However, the model cannot accurately recapitulate the response that occurs within the human body. 3T3-L1 cells are a uniform population whereas composition of cells within tissue is quite diverse within humans.

Another limitation of this study is the acute exposure of these cells to the BPA analogues. While this short exposure allows us to view the acute effects of certain concentrations, humans have been exposed to these chemicals for a much longer time. Thus, concentrations being tested may not accurately represent what humans are exposed to. In addition, BPA and its analogues may

accumulate in adipose tissue over time causing predominant effects in certain tissues versus others. There is a lack of research pertaining to the accumulation of BPA and its analogues in tissue, however a recent study by Keshavarz-Maleki and colleagues investigated amount of BPA in breast adipose tissue samples. The study found BPA to accumulate in 73% of breast adipose tissue samples with an average of 0.84ng/mL detected (Keshavarz-Maleki et al., 2021). Future studies in other cells lines may be better equipped to tackle these limitations.

CONCLUSION

In conclusion, our data revealed that BPF and TMBPF along with BPA were found to be adipogenic. This was done through assessing mRNA and protein expression of adipogenic markers within mature murine adipocytes. TMBPF was found to be more potent than BPA and the other analogues at inducing mRNA expression of *Fabp4*, *Lpl* and *Adiponectin*. TMBPF, BPA and BPF increased expression of FABP4, LPL and PLIN for both protein and mRNA. TMBPF induced greater lipid accumulation compared to BPA and other analogues. It is not quite clear how these substitute bisphenols mediate their effects, however it is not through GR activation for BPA or TMBPF. This is the first study to look at the adipogenic effects of BPAP and BHPF. We found they do not affect adipogenesis in this cell model. This is also the first study to look at the effects of TMBPF within the 3T3-L1 cells model. More research is necessary to deduce the mechanism of action for BPA, TMBPF and BPF.

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APPENDICIES

Appendix A Primers

Table 3: Primers used for real time PCR reactions

Primer name	Forward and Reverse Primer Sequence
Beta Actin	Forward: 5'-GACTTCGAGCAAGAGATGGC-3'
	Reverse: 5'-CCAGACAGCACTGTGTTGGC-3'
Pparδ	Forward: 5'GCCTGCGGAAGCCCTTTGGT-3'
	Reverse: 5'-GCAGTTCCAGGGCCTGCAGC-3'
C/ebpα	Forward: 5'-TGCGCAAGAGCCGAGATAAA-3'
	Reverse: 5'-CCTTGACCAAGGAGCTCTCA-3'
C/ebpβ	Forward: 5'-GAGCGACGAGTACAAGATGC-3'
	Reverse: 5'-AGCTGCTCCACCTTCTTCTG-3'
C/ebpδ	Forward: 5'-GCCCAACTTGGACGCCAG-3'
	Reverse: 5'-CCCTGCCTGGCTCGTAGAA-3'
Adipsin	Forward: 5'-CCTGAACCCTACAAGCGATG-3'
	Reverse: 5'-CAACGAGGCATTCTGGAATA-3'
Adiponectin	Forward: 5'-TGACGACACAAAAGGGCTC-3'
	Reverse: 5'-CACAAGTTCCCTTGGGTGGA-3'
Glucocorticoid Receptor	Forward: 5'-AAAGACCTAGGAAAAGCCATTGTC-3'
	Reverse: 5'-TCAGCTAACATCTCTGGGAATTCA-3'
Lipoprotein Lipase	Forward: 5'-GATCCGAGTGAAAGCCGGAG-3'
	Reverse: 5'-TTGTTTGTCCAGTGTCAGCCA-3'
Perilipin	Forward: 5'-TTGGGGATGGCCAAAGAGAC-3'
	Reverse: 5'-CTCACAAGGCTTGGTTTGGC-3'
Fatty acid binding protein 4	Forward: 5'-GGAAGCTTGTCTCCAGTGAA-3'
	Reverse: 5'-GCGGTGATTTCATCGAATTC-3'

Appendix B Antibodies

Table 4: Antibodies used for Western Blot analysis

1º Antibody	Supplier	Catalog Number	Dilution factor for Western Blotting
Beta Actin	Cell Signalling	4970	1:1000
Pparδ	Cell Signalling	2443	1:500
C/ebpδ	Cell Signalling	2318	1:1000
Lipoprotein Lipase	R&D	AF7197	1:500
Perilipin	Cell Signalling	9349	1:1000
Fatty acid binding protein 4	R&D	AF3150	1:500

Misha Singh

EDUCATION AND RESEARCH EXPERIENCE

MSc Biochemistry **(Sept 2018 – Present)**
University of Ottawa, Ottawa, Ontario
Thesis: The Effects of BPA and its Structural Analogues on Adipogenesis
Supervisor: Prof. Ella Atlas, Co-supervised by Prof. Alexander Sorisky

BSc Honours Chemistry **(Sept 2013 – May 2018)**
Wilfrid Laurier University, Waterloo, Ontario
Project: Efficient formation of light-absorbing polymeric nanoparticles from the reaction of soluble Fe (III) with C4 and C6 dicarboxylic acids.
Supervisor: Prof. Hind A. Al-Abadleh

PROFESSIONAL EXPERIENCE

Biologist at Health Canada **(Jul 2021 – Present)**

- Leveraged critical thinking skills to collect and analyze relevant literature as a tool for designing experiments and contextualizing the findings of our research
- Maintained an accurate biological and chemical inventory by updating supply counts weekly allowing laboratory technician to effectively order the required supplied resulting in efficient laboratory operations
- Conducted statistical analysis of large data sets generated from experiments using Microsoft Excel and Sigma Plot software's in order to organize data and draw conclusions from results

Masters of Biochemistry Candidate at University of Ottawa: **(Sept 2018 – Present)**

- Successfully trained and supervised progress of one lab member and undergraduate student to ensure adequate understanding of protocols, project and lab safety resulting in their ability to work independently
- Effectively planned and executed 10 cell culture experiments using sterile techniques within 4 weeks ultimately delivering ample data for analysis and advancing project objectives
- Chosen by conference committee members to give an oral presentation conveying technical information in an easy-to-understand manner to over 30 scientists at the Health Canada Science Forum 2 years in a row

REASEARCH EXPERIENCE

Graduate Research (MSc Biochemistry) **(Sept 2018 – Present)**

Supervised by Dr. Ella Atlas, Co-supervised by Dr. Alexander Sorkisky

- Goal: The aim of this project was to better understand the effects of BPA and its replacement analogues on adipogenesis as well as to better understand how these chemical may be mediating their effects
- Hypothesis:
    - BPA analogues may also have an effect of adipogenesis similar to BPA
- Summary of Skills and Methods:
    - Knowledge in the area of multiple discipline such as adipogenesis, endocrine disruptors, cell signalling, developmental biology, cell metabolism, cellular respiration, transcription and translation
    - Proficiency with multiple software applications such as Microsoft word, Microsoft Excel, Microsoft PowerPoint, Sigma Plot among others
    - Developed technical skills required for:
        - Cell culture, cell viability assay, qPCR, Protein extraction and Western analysis, Luciferase binding assays

COMMITTEE INVOLEMENT AND INTERESTS

Unity for Action, Executive Team Member **(Sept 2019 – 2021)**

- Created and managed and participated in fundraising events for local charities resulting in $1200 of donations
- Acquired more than 50 sponsorships in support of local fundraising events by leveraging communication and interpersonal skills
- Resiliently continued fundraising efforts during COVID-19 by implementing alternative fundraising strategies such as virtual yoga sessions and escape rooms resulting in $575 profit over 5 months from fundraising efforts

Let's Talk Science: **(Sept –Dec 2019)**

- Mentored 30 high school students within 4 months' time to improve and foster enthusiasm for STEM field
- Lead classroom through discussion and trained students to think critically by performing various experiments such as matter and materials type demonstrations

Grand River Hospital, Hospital Elderly Life Program Volunteer **(Aug 2016 – 2018)**

- Participated in Daily Visitor Program to encourage cognitive orientation, communication, and social support in patients allowing for accelerated reintegration into society
- Implemented Therapeutic Activities Program for cognitive stimulation and socialization allowing for personalized interactions with individual patients
- Reduced mobility recovery time by performing exercise and walking assistance with patients in adherence to Early Mobilization Program

Deaf Blind Ontario Services, Planning Committee Volunteer (Sept 2014 - Feb 2016)

- Planned, organized and recruited volunteers for Dining in the Dark event consisting of over 100 attendees
- Directed teams through blindfolded dinner while maintaining safety protocols ultimately delivering an exceptional experience
- Leveraged knowledge of marketing to promote and advertise the event resulting in selling out of tickets to capacity

HONOURS AND AWARDS

- **Graduate Student Intertek Award Finalist (Nov 2020 – Dec 2020)**
  for the 52nd Annual Symposium of the Society of Toxicology of Canada

CONFERENCE PRESENTATIONS

- **Health Canada Science Forum, Ottawa, Canada, Oral and Poster (Jan 18-22, 2021).** Misha Singh, Alexander Sorisky, Ella Atlas. The Effects of BPA and its Structural Analogues on Adipogenesis.
- **Society of Toxicology Canada, Ottawa, Canada, Oral and Poster (Nov 30 – Dec 2, 2020).** Misha Singh, Alexander Sorisky, Ella Atlas. The Effects of BPA and its Structural Analogues on Adipogenesis.
- **Health Canada Science Forum, Ottawa, Canada, Oral (Jan 20-21, 2020).** Misha Singh, Alexander Sorisky, Ella Atlas. The Effects of BPA and its Structural Analogues on Adipogenesis.
- **Endocrine Disrupting Chemicals: Towards Responsible Replacements, McGill University, Montreal, Canada, Oral and Poster (Oct 25, 2019).** Misha Singh, Alexander Sorisky, Ella Atlas. The Effects of BPA and its Structural Analogues on Adipogenesis.

SPECIAL TRAINING AND CERTIFICATION

- **Standard First Aid with CPR "C"**, Waterloo Ontario **(Sept 9-10, 2017)**
- **First Responder**, Waterloo Ontario **(Oct 27-29, Nov 3-5, 2017)**