INVESTIGATING THE EFFECTS OF *IN UTERO* BENZENE EXPOSURE ON MURINE FETAL PLACENTA DNA REPAIR GENE EXPRESSION

by

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Abstract

Benzene is a ubiquitous pollutant found in the environment, in car emissions, cigarette smoke, and at elevated levels in the industrial manufacturing of plastics, resins and dyes. Exposure to benzene results in carcinogenic and hematotoxic effects. In humans, adult exposure to the environmental pollutant is positively correlated with the development of leukemia and benzene may be associated with childhood leukemia following *in utero* exposure. The evidnce suggests that benzene and its metabolites are more toxic to the developing fetus, since the fetus has decreased detoxification and DNA repair abilities. While numerous studies implicate benzene crossing the placenta, being metabolized and causing oxidative stress and DNA damage ultimately leading to childhood leukemia, the mechanism by which it does this is unclear. It is possible that fetal exposure to benzene results in increased DNA damage that may be left mis repaired or unrepaired, leading to genotoxicity. By using a molecular toxicological approach, employing a CD-1 mouse in utero model, we tested whether in utero benzene exposure alters gene expression of critical DNA repair genes in fetal placentas. By conducting qRT-PCR tests for mRNA expression of critical NHEJ (non-homologous end joining) and HR (homologous recombination) genes, we showed that *in utero* benzene affects the regulation of 5 DNA repair genes following *in utero* exposure using two-way ANOVA. *Xrcc4* showed significant increased gene expression at 24h, *Xrcc5* showed significant increased expression at 6h, *Xrcc6* showed no changes at any time point, *Rad51* showed significant increased expression at 24h and *Brca2* showed significant decreased expression at 2h and 6h. Results summarized in this thesis add to the growing evidence of the effects of *in utero* benzene exposure on fetal and placental DNA repair genes.

Co-Authorship

This research was conducted by the candidate Christian Taylor Bilé, under the supervision of Dr. Louise M. Winn.

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List of Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
ANOVA	Analysis of variance
BQ	Benzoquinone
BRCA2	Breast cancer 2
cDNA	Complementary DNA
CLL	Chronic lymphoblastic leukemia
CML	Chronic myelogenous leukemia
CYP	Cytochrome P450
CYP2E1	Cytochrome P450 2E1
DNA	Deoxyribonucleic acid
DNA-PKCs	DNA-dependent protein kinase, catalytic subunit
DSB	Double-stranded break
GD	Gestational day
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
HOO•	Hydroperoxyl radical
HSC	Hematopoietic stem cells
mRNA	Messenger ribonucleic acid
NAC	N-acetyl-l-cysteine
NF-κB	Nuclear factor kappa B

NHEJ	Non-homologous end joining
OH^-	Hydroxyl radical
$O_2\bullet^-$	Superoxide anion radical
PPM	Parts-per-million
qRT-PCR	Quantitative real-time polymerase chain reaction
Rad51	RAD51 Recombinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDSA	Synthesis-dependent strand annealing pathway
ssDNA	Single-stranded DNA
TBP	TATA-binding protein
TGB	1,2,4-trihydroxy-5-glutathionyl benzene
Topo IIα	Topoisomerase IIα
XLF	XRCC4-like factor
Xrcc4	X-ray repair cross-complementing protein 4
Xrcc5	X-ray repair cross-complementing protein 5
Xrcc6	X-ray repair cross-complementing protein 6

Chapter 1

Introduction

1.1 BENZENE

1.1.1 Benzene

Benzene is an environmental pollutant that can be found both naturally and due to human activity. It is found at elevated levels in the production of a wide range of products, including plastics, dyes, and pesticides. Therefore, workers in the manufacturing of these products are at elevated risk of exposure (De Maria et al., 2020). Additionally, benzene is a component of crude oil, gasoline and cigarette smoke (Korte et al., 2000; Pankow et al., 2017; Brosselin et al., 2009). Benzene is a volatile liquid, and thus in humans, the primary route of exposure is via inhalation.

Environmental levels of benzene are monitored yearly by Environment Canada in the Analysis and Air Quality Division of the Environment Protection Service. The collected data has shown higher levels of benzene in areas near industrial sources and roadways (Environment Canada, 2021). A study comparing benzene exposures between female non-smokers in rural vs urban environments showed significant increases in benzene exposure in workers in urban high traffic environments compared to those rural areas (Ciarroca, 2012). This study also showed that in urban workers, airborne benzene levels and blood benzene levels were positively correlated (Ciarroca, 2012).

Until recently, the largest source of non-occupational exposure to benzene was cigarette smoke, however recent increases in e-cigarette use poses a new risk. In fact, a study by Pankow et al. (2017), showed that e-cigarettes delivered about 86 μg benzene/cigarette, resulting in about 200,000 μg/m^3 of smoke benzene concentration, significantly higher than ambient air benzene concentrations of ~1 μg/m^3. Through known exposures to benzene, it has been shown by researchers that benzene can have carcinogenic and hematotoxic effects in humans and mice (Nordlinder and Järvholm,1997; Steffen et al., 2004; Snyder, 2000; Badham et al., 2010). Despite years of research, the specific mechanisms of how benzene causes toxicity remains unknown.

1.1.2 Benzene toxicity

Benzene is associated with multiple different toxicities in humans that include acute and chronic health effects (Snyder, 2012). Benzene can act as a neurotoxicant and acute exposures can cause common symptoms such as irritation of mucous membranes, restlessness, headaches, nausea, dizziness and respiratory depression that in some extreme cases has led to induction of comas and death (Duarte-Davidson et al., 2001, Ahmad 2007). Chronic exposures to benzene are commonly known to be associated with hematological health effects such as aplastic anemia and leukemia (Ahmad 2007).

Aplastic anemia results from benzene toxicity in the bone marrow and the subsequent decreases in erythrocytes, thrombocytes and leukocytes. Incidence rates of aplastic anemia reported at high levels of benzene exposure (>100ppm) drops 10-fold at lower exposure levels of 10-20 ppm (Smith, 1996). However, working atmospheric conditions where benzene concentrations exceeded 5 ppm were shown to significantly reduce hemoglobin levels and mean corpuscular hemoglobin concentration (Bogadi-Sare, Turk, Zavalic, 1995), and importantly, suppression of hemoglobin can still occur at <5 ppm (Ward et al., 1996). Additionally, it has been suggested with low levels of benzene there may exist a synergistic effect with other low-level contaminants such as toluene and xylene, that promote myelotoxicity (Molinini et al., 1997). Furthermore, benzene and its metabolites function well as highly clastogenic agents that result in chromosomal aberrations, sister chromatid exchange and micronuclei (Snyder, Witz, Goldstein, 1993)

1.2 HEMATAPOIESIS AND BENZENE

1.2.1 Normal hematopoiesis

Hematopoiesis entails the self-renewal of progenitor cells to give rises to blood cells of multiple lineages by progressive differentiation as seen in figure 1. This process demands the self-renewal of hematopoietic stem cells (HSC), proliferative expansion of progenitor cells and the progressive commitment to a single lineage (Evans, Sano and Walsh, 2020). During the proliferation of HSCs, a dividing cell will give rise to two daughter cells. One daughter cell remains as an HSC capable of self-renewal and thus contributes to the proliferative expansion of progenitor cells, while the other daughter cell will differentiate into a committed lineage of myeloid or lymphoid cells. Myeloid cells will continue to differentiate to erythrocytes, platelets and granulocytes that can further progress to neutrophil, eosinophils, macrophages and basophils. Lymphoid progenitor cells will give rise to cells involved in cell-mediated immunity called T and B cells (Jagannathan-Bogdan and Zon, 2013) (Figure 1.1).

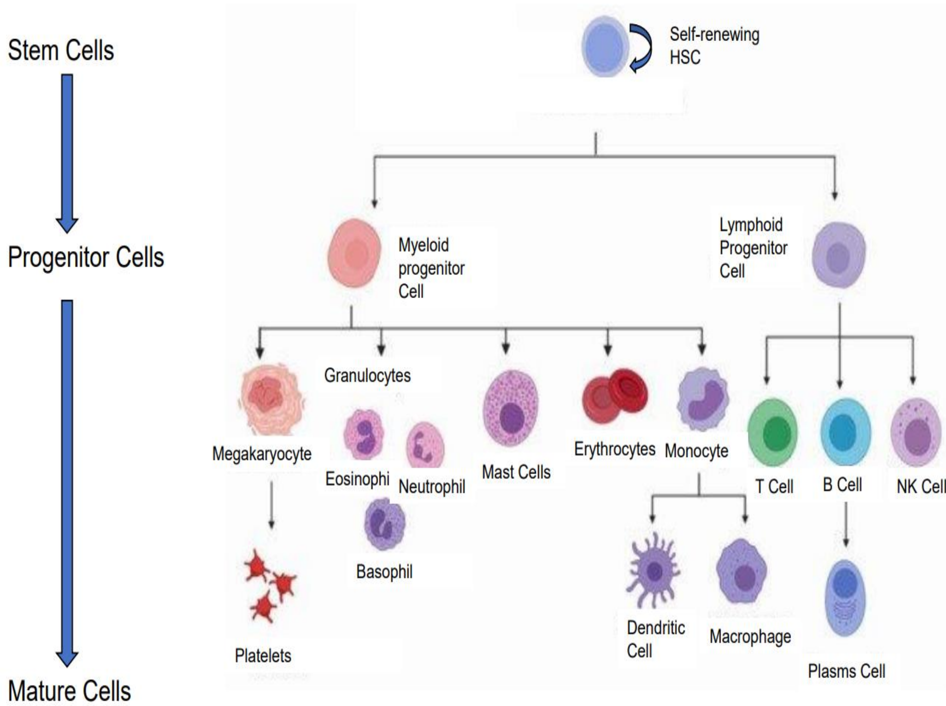

Figure 1.1 Normal hematopoiesis

Hematopoiesis entails the self-renewal of progenitor cells and the subsequent differentiation into multiple lineages of cells. Hematopoietic stem cells are a preferential target for benzene toxicity due to their immaturity. Modified from Raza, Salman and Luberto, 2021.

In vertebrates, blood development occurs in two waves: the primitive wave and the definitive wave (Galloway and Zon, 2003). The primary purpose of the primitive wave is to facilitate tissue oxygenation by producing red blood cells as the embryo is undergoing rapid growth (Orkin and Zon, 2008). The primitive wave involves the division of an erythroid progenitor cell to give rise to erythrocytes and macrophages (Palis and Yoder, 2001). In mammals, erythroid progenitor cells are present in early development in blood islands in the extra-embryonic sac (Paik and Zon, 2010). This primitive wave is only temporary as the erythroid progenitors do not possess self-renewal capabilities. Following the primitive wave, a transition to the definitive wave occurs later in development when HSCs are born in the aorta-gonad-mesonephros region of the developing embryo. These HSCs will eventually migrate to the fetal liver and then the bone marrow in adults (Cumano and Godin, 2007).

1.2.2 Development of hematopoiesis

The development of hematopoiesis in the mouse model can be studied to better understand the complexities of hematopoiesis in humans, as the mechanism is comparable to that observed in developing humans. In murine models, hematopoiesis begins in the extra-embryonic yolk sac, where early detection of primitive hematopoietic cells, also known as “blood islands” aggregate on gestational day (GD) 7.5 (Auerbach, 1996). The accumulation of primitive HSCs capable of producing erythrocytes begins the establishment of a functional circulatory system between the developing embryo and the extra-embryonic sac at GD 8.5 (Moore, Metcalf, 1970). This event coincides with the commencement of the heart beating and primitive erythrocytes being released into the circulation (Fraser, Isern, Baron, 2007). Shortly after on GD 9, definitive erythroid and

HSCs travel to the developing embryo (Baron, 2012). Each consecutive generation provides signals that contribute to the maintenance of HSCs and the continued formation, proliferation and differentiation of HSCs. While in the embryo, definitive HSCs will travel to the dorsal aorta (GD 10), the fetal liver (GD 11) where the primary site of fetal hematopoiesis occurs for the remainder of gestation, as well as other secondary sites including the spleen and thymus (GD 14.5) and the bone marrow (GD 17) (Yokomizo and Dzierzak, 2010; Yoder, 2002).

Human embryonic hematopoiesis similarly begins in the yolk-sac where “blood-islands” can be observed as early as GD 15 (Dzierziak, 2005). Between 4-6 weeks post conception, HSCs are detected in the aorta, and are definitively found in the liver during week 5, which becomes the primary site of hematopoiesis for the first half of gestation (Tavian et al, 2010). In humans, hematopoiesis moves to the bone marrow by week 20, which is the final site where hematopoiesis takes place.

1.2.3 Leukemia

Leukemia is a group of cancers that start in blood stem cells originating in the bone marrow, characterized by the uncontrolled proliferation of a population of white blood cells (Canadian Cancer Society). Lymphoid stem cells develop into lymphocytes, where myeloid stem cells develop into erythrocytes, platelets and granulocytes. As the stem cells develop, they become immature blood cells known as blast cells. During a leukemic change, there is an overproduction of blast cells that fail to develop into mature blood cells and will overtime crowd out normal cells, preventing them from functioning properly. In some cases, blasts may overcrowd the bone marrow to the extent they leak into circulation and populate other organs. Leukemias are diagnosed based on the cell they originated from (lymphoblastic or myelogenous) as well as the rate at which cell proliferation occurs. Acute leukemia involves the rapid proliferation of immature blasts, within days or weeks, and requires immediate treatment. Chronic leukemias on the other hand develop much more slowly over months or even years. Leukemia can therefore be identified by four primary categories: acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML) and chronic lymphoblastic leukemia (CLL). In adults, CML and AML are the most common leukemias.

1.2.4 Childhood leukemia

In Canada, cancer remains the number one leading cause of death in all ages (Canadian Cancer Society, 2021). In children, aged 0-14, leukemia is the most diagnosed form of cancer (35%), followed by central nervous system (17%) and lymphomas (13%), and has been increasing over the last decade. In the same cohort of children, leukemia is the second leading cause of cancer-related deaths (23%). The etiology of many cases of childhood leukemia remains unknown but it has been suggested that they could be related to changes in environmental exposures (benzene), lifestyle (smoking) and parental behaviors (Dong et al., 2020; Brenner et al., 2021).

1.2.5 Leukemia and benzene

In developing countries, the incidence of childhood leukemia is increasing, and it is believed that *in utero* exposures to environmental toxicants such as benzene are primary causes for this increase (Filippini et al., 2019). Key changes necessary for the development of childhood leukemia could therefore occur before birth in humans. Benzene has been implicated with various forms of leukemia (Mallory, Gall, Brickley, 1939; Snyder, 2002; Vigliani and Forni, 1976) and is classified as a Group 1 known human carcinogen by the International Agency for Research on Cancer (Golding and Watson, 1999). With there being many sources of benzene in parts of our everyday lives from service industries such as dry cleaning, printing, oil and gasoline, to consumer products including stain removers, paint thinners and cigarettes (Belingheri et al., 2019), the exposure of pregnant women to benzene is a growing cause for concern.

1.3 POTENTIAL MECHANISMS OF BENZENE-INDUCED TOXICITY

1.3.1 Benzene metabolism

The metabolism of benzene is critical for benzene to exert its toxicity (Snyder, 2000). Benzene enters the body primarily through inhalation, and following exposure, diffuses into the lungs and is absorbed into the bloodstream. As shown in figure 1.2, benzene metabolism is primarily mediated by cytochrome P450 (CYP) 2E1 (CYP2E1) in the liver to benzene oxide (Snyder and Hedli, 1996). Benzene oxide can then follow several different metabolic pathways, importantly, spontaneous rearrangement to form phenol or detoxification via glutathione conjugation (Snyder and Hedli, 1996). Phenol can then be further hydroxylated to hydroquinone and catechol. The resulting metabolites are thought to be critically important for benzene to exert its toxicity (Snyder and Kocsis, 1975, Snyder, Witz, Goldstein, 1993). These metabolic intermediates can be transported in the bloodstream to the bone marrow to be further metabolized by myeloperoxidase to yield 1,4-benzoquinone (BQ) and 1,2-benzoquinone. Importantly, BQ has been shown to be a more potent toxicant that can produce more oxidative stress than the other metabolites (Ross, 2000). Some detoxification pathways of benzene attempt to increase the water solubility of benzene, including glutathione conjugation, sulfation and glucuronide conjugation (Schrenk and Bock, 1990; Schrenk et al., 1996). Additionally, competitive inhibitors of benzene such as toluene show a decrease in benzene toxicity when there is dual exposure (Sammett et al., 1979). Furthermore, it has been shown that benzene toxicity in fetal hematopoietic cells, measured by altered progenitor cell growth, can be attenuated with a pretreatment of catalase, an antioxidative enzyme (Badham and Winn, 2010). While the mechanisms of benzene toxicity remain unclear, several studies have shown the accumulation of benzene metabolites that result in an increase in reactive oxygen species (ROS) *in vitro* (Shen et al., 1996; Badham and Winn, 2010) and *in utero* (Wan and Winn, 2008; Badham and Winn, 2010). In the developing fetus, xenobiotic exposure is dependent on the metabolism capabilities of the mother, where lower metabolisms result in higher exposure to the fetus (Jennings-Gee et al., 2006). It is proposed that murine fetal metabolizing enzyme systems exist *in utero* as early as GD 14 (Badham and Winn, 2010). As described below, baseline levels of endogenous ROS

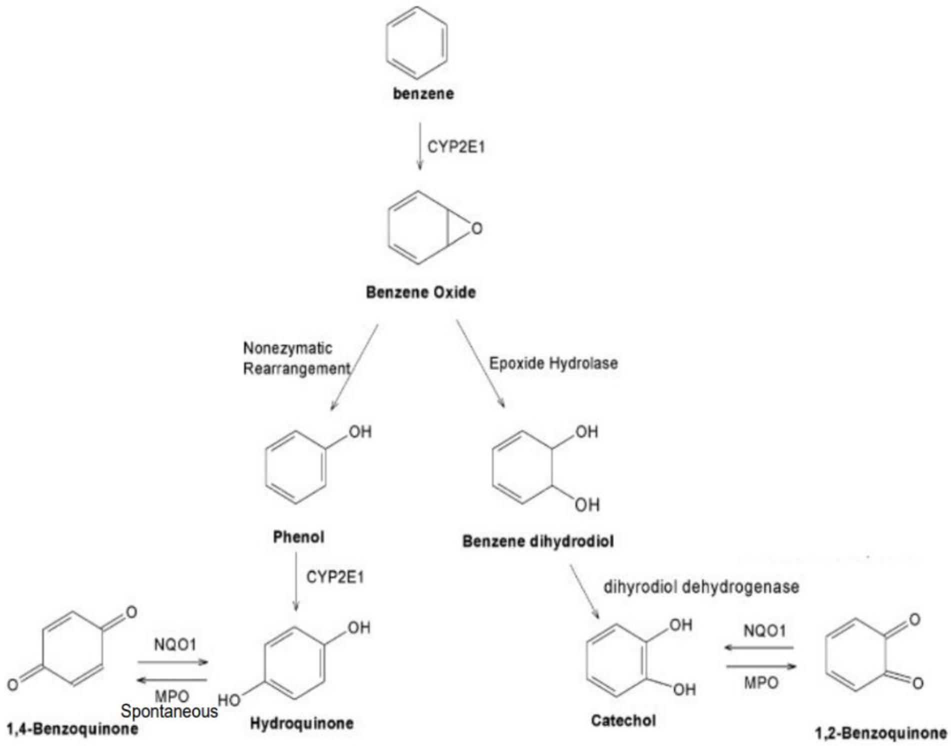

Figure 1.2 Simplified schematic of benzene metabolism

To exert its toxicity, benzene must be metabolized to several different metabolites. Benzene is primarily metabolized in the liver by cytochrome P450 2E1 but is further metabolized in target organs including the bone marrow. Modified from Rappaport et al., 2010.

CYP2E1: Cytochrome P450 2E1; NQO: NAD(P)H quinone dehydrogenase 1; MPO: Myeloperoxidase remain important for various cellular functions but can be perturbed by increased levels of ROS that can damage macromolecules such as DNA (Wells et al., 1997).

1.3.2 Reactive oxygen species and oxidative stress

Upon reacting with free electrons, oxygen molecules are transformed into ROS. ROS include hydrogen peroxide (H_2O_2), the hydroxyl radical (OH^-), the superoxide anion radical ($O_2\bullet^-$) and hydroperoxyl radical (HOO•) that are endogenous molecules produced during regular cell life and normal immune activation and have roles in cell signaling and homeostasis (Simic et al., 1989). ROS can also be produced via the metabolism and redox cycling of external molecules such as benzene. The damage these species can cause to the cell is dependent not only on their intracellular concentrations, but also on the equilibrium between ROS and endogenous antioxidant species. When the pro-oxidant/antioxidant equilibrium is lost oxidative stress occurs and has the potential to damage DNA/RNA, lipids and proteins (Veskoukis et al., 2012). The reactive nature of these species dysregulates sensitive redox-sensitive pathways. These species can also cause ‘nicks’ in DNA and alter DNA repair mechanisms. When DNA becomes oxidized by these species the production of 8-hydroxy-2′-deoxyguanosine and other oxidized bases can result, these oxidized base products can result in the generation of mutations in DNA in a process that promotes carcinogenesis (Matsui et al., 2000). Furthermore, cells may be injured by ROS as they are rich in polyunsaturated lipids, that when oxidized release lipid peroxidation reactions and increase cell permeability which can lead to cell death (Halliwell and Chirico, 1993).

Natural endogenous antioxidants have evolved in cells as mechanisms to detoxify ROS and avoid potential damage caused by ROS. While there are many antioxidants including catalase, superoxide dismutase and glutathione reductase, they can be overwhelmed by increases in ROS and a state of oxidative stress can occur. Oxidative stress is crucial from a biomedical point of view due to the large number and variety of related human diseases shown to be associated with increases in oxidative stress. Neurodegenerative diseases, inflammatory diseases, cardiovascular disease, immune dysfunctions and cancer all have some basis on oxidative stress (Sosa et al., 2013). ROS promote tumor development through four biological processes: cellular proliferation, evasion of apoptosis or anoikis, tissue invasion or metastasis and angiogenesis (Sosa et al., 2013). Evidence suggests a role for ROS and oxidative stress having deleterious effects *in utero*, as the developing fetus is more sensitive to chemicals compared to adults (Votavova, 2011). In fetal mouse explant cultures, treatment with cigarette smoke extracts showed increases in oxidative stress markers and a decrease in anti-apoptotic markers (Menon et al., 2011). In another study, maternal exposure to cigarette smoke extracts in rats led to fetal pancreatic oxidative stress (Bruin et al., 2008). Together these studies support the notion that maternal smoking, a source of benzene, induces oxidative stress in both the mother and the fetus.

1.3.3 Benzene and oxidative stress

Although this project did not directly measure ROS and oxidative stress markers, benzene's ability to cause carcinogenesis is well studied and evidence implicates metabolite-generated ROS in benzene's toxicity (Sun et al, 2018; Salimi et al., 2021; Badham et al., 2010; Badham and Winn, 2010). *In vitro* studies demonstrate that various metabolites of benzene can increase ROS production in multiple cell lines. One study showed that HL-60 cells, a leukemic cell line, exposed to BQ presented with ROS-mediated mitochondrial damage as characterized by mitochondrial membrane potential disruption and induction of apoptosis as measured by activated caspase-3 and caspase-9 (Sun et al., 2018). In addition, this study also showed that pretreatment with N-acetyl-l-cysteine (NAC) blocked BQ induced production of ROS. Another study from our lab showed that HD3 cells, an erythroid blast cell, exposed to the benzene metabolites catechol, BQ and hydroquinone resulted in increased c-Myb activity (one of the earliest steps leading to the formation of leukemic cells), phosphorylation of c-Myb and increased production of ROS (Wan, Badham and Winn, 2005; Badham et al., 2010). These studies also demonstrated that BQ exposure resulted in increased amounts of oxidative stress as compared to exposure to other metabolites. A third study demonstrated benzene in its pure unmetabolized form increased oxidative stress in HL-60 cells (Nishikawa et al., 2011). Other i*n vivo* studies show an increase in ROS via administration of hydroquinone to mice and rats (Lau et al., 2010) and increases in oxidative damage following benzene exposure in humans (Buthbumrug, 2008). Evidence of ROS formation has been obtained *in utero* in a study by Badham *et al* in 2010 and has become a foundation for future studies on *in utero* exposure to benzene. Their data revealed an increase in ROS production in fetal livers following maternal exposure to benzene that also correlated with increased incidence of carcinogenesis in pups one year after birth.

Benzene is myelotoxic and leukemogenic in humans, but precise mechanisms by which benzene induces ROS to yield leukemia is not yet clear. An experiment determining the levels of ROS formation induced by the five most potent benzene metabolites suggested that an interaction between benzene metabolites and lipid peroxidation enhances oxidation and DNA damage leading to carcinogenesis (Uysal et al., 1986). Others have proposed a mechanism that involves the recruitment of GSH (glutathione) or protein thiol groups in the production of ROS after benzene exposure (Stoyanovsky et al., 1995). They postulate that redox cycling of phenoxyl radical initiated GSH oxidation and subsequent generation of superoxide anions resulted in oxidative damage by benzene metabolites Another possible mechanism involving the conjugation of GSH involves redox cycling of the BQ-GSH conjugate 1,2,4-trihydroxy-5-glutathionyl benzene (TGB) that produces superoxide radicals and hydroxysemiquinone radicals (Brunmark and Cadenas, 1988; Rao 1996).

1.4 DNA DOUBLE-STRAND BREAKS AND TISSUE OF INTEREST

1.4.1 The placenta

The placenta is an organ that sits at the boundary between fetal and maternal vascular beds that helps mediate the exchange of nutrients and waste, allowing for growth and development of the fetus (Maltepe and Fisher, 2015). The placenta is the first organ to develop in pregnant mammals, as it is necessary to create a vascular interface between mother and fetus to supply bioenergetics to support fetal development (Maltepe et al., 2010). In rodents and humans, prior to implantation, the fertilized embryo begins to divide into eight nearly identical cells known as blastomeres. Three following divisions creates a grouping of cells known as the blastocyst. Surrounding the blastocyst is the trophectoderm which will give rise to the placenta (Rossant & Tam 2009; Sasaki 2010). The placenta is a fetal organ made up of the parenchyma, chorion, amnion, and umbilical cord, all structures which derive from the zygote (Herrick and Bordoni, 2021) separating it from the maternal endometrium. The placenta is composed of three layers: the innermost amnion that surrounds the fetus, the middle layer called the allantois which highly dense with fetal blood vessels and the outermost chorion which contacts the maternal endometrium (Portilo and Pelajo-Mechado, 2018).

The placenta is a complex and diverse organ that is a high-yield source for stem cells (Lobo et al., 2016) and so can be a target for benzene toxicity and can be a representation of benzene's effects *in utero*. Hematopoietic activity in the placenta occurs between GD 10.5-12.5 and aligns with the dosing and organ collection protocol followed in this thesis. The placenta plays a significant role in mouse and human blood cell development (Portilo and Pelajo-Mechado, 2018). *In vivo* studies discovered that the placenta is capable of long-term HSC repopulation (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). Flow cytometry indicated that all HSC cells at GD 12.5 exhibited phenotypes and population comparable to HSC in the fetal liver (Ottersbach and Dzierzak, 2005, which is a fetal tissue that has been shown to be subject to benzene toxicity (Holmes and Winn, 2019; Badham and Winn, 2010). Additionally, the placenta can produce autonomous generation of HSCs in isolation from the chorion (Corbel et al., 2007). This data indicates the placenta has a role in HSC generation between the dorsal aorta and the fetal liver, thus making it a target for benzene toxicity and a potential indicator of benzene toxicity.

Commonly referred to as the afterbirth and is bluntly discarded after delivery, new research on placental biology is changing the perception on the utility of the placenta and revealing previously unknown biology regarding the developing fetus. Furthering our understanding of placental development and its relation to the developing fetus will improve our ability to predict and treat pregnancy complications and potential threats to *in utero* development.

1.4.2 Benzene and double stranded breaks

DNA is at constant risk of being damaged by endogenous sources, ionizing radiation and chemical substances (Xu and Xu, 2020). Double stranded DNA breaks (DSBs) are the most toxic kind of DNA damage and misrepair of these breaks can lead to severe mutations such as deletions and chromosomal translocations (Shibata, 2017). Benzene and benzene metabolites are known to cause DSBs as evidenced by studies demonstrating biomarkers of DNA damage such as γ-H2A.X foci. Tung et al., (2012) showed that BQ could induce double stranded breaks in murine fetal hematopoietic cells from pZK1 mice and implicated ROS as an important mechanism. Additionally, treatment of human HSCs with hydroquinone induced DSBs and induced the NF-κB pathway to promote homologous recombination. Another potential mechanism by which benzene may cause DSBs without direct production of ROS could be through its inhibition of topoisomerase IIα (Topo IIα), an enzyme that relieves strain on double-stranded helices (Holmes and Winn, 2019). Topo IIα activity decreases following exposure to BQ *in vitro* and disrupts the replication fork causing DNA damage and implicating it as a target for benzene toxicity. The ability for benzene and its metabolites to cause DSBs through ROS and other pathways begs the questions how the cell’s repair pathways are affected and how the developing fetus, which is more sensitive to benzene toxicity, handles these breaks.

1.4.3 Double strand break repair *in utero*

The two main double-stranded DNA repair pathways during fetal development are non-homologous end joining (NHEJ) (figure 1.3) and homologous recombination (figure 1.4). DSBs are the most dangerous kind of DNA damage as they can result in the loss of large portions of chromosomes (Xu and Xu, 2020). These breaks require nucleases for end-processing and DNA polymerases to repair the section of damaged DNA. In NHEJ, the DSBs are initially recognized by the Ku70-Ku80 heterodimer, which acts as a recruiter and attachment point for other repair proteins, such as DNA-dependent protein kinase catalytic subunit (DNA-PKcs) which have a high affinity for the Ku-DNA ends (Meek, Dang and Lees-Miller, 2008). The process of NHEJ requires the incorporation of nucleotides in the segment of broken DNA following the limited processing of the DNA strand ends (Chang et al.,2017). Although rapid, NHEJ is error prone and evidence suggests that increased NHEJ is associated with increases in somatic intrachromosomal recombination and chromosomal mutations (Hooker et al., 2004). Furthermore, errors in DSB repair have been shown to lead to many forms of cancer including leukemia, and chromosomal aberrations resulting from loss of NHEJ proteins is thought to be a key contributor (Bast, Hennessey and Mills, 2009; Ceccaldi, Rondinelli and D'Andrea, 2015). Critical genes for the function of NHEJ include *X-Ray Repair*

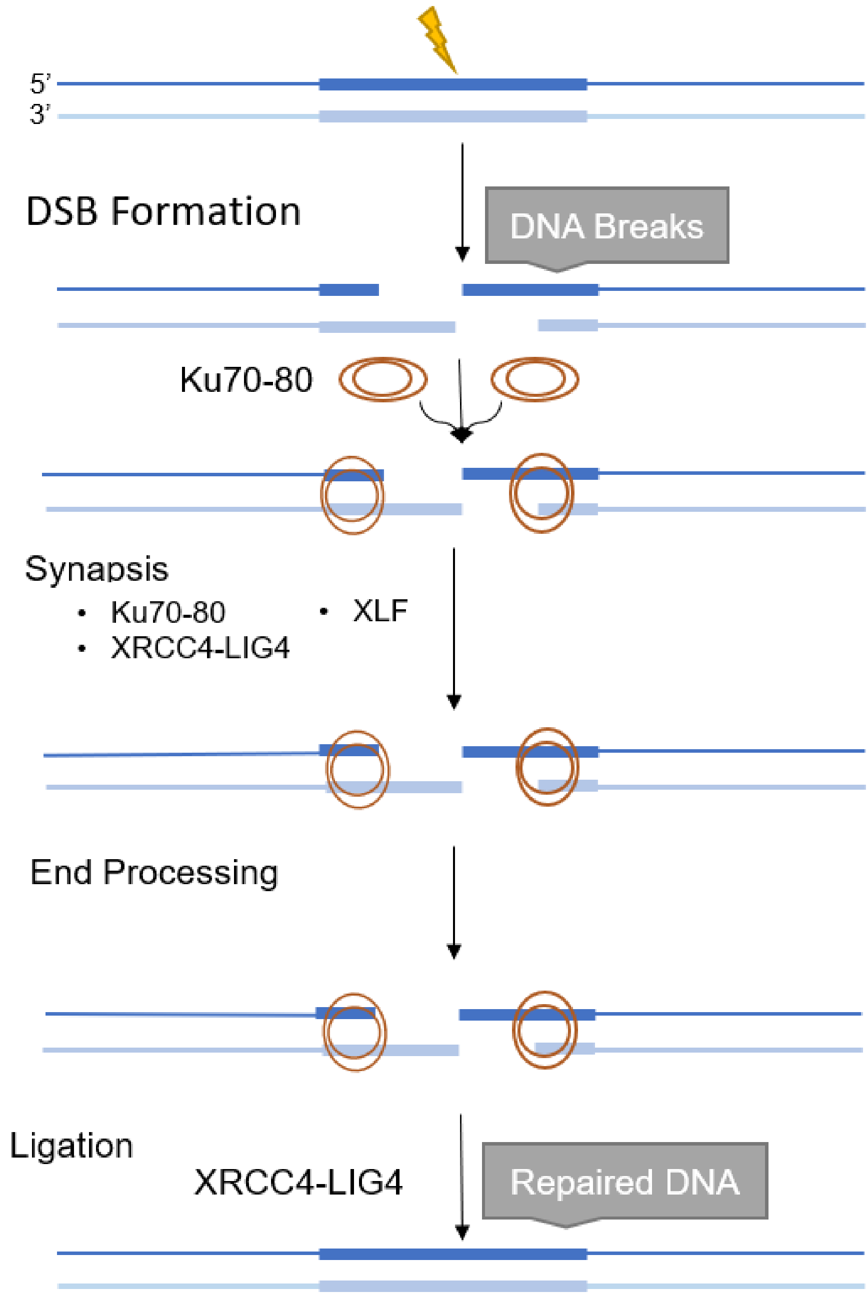

Figure 1.3 Simplified schematic of NHEJ

NHEJ is a predominant DSB repair pathway in mammalian cells and the developing fetus. NHEJ is a rapid but error prone process that does not require a DNA template to initiate repair. As seen above, open DNA ends are recognized by Ku70-Ku80 proteins begins loading DNA PKCs and other repair proteins such as XRCC4-LigaseIV which ligates the DNA. Modified from Zhao et al., 2020.
XRCC4-LIG4:XRCC4-LigaseIV; XLF: XRCC4 Like Factor

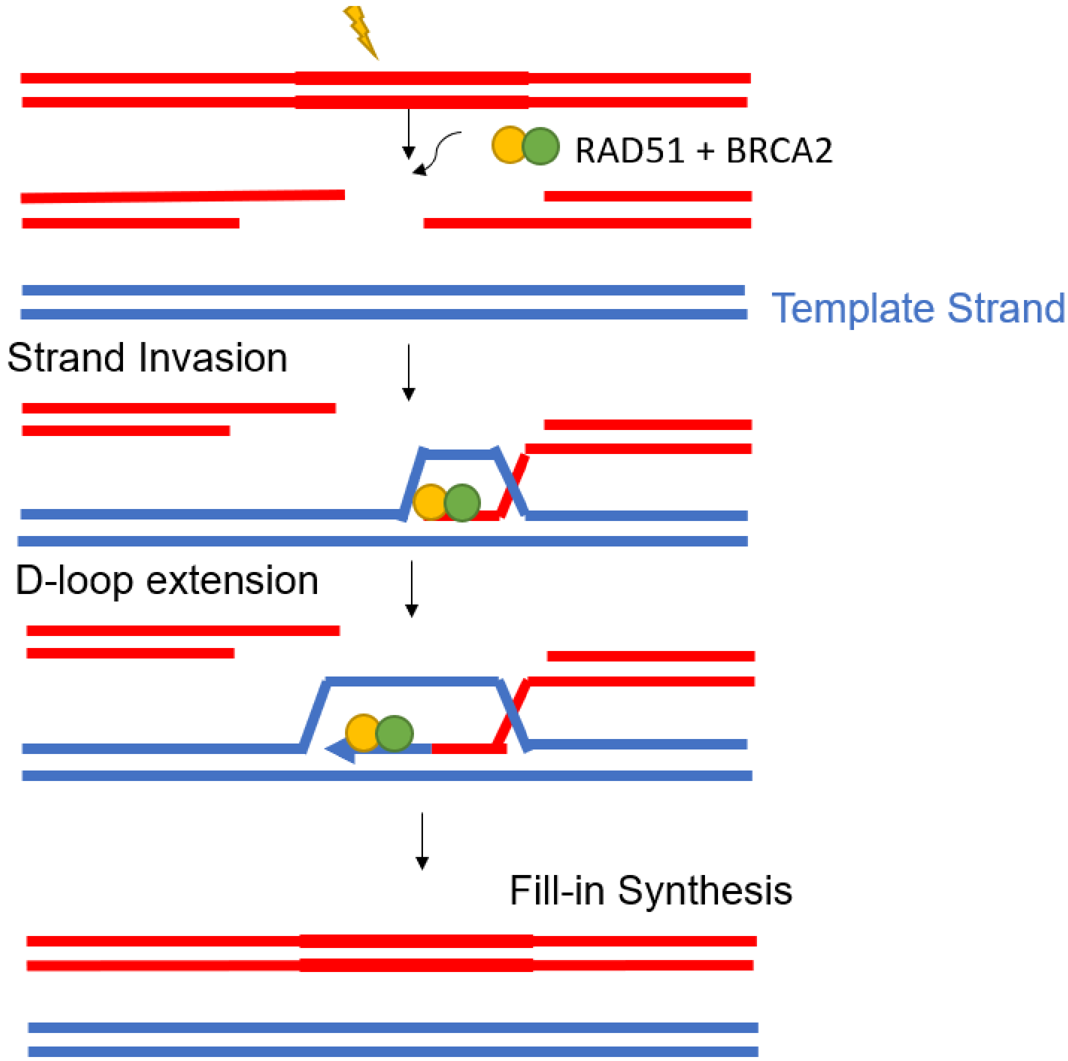

Figure 1.4 Simplified schematic of homologous recombination

Homologous recombination is responsible for the repair of the majority of DSBs induced through regular cellular processes of replication. This process requires the invasion of a template DNA strand facilitated by homologous recombination proteins such as RAD51 and BRCA2. Modified from Renodon-Cornière et al., 2012.

Cross Complementing (XRCC) *4,5* and *6*, which code for the proteins Xrcc4, Ku80 and Ku 70 respectively (Chang et al., 2017).

Homologous recombination is the alternative method of DSB repair *in utero*. Homologous recombination uses a sister DNA strand as a template that will synapse with one of the DSB ends and invade to prime DNA repair synthesis and creates a D-loop. This loop is created by the ‘D’ like orientation that the invading strand adopts before it is resolved and removed. DSB repair favors the use of the sister chromosome over the homologous chromosome as a template (Kadyk and Hartwell, 1992) and will primarily resolve D-loops through the synthesis-dependent strand annealing pathway (SDSA) (Pâques and Haber, 1999). DNA synthesis creates homology to the other broken end so that once the D-loop is resolved the two strands may anneal and the strand is repaired. The process of homologous recombination is aided by the Rad51 family of proteins and proteins that aid Rad51 such as Brca2 (Wright, Shah and Heyer, 2018).

In mammalian cells, there are an estimated ten DSBs per day per cell (Lieber, 2010). These daily occurrences are caused by ionizing radiation, ROS and replication errors, and any changes to these factors has the potential to cause increased damage to the cell and cause disease states. Ultimately, if benzene induces DSBs, and prevents appropriate repair of breaks, then the fetus may be at risk of chromosomal instability and result in leukemogenesis.

1.4.4 Genes of interest

Given the evidence that benzene can cause DNA damage, understanding the impacts of benzene exposure on fetal DNA repair genes is important. This is particularly relevant given that very little is known about fetal DNA repair capacity. To begin to understand the impacts of benzene this thesis looked at a number of selected genes that function under the aforementioned main pathways of double-stranded DNA repair. The genes *Xrcc4*, *Xrcc5* and *Xrcc6* participate in NHEJ via formation of the proteins Xrcc4, Ku80 and Ku70 respectively.

Xrcc4 alongside ligase IV form the most central part of NHEJ in eukaryotes (Lieber, 2010). Xrcc4 stimulates ligase IV activity and forms the Xrcc4- DNA ligase IV complex which is critical for efficient reconstitution of the NHEJ pathway (McElhinny et al., 2000). Furthermore, biochemical evidence shows that DNA end bridging is dependent on the presence of Xrcc4 (Chang et al., 2016).

Xrcc5 codes for the protein Ku80 which makes up part of the Ku70-Ku80 heterodimer. This heterodimer has a critical function in NHEJ by acting as a 'tool belt' that enables the loading and recruitment of other proteins to the open DNA ends. *Xrcc6* codes for the smaller subunit in the heterodimer, Ku70. Both Xrcc5 and Xrcc6 are critical for recognition of the open DNA ends and protecting the DNA from degrading, which would result in chromosomal instability (Hristova, Lauer and Ferguson, 2020).

In homologous recombination, *Rad51* is the major regulator and key contributor to the formation of the nucleoprotein filament scaffold on single stranded DNA (Bagnolini et al., 2022). The Rad51 protein forms a filament composed of nucleic acids and protein on the single strand of ssDNA. The filament functions to search for DNA sequences similar to the 3' overhang and invades in to allow homologous recombination to take place. *Brca2* is a gene coding for the Brca2 protein and is a direct mediator of the function of Rad51. Biochemical evidence suggests that BRCA2 creates a complex

(Brca2-DSS1) critical for RAD51 filament nucleation (Jensen, Carreira and Kowalczykowski, 2010; Liu et al., 2010).

1.4.5 CD-1 mouse model

The CD-1 mouse model used to carry out the experiments described in this thesis is based on the *in vivo* model that was previously established in this laboratory (Badham et al., 2010). The model and dosage are reflective of high exposure in humans who are exposed to benzene via inhalation. In this model, female dams are intraperitoneally injected with 200mg/kg of benzene diluted in corn oil (or only corn oil as a control) on days 8, 10, 12 and 14 of gestation. This model has been shown to produce tumors one year after birth (Badham et al., 2010) and confirms that the pups were subject to *in utero* benzene toxicity.

1.5 HYPOTHESIS AND OBJECTIVES

1.5.1 Hypothesis

The etiology of the majority of childhood leukemias remains unclear, however new research has proposed that proximity and exposure to environmental carcinogens in early stages of life may be a contributing factor. Benzene is a ubiquitous environmental pollutant that has been acknowledged as an environmental carcinogen and is a known contributor to the development of leukemia in adults. We propose that maternal benzene exposure will lead to an increase in DSBs in the placenta and will be evidenced by changes to gene expression of critical DNA repair genes. Specifically, we hypothesize that maternal benzene exposure will cause increases in gene expression of *Xrcc4, Xrcc5, Xrcc6, Rad51* and *Brca2* in the placenta of CD-1 mice.

1.5.2 Objective

Objective 1. To measure placenta gene expression of critical genes related to non-homologous end joining and homologous recombination.

Chapter 2

Methods and Materials

2.1 ANIMAL TREATMENT AND TISSUE COLLECTION

Four- to six-week-old female CD-1 mice (Charles River, Montreal, QC, Canada) were housed in the Queen's University Animal Care facility. Mice were fed a standard rodent chow (Purina, Strathroy, ON, Canada) and provided tap water *ad libitum*. Animal handling and tissue extractions were performed according to the Queen's University Animal Care Committee and the Canadian Council on Animal Care guidelines. For breeding, two female mice were housed overnight with one male mouse, with the presence of a vaginal plug the next morning indicating gestational day (GD) 1. Pregnant CD-1 dams were injected intraperitoneally with 200 mg/kg of benzene dissolved in corn oil or corn oil alone (control), on GD 8, 10, 12 and 14. At times 2, 6 and 24 hours following the final dosing on GD 14, pregnant dams were euthanized via cervical dislocation and pneumothorax and GD 14 placentas were collected and fetal limbs were collected for sex determination. Tissues were stored in a -80 °C freezer until processed.

2.2 GENE ANALYSIS

Placentas were cut in half along the major axis according to their elliptical shape using a razor blade. One half was placed back in storage while the other was homogenized using a 25-gauge needle to create a solution for RNA isolation via a QIAGEN kit (QIAGEN, RNEasy, Mississauga, Ontario, Canada). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit (Thermofisher) and was subsequently used for qRT-PCR in 96 well plates to determine transcription levels of genes of interest. qRT-PCR reactions for genes involved in DNA repair used Qiagen's RT^2 qPCR primers. Probes detected gene transcript levels for Breast cancer resistance 2 (*Brca2*, PPM03704E), Rad51 Recombinase (*Rad51*, PPM03278C-200), X-ray repair complementing defective repair in Chinese hamster cells 4 (*Xrcc4*, PPM04389A-200), X-ray repair complementing defective repair in Chinese hamster cells 5 (*Xrcc5*, PPM04373A-200) and X-ray repair complementing defective repair in Chinese hamster cells 6 (*Xrcc6*, PPM04966A-200). TATA-binding protein (*TBP*, PPM03560F-200) was used as a control gene in these samples and analyzed in triplicate using the delta-delta Ct method. For this study, each time point had an N value of 5, where an N represents the average of three placentas from a litter. Five litters make up a time point from dosed and undosed mice.

2.3 FETAL SEX DETERMINATION

In attempt to avoid litters with only male or only female offspring and ensure at least one male or female placenta was collected per litter, the sex of each offspring was determined using a method designed by Tunster (2017) using PCR for the *Rbm31x/y* exon. This chromosomal region exists as an exon 1775 bps in length on *Rbm31x*, whereas that of *Rbm31y* is an exon of 1958 bps in length. *Rbm31x* features an 84 bp deletion. Primers were designed to flank homologous regions of the 84 bp deletion to yield a 269 bp product from the X chromosome and a 353 bp product from the Y chromosome. Limbs were lysed overnight at 55°C in lysis buffer (50 mM Tris (pH 8), 10 mM EDTA, 20 mM NaCl and 0.031% SDS) supplemented with 400 μg/ml Proteinase K (Promega).

Lysates were vortexed, heated at 95°C for 15 min, cooled for 10 min and 0.6 μl used as template in a 15 μl PCR reaction containing 1X buffer, 0.2 mM each dNTP, 0.17 μM each primer, and 0.5U DreamTaq HotStart DNA Polymerase (ThermoFisher). Samples were run in the thermocycler at 94°C for 2 min, followed by 30 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 30 seconds, with a final elongation at 72°C for 5 min. PCR reactions were mixed with 6X Orange G Gel Loading Dye, loaded on to a 1% (*w*/*v*) agarose gel containing 10 μl SafeView (NBS Biologicals), electrophoresed in 1X tris acetate edta (40 mM Tris acetate, 2 mM Na_2EDTA) at 10 V/cm for 20 min and visualized on a UV transilluminator. Fetal sex could then be confirmed by examining different sized base pair products (Tunster, 2017)

2.4 DATA ANALYSIS

qRT-PCR analysis included an $n = 5$ for each group, where each n consisted of at least one sex from each litter and represents one placenta or pup. Statistical analysis was performed using two-way ANOVA and Tukey post-hoc test from GraphPad PRISM 9, to examine the effect of dose of benzene versus control across three time points (2, 6 and 24 hours following dosing). Statistical significance was determined at $p < 0.05$.

Chapter 3

Results

3.1 *Xrcc4* mRNA EXPRESSION

We have previously shown that benzene is able to cross the placenta and have *in utero* carcinogenic effects in mice (Badham et al., 2010). While the mechanism of benzene toxicity is still being investigated, one hypothesis proposes that benzene exerts it toxicity through the production of ROS (Sun et al, 2018; Salimi et al., 2021) leading to an increase in DSBs. Increased rates of DSBs can result in various disease states, including cancer (Shibata, 2017). Investigations into DSB repair mechanisms of damage caused by benzene *in utero* can potentially increase our understanding of benzene-induced carcinogenesis in the developing fetus and may lead to future clinical outcomes.

Xrcc4 has been shown to be a critical gene for DSB repair via NHEJ (Ruis et al., 2020). To provide evidence that benzene contributes to fetal carcinogenesis by disrupting DSB repair in CD-1 mice, mice were treated with either benzene or vehicle and qRT-PCR was then conducted using RNA collected from GD14 placentas for the key DSB repair gene *Xrcc4*. After analysis, significant upregulation of mRNA expression was observed at the 24h time-point ($p < 0.01$) (Figure 3.1a) using two-way ANOVA. Additionally, the dose of benzene was a significant source of variation ($p < 0.01$).

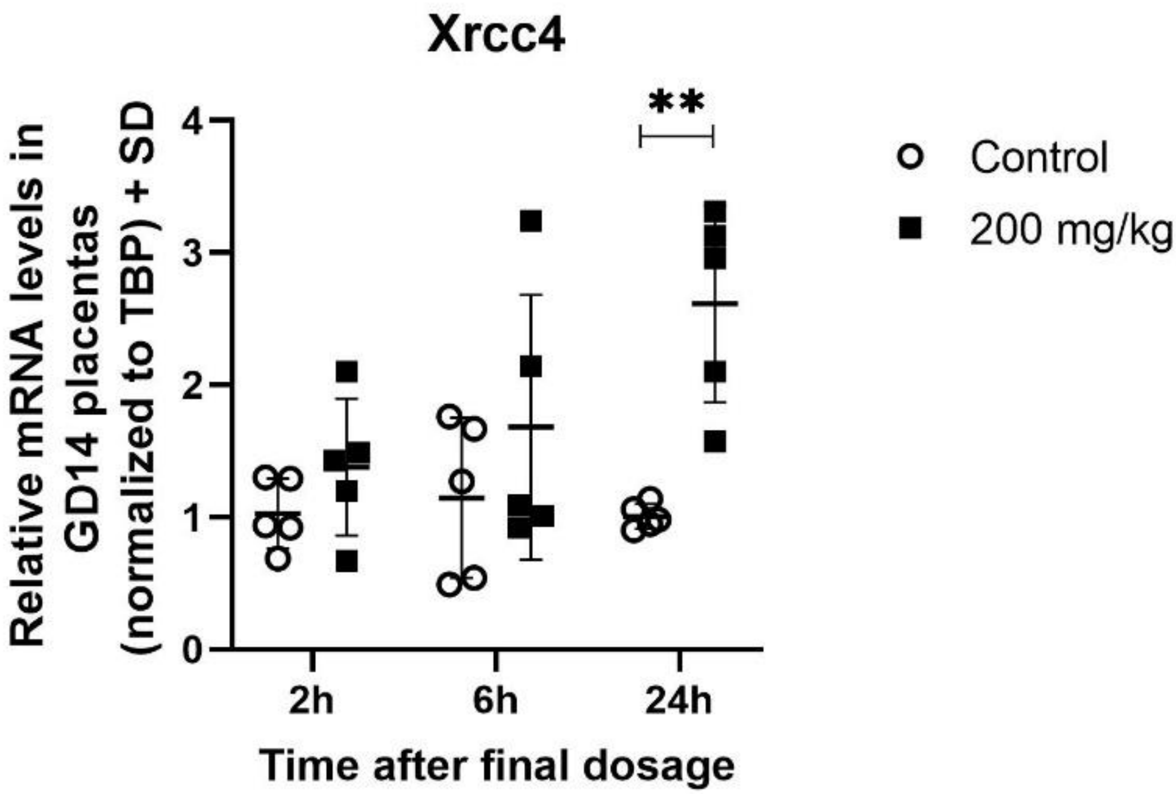

Figure 3. 1: **qRT-PCR analysis of *Xrcc4* mRNA levels from gestational day (GD) 14 placentas exposed to either control or benzene (200 mg/kg) on GD8, GD10, GD12 and GD14**. Total RNA from GD14 placental tissue was extracted after treatment with either benzene or control. Expression of mRNA after the final benzene exposure was analyzed and transcript levels normalized to TBP. *Xrcc4* mRNA levels at 2h and 6h remained unchanged, however increased at 24h ($p < 0.01$) following the final benzene exposure in placentas ($n = 5$) compared to control ($n = 5$). Statistical analysis was performed using two-way ANOVA, post hoc Tukey test. Dose was a significant source of variation ($p < 0.01$).

3.2 *Xrcc5* mRNA EXPRESSION

Xrcc5 plays an important role in DSB repair by binding to open DNA ends and initiating NHEJ (Yang et al., 2011). To provide evidence that benzene contributes to fetal carcinogenesis by disrupting DSB repair in CD-1 mice, mice were treated with either benzene or vehicle over a 14-day period and qRT-PCR was then conducted from RNA collected from GD14 placentas for the key DSB repair gene *Xrcc5*. After extraction, significant upregulation of *Xrcc5* mRNA expression was observed at 6h ($p < 0.05$) while performing statistical analysis with two-way ANOVA (3.2). The other time points showed non-significant changes to mRNA expression for *Xrcc5* (3.2) Additionally, the dose of benzene exposure was found to be a significant ($p < 0.0001$) source of variation.

3.3 *Xrcc6* mRNA EXPRESSION

Xrcc6 complements with Xrcc5 and binds to open DNA ends and recruits and begins recruitment to the site of the break (Yang, 2011). To provide evidence that benzene contributes to fetal carcinogenesis by disrupting DSB repair in CD-1 mice, mice were treated with either benzene or vehicle and qRT-PCR was then conducted using RNA collected from GD14 placentas for the key DSB repair gene *Xrcc6*. After extraction, no significant changes at any time points between exposed and control mice was observed when a two-way ANOVA was used for statistical analysis (Figure 3.3).

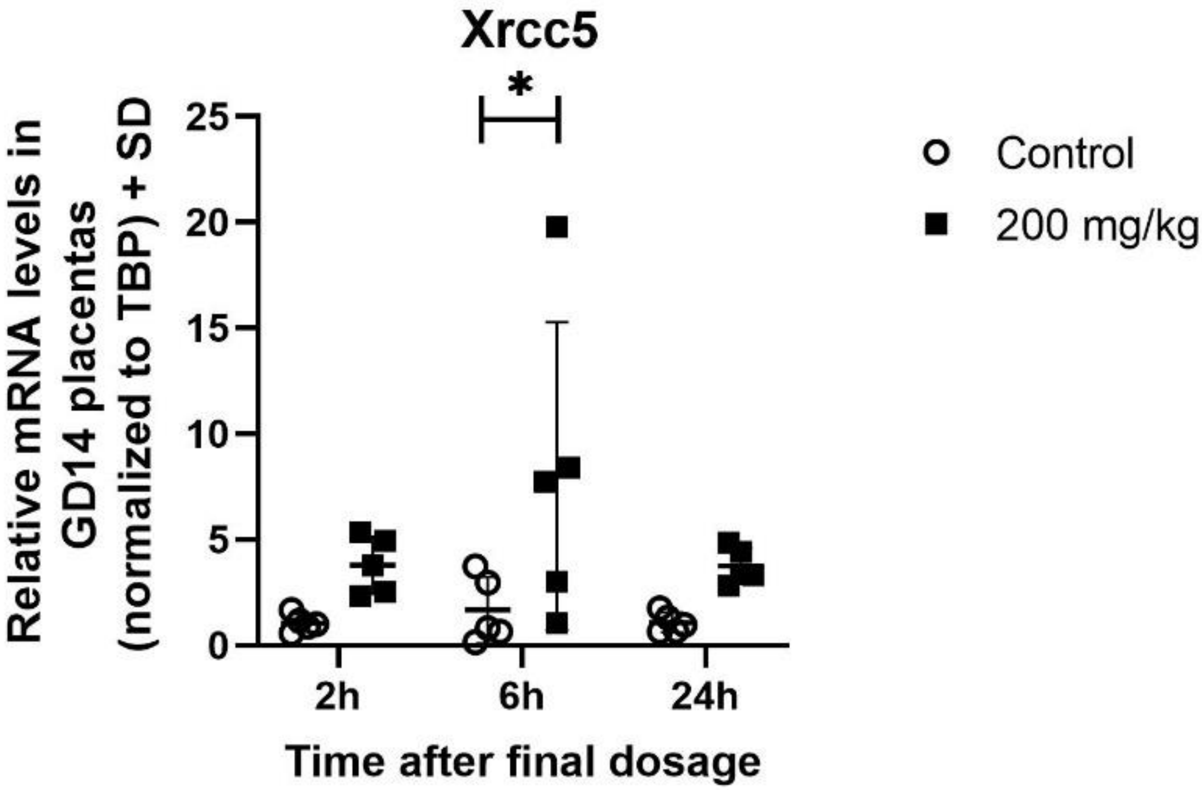

Figure 3.2: **qRT-PCR analysis of *Xrcc5* mRNA levels in gestational day (GD) 14 placentas exposed to either control or benzene (200 mg/kg) on GD8, GD10, GD12 and GD14**. Total RNA from GD14 placental tissue was extracted after treatment with either benzene or control. Expression of mRNA after the final benzene exposure was analyzed and transcript levels normalized to TBP. mRNA levels at 2h, 24h remain unchanged and increase significantly at 6h ($p < 0.05$) following final benzene exposure (200 mg/kg) in fetal placentas ($n = 5$) compared to control ($n = 5$). Statistical analysis was performed using two-way ANOVA, post hoc Tukey. Additionally, the level of benzene exposure was found to be a significant ($p < 0.0001$) source of variation.

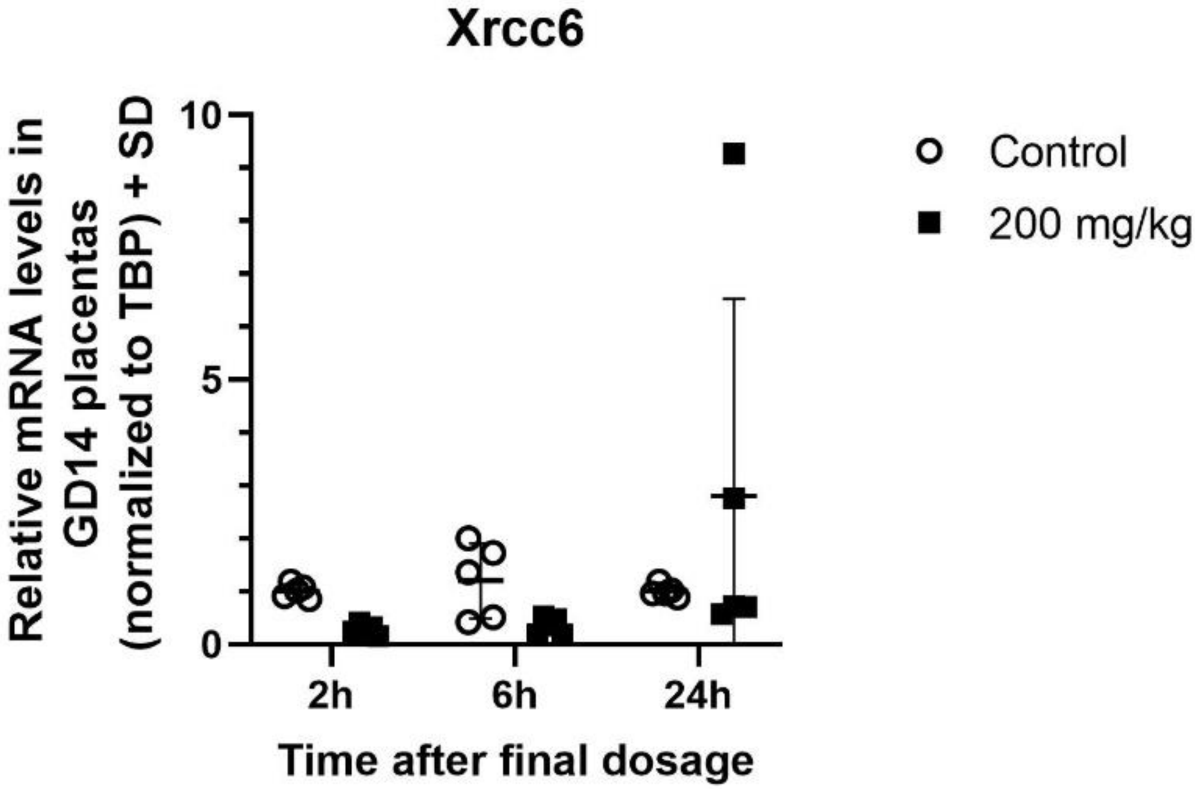

Figure 3.3: qRT-PCR analysis of *Xrcc6* mRNA levels in gestational day (GD) 14 fetal placentas exposed to either control or benzene (200 mg/kg) on GD8, GD10, GD12 and GD14. Total RNA from GD14 placental tissue was extracted after treatment with either benzene or control. Expression of mRNA after the final benzene exposure was analyzed and transcript levels normalized to TBP. mRNA levels at 2h, 6h and 24h remain unchanged following final benzene exposure (200 mg/kg) in fetal placentas ($n = 5$) compared to control ($n = 5$). Statistical analysis was performed using two-way ANOVA, post hoc Tukey test. Additionally, the dose of benzene exposure was found to be a significant source of variation ($p < 0.001$).

.Additionally, the dose of benzene exposure was found to be a significant source of variation ($p < 0.001$).

3.4 *Rad51* mRNA EXPRESSION

In homologous recombination, Rad51 is the major regulator and key contributor to the formation of the nucleoprotein filament scaffold on single stranded DNA (Bagnolini et al., 2022). To provide evidence that benzene may contributes to fetal carcinogenesis by disrupting DSB repair in CD-1 mice, mice were treated with either benzene or vehicle and qRT-PCR was then conducted using RNA collected from GD14 placentas for the key DSB repair gene *Rad51*. After extraction, significant upregulation of *Rad51* mRNA was observed at the 24h time-point ($p < 0.001$) whereas no significant changes were observed at the 2h or the 6h time-points using a two-way ANOVA (Figure 3.4). The level of benzene exposure was a significant source of variation ($p < 0.05$) and time following the final benzene exposure was also a significant source of variation ($p < 0.01$), resulting in a statistically significant interaction ($p < 0.001$)

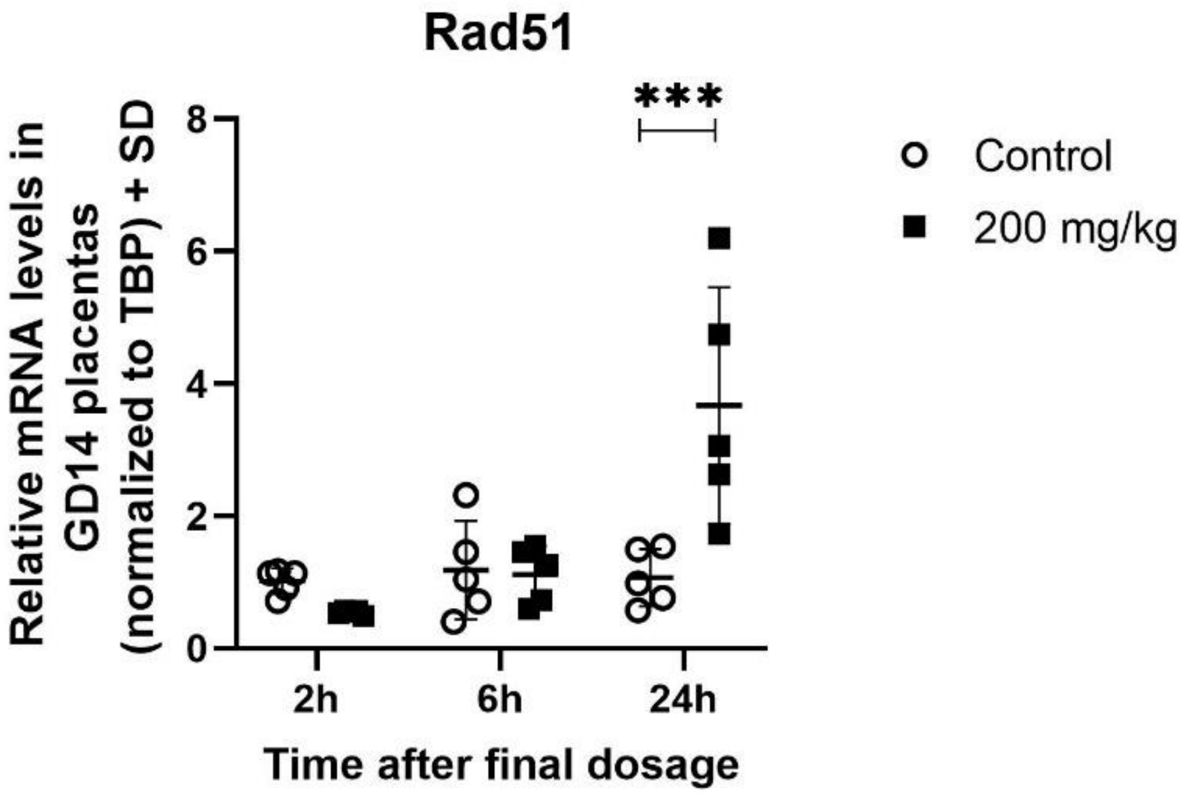

Figure 3.4: qRT-PCR analysis of *Rad51* mRNA levels in gestational day (GD) 14 fetal placentas exposed to either control or benzene (200 mg/kg) on GD8, GD10, GD12 and GD14. Total RNA from GD14 placental tissue was extracted after treatment with either benzene or control. Expression of mRNA after the final benzene exposure was analyzed and transcript levels normalized to TBP. mRNA levels at 2h and 6h remain unchanged following final benzene exposure (200mg/kg) in fetal placentas ($n = 5$) compared to control ($n = 5$) and increase significantly ($p < 0.001$) at 24h. Statistical analysis was performed using two-way ANOVA, post hoc Tukey test. The level of benzene exposure was a significant source of variation ($p < 0.05$) and time following the final benzene exposure was also a significant source of variation ($p < 0.01$).

3.5 *Brca2* mRNA EXPRESSION

Brca2 in HR is a mediator of Rad51 and has additional roles in tumor suppression (Prakash et al.,2015). To provide evidence that benzene contributes to fetal carcinogenesis by disrupting DSB repair in CD-1 mice, mice were treated with either benzene or vehicle and qRT-PCR was then conducted using RNA collected from GD14 placentas for the key DSB repair gene *Brca2*. After analysis, a significant decrease ($p < 0.01$) in *Brca2* mRNA expression was observed in placenta from benzene exposed animals relative to control mice at 2h and at 6h post final benzene exposure (Figure 3.5). Benzene exposure level was found to be a significant source of variation ($p < 0.0001$).

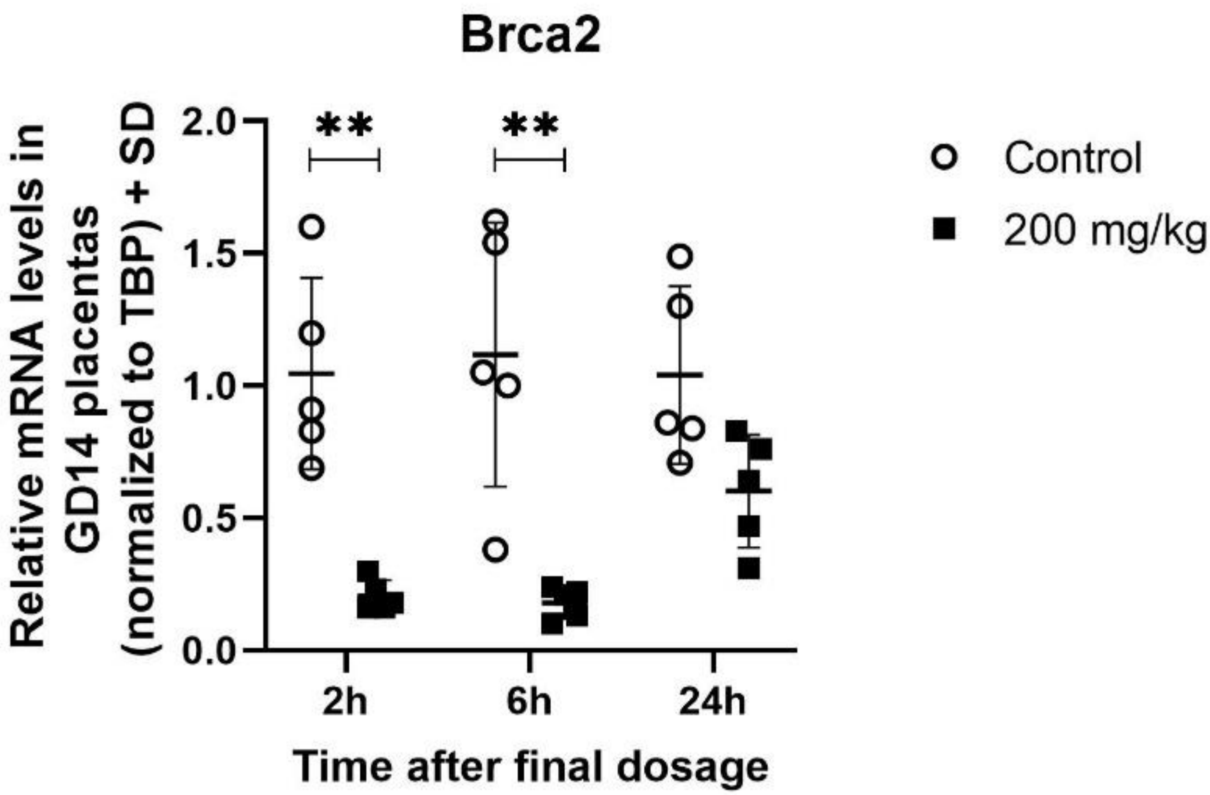

Figure 3.5: qRT-PCR analysis of *Brca2* mRNA levels in gestational day (GD) 14 fetal placentas exposed to either control or benzene (200 mg/kg) on GD8, GD10, GD12 and GD14. Total RNA of GD14 placental tissue was extracted after treatment with either benzene or control. Expression of mRNA after the final benzene exposure was analyzed and transcript levels normalized to TBP. mRNA levels at 24h remain unchanged following final benzene exposure (200 mg/kg) in fetal placentas (n = 5) compared to control (n = 5), whereas are significantly decreased at 2h ($p < 0.01$) and 6h ($p < 0.01$). Statistical analysis was performed using two-way ANOVA, post hoc Tukey test. Additionally, benzene exposure level was found to be a significant source of variation ($p < 0.0001$).

Chapter 4

Discussion

4.1 GENERAL DISCUSSION

Benzene is a ubiquitous environmental pollutant with known carcinogenic and more specifically, leukemogenic effects. Furthermore, *in-utero* exposure to benzene in humans has been shown to increase the risk of childhood leukemia (Snyder, 2012, Shallis *et al.,* 2021). It has been postulated that maternal exposure to environmental chemicals, such as benzene, while pregnant may initiate fetal cellular changes that lead to the progression of childhood leukemia before birth (Rassschou-Nielsen *et al.,* 2018; Heck *et al.,* 2014; Filippini *et al.*, 2015). To further our understanding of the potential role of benzene in the *in utero* initiation of child leukemogenesis, we investigated benzene's potential to cause toxicity in the mouse placenta. Specifically, this study aimed to analyze the effects that maternal benzene exposure had on the gene expression of DSB repair genes in the mouse placenta. Placentas were collected from GD 14 mouse fetuses following maternal exposure to benzene and were subsequently assessed for changes in gene expression of key DSB repair genes involved in NHEJ and HR, including *Xrcc4, Xrcc5*, *Xrcc6*, *Rad51* and *Brca2.* While benzene is known to cause DNA damage, it is also well known that the fetal capacity to repair DNA damage is significantly lower than the adult (Vinson and Hales, 2002; Pachowski *et al.*, 2011). Benzene-induced DNA damage in the developing fetus may thus go unrepaired and continued inability to correctly repair DNA breaks can be extremely detrimental to the developing fetus

(Pachkowski *et al*., 2011), thus differences between maternal and fetal abilities to repair DNA damage warrants further investigation into fetal DNA repair pathways (Lau *et al*., 2009).

The objective of this study was to determine whether benzene-induced changes in placental gene expression of critical genes related to NHEJ and HR, given that DSBs are primarily repaired via these two main pathways. Both NHEJ and HR, whilst efficient at repairing DNA DSBs, are error prone processes (Chen *et al*., 2021). Studies have shown that increases in NHEJ activity can yield products associated with genetic deletions (Seol, Shim and Lee, 2017). Furthermore, unreliable and erroneous DNA repair may be a possible mechanism through which chromosomal aberrations that are commonly seen in childhood leukemias occur (Rodgers and McVey, 2016; Maher *et al.,* 2011). The determination of whether *in utero* benzene exposure causes changes in the gene expression of key NHEJ and HR genes can provide further understanding of the mechanisms of benzene-initiated toxicity.

The first key gene we studied was *Xrcc4*, which codes for the protein Xrcc4 and participates in the Xrcc4 – DNA Ligase IV complex (Ruis *et al*., 2021). One previous study showed an insignificant interaction between *Xrcc4* and benzene exposure in workers compared to controls (Shen *et al*., 2006), where single nucleotide polymorphisms (SNPs) for genes of interest (*Xrcc4)* were analyzed in white blood cells following benzene exposure. However another study showed a significant association of *Xrcc4* polymorphisms with childhood leukemia (Wu *et al*., 2010). In the latter study, mutated variants of the human *Xrcc4* gene were found to resemble those that are commonly seen in childhood leukemias. It can therefore be hypothesized that benzene-exposure could potentially lead to *Xrcc4* mutations, which may be a cause of childhood leukemia. Herein we showed that *in utero*, 14-day benzene exposure caused a significant time-dependent increase in *Xrcc4* gene expression levels in the placenta, most prominently at the 24h time-point. Our results therefore demonstrate a potential link between benzene exposure and changes to DNA DSB repair.

The next key gene we studied was *Xrcc5*, which codes for the protein Ku80, and forms part of the main heterodimer required for NHEJ in vertebrates (Chang *et al*., 2017). We observed a significant upregulation in mRNA expression of *Xrcc5* at 6h, indicating that *Xrcc5* shows an increased sensitivity to benzene exposure rapidly after extraction when compared to other examined genetic markers. Several studies have implicated *Xrcc5* as crucial for NHEJ mechanisms, so much so that the absence of *Xrcc5* has been shown to cause a complete stop to DNA repair (Fattah, Ruis and Hendrickson, 2003; Uegaki *et al*., 2006; Ghosh, Myung and Hendrickson, 2007). The increased *Xrcc5* mRNA expression observed in our study is consistent with the trend of increasing *Xrcc5* expression in subjects living in highly polluted areas with exposure to high levels of benzene (Rossner Jr *et al*., 2011).

With respect to NHEJ, the final gene we assessed in this DNA repair pathway was *Xrcc6*, which codes for the protein Ku70 and completes the heterodimer required for NHEJ in vertebrates (Chang *et al*., 2017). We observed no significant changes in mRNA expression of the gene at all time points. This observation is in contrast with research that concluded that *Xrcc6* is increased in pre-leukemic states (Pei *et al*., 2013) and is elevated in lung cancer patients to preserve genomic integrity (Singh *et al*., 2018). While our *Xrcc6* findings contrast with *Xrcc5* and *Xrcc4*, this may be indicative of a yet unknown mechanism controlling Xrcc6 independently of its heterodimerization function with Xrcc5.

Several studies have implicated altered NHEJ DNA repair genes as having the potential to cause childhood leukemia through erroneous DNA DSB repair leading to genomic instability (Rodgers and McVey, 2016; Yoshioka *et al.*, 2021; Audoynaud, Vagner and Lambert, 2021). To the best of our knowledge, ours is the first study directly determining the presence of altered NHEJ genes in the developing placenta post-maternal benzene exposure. Here we demonstrate that exposure to benzene *in utero* causes various alterations to fetal genes involved in DNA DSB repair via both NHEJ and HR. Interestingly, the genes related to NHEJ show differing responses to *in utero* benzene exposure. Although together, they form the Ku70/80 heterodimer and its recruitment control mechanism, they all demonstrate differing gene expression changes after maternal exposure, occurring at different time points. The increased gene expression of both *Xrcc4* and *Xrcc5* in our study aligns with the study conducted by Schulte-Uentrop, *et al*., (2008), in which they demonstrated that functional *Xrcc4* and *Xrcc5* were essential for repair of DSBs in mice. These results are also validated by another study that determined that benzene-exposed workers showed increased DSB repair by inducing DNA-PKCs, which completes the NHEJ catalytic subunit and concluded that increased NHEJ led to increased mutations (Bi *et al*., 2009). An additional study confirmed this observation by demonstrating that benzene induces DNA-PKCs at the protein and mRNA level (Xiao *et al*., 2010). Together these findings indicate that the induction and subsequent activation of DNA-PKCs in NHEJ may contribute to benzene carcinogenesis. What remains unknown however is the relationship of these DNA PKCs to *Xrcc4, Xrcc5* and *Xrcc6*.

Specifically, in the current study we observed contrasting expressions of important NHEJ genes including the decrease in *Xrcc6*, which is an interesting finding given that it forms a heterodimer with the induced *Xrcc5*. The differing relative expression of these genes after maternal benzene-exposure may be indicative of a yet unknown relationship between the gene expression response and the increased rates of DSBs. This also provides incentive to determine more information on the relationship between these proteins and whether they have differing contributions to benzene-induced DNA DSB repair.

Changes to gene expression might be influenced by the increased number of DSBs caused by benzene exposure but may also be influenced by epigenetic marks including histone modification, DNA methylation and microRNA expression, which can modify the expression of genes by gene activation or repression (Guil *et al.*, 2009). One review argues for the mechanism of benzene toxicity through the involvement of epigenetic changes (Baccarelli *et al.*, 2009). Further studies are required to determine if changes to DSB repair gene expression is influenced by more than an increase in DSBs.

In utero, the other preferred method of DSB repair is HR. The Rad51 protein creates a single strand filament to search for 3' DNA overhangs which assists in strand invasion and DNA repair protein recruitment (Bagnolini *et al*., 2022). In the present study, we found that between the 2h and 24h time-points, *Rad51* mRNA gene expression increases, where at 2h it is non-significantly decreased relative to controls, expression increases significantly towards the 24h point. This may reflect an unknown mechanistic response by *Rad51* gene expression that is more dynamic 24h after benzene exposure. Furthermore, other studies have implicated HR with benzene toxicity (Winn, 2003; Yang *et al.,* 2019), stipulating that benzene-induced promotion of HR leads to hyper-recombination and harmful genetic changes. In addition to *Rad51*, *Brca2* mRNA expression was also measured to evaluate changes to HR after benzene-induced DNA lesions. *Brca2* is a mediator of the Rad51 microfilament (Saeki et al., 2006*)* and was found to colocalized with *Rad51* (Chen *et al.,* 1998). In the current study, *Brca2* mRNA expression was decreased at 2h and 6h and appears to demonstrate a decreased response of HR to DSBs. However, it should be noted that *Brca2* participates in several biological processes, including DNA transcription, recombination and cell cycle control (Shivji and Venkitaraman, 2004; Abaji *et al.,* 2005). Some studies have observed genetic changes to *Brca2* after benzene exposure in adults chronically exposed to benzene (Shen *et al.,* 2006). It was further suggested that low levels of white blood cells, specifically granulocytes and B-lymphocytes are associated with observed low levels of *Brca2* in low- level benzene exposed workers (Galvan *et al.,* 2006). Together this links genetic instability to the loss of *Brca2.* Finally, it is known that cancers, including leukemia, experience hypermethylation of promoter regions for tumor-suppressing genes such as *Brca2,* leading to genomic instability (Shames *et al.,* 2007).

Other studies have attempted to classify changes to gene expression after benzene exposure *in utero*. Interestingly, they found that circadian rhythm plays an important role in benzene toxicity (Park *et al.,* 2008). A hypothesis built upon a study by Rana *et al.* (2007) showed benzene induced lipid peroxidation was affected by the circadian rhythm. Circadian rhythms are also governed by protein synthesis, and Park *et al.* (2008) show that genes that regulate cell cycle and circadian rhythm are influenced by benzene in the mouse liver. Additionally, rats and humans alike are differentially susceptible to benzene at different times of the day (Leung and Harrison, 1998). With regards to the present study, changes in gene expression over the 24h period may be influenced by circadian rhythms as well as the collection time after final dosage.

All genes demonstrated that the dosage of benzene was a significant source of variation, indicating the presence of benzene was responsible for the observed changes in gene expression between control and benzene-dosed mice.

4.2 FUTURE STUDIES

Sex differences have been noted in CD-1 mice in benzene toxicity. A study in our lab showed female CD-1 mice had a significant increase in hematopoietic tumours, and male CD-1 mice had a significant increase in liver tumours (Badham *et al*., 2010). Due to differences in the metabolism in CD-1 GD14 pups, it is possible that this may result in cellular damage differences and therefore different outcome of cancer. Further work is necessary to determine if changes to DNA repair gene expression is sex-dependent *in utero*.

While we examined multiple genes affected by benzene, the most surprising was the differences in *Xrcc5* and *Xrcc6*. It would be expected that these genes would change synonymously, but instead we observed the upregulation of *Xrcc5*, and downregulation of *Xrcc6*. Potentially a BioID (Sears *et al.*, 2019), co-immunoprecipitation experiment or western blot analysis for protein-protein interaction assay could be conducted to determine any independent control of *Xrcc6*. Functional changes in DSB repair genes should also be investigated further to improve our understanding of how genes within

NHEJ and HR can be expressed differently to one another. Given that there were limitations to the selection of genes assessed in this study, a larger scale gene analysis is warranted.

The placenta offers a unique opportunity to provide knowledge of the consequences of *in utero* exposures and may provide new biomarkers for the presence of environmental carcinogens. Different external carcinogens produce unique gene expression patterns in target organs (Minami *et al.*, 2005). Smith *et al.* demonstrated through toxicogenomic studies that benzene produces a specific array of over 100 differently expressed genes related to cell cycle control (Smith *et al.*, 2005). Further studies on the placenta should attempt to separate fetal and maternal components and compare how mRNA expressions compare to fetal and maternal livers respectively. This may validate the placenta as an important tool for anticipating *in utero* damage.

Further studies should also examine benzene toxicity in human cell cultures, to link observed toxicity mechanisms in different strains of mice, with downstream carcinogenic effects in humans. Human hematopoietic stem cells from the bone marrow could be used to validate findings *in utero* as they are susceptible to benzene toxicity, die to their production of myeloperxoxidase. This would aid in determining the effect of this exposure on similar parameters of mutations. Ultimately, the potential of benzene to cause damage and alter cellular function in humans is of great importance and continuing to explore the mechanism of benzene toxicity should lead to better outcomes.

4.3 CONCLUSION

The current study demonstrates that *in utero* benzene exposure in CD-1 mice, where pregnant CD-1 dams were injected intraperitoneally with 200 mg/kg of benzene dissolved in corn oil or corn oil alone (control), on GD 8, 10, 12 and 14 results in altered gene expression of DSB repair genes in the placenta. Select genes involved in NHEJ and HR showed changes in mRNA expression in response to in utero benzene exposure and demonstrates a placental response. The temporal relationship between damage and repair response with individual genes suggests differential gene response. The generation of DSBs and interference with proper DSB repair is harmful to the developing fetus. As the developing fetus has limited DNA repair ability (Pachowski *et al.,* 2011), compounded with the error-prone nature of NHEJ and HR, any increases in DSBs increases the susceptibility of the fetus to benzene toxicity. Without the proper ability to manage these DSBs, this may allow the progression of disease to occur. This study expands our understanding of the damage occurring in the placenta, linking the observations of DNA damage in the fetus to the mother.

4.4 SIGNIFICANCE

Exposure to environmental pollutants such as benzene is considered a major contributor to the rise of childhood leukemia. With childhood leukemias representing the most diagnosed form of cancer in Canada, and the leading cause of death (Canadian Cancer Society), it is hypothesized that many of these leukemias are initiated *in utero* (Filippini *et al*., 2019). The field of risk assessment would benefit significantly from identifying robust biomarkers of early exposure and susceptibility. Furthermore, understanding the mechanisms through which benzene induces genomic instability can provide insight into other mechanisms of environmental carcinogen toxicity.

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