INVESTIGATING BENZENE-INITIATED DNA DOUBLE-STRAND BREAKS AND RECOMBINATION AFTER ACUTE AND *IN UTERO* EXPOSURE IN MICE

by

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Abstract

Benzene is an ubiquitous pollutant and industrial solvent that has been identified as a human leukemogen. Early exposure to environmental carcinogens such as benzene has been postulated to play a role in the etiology of childhood leukemia, however the association remains controversial. Genotoxic agents such as benzene can cause an increase in the frequency of DNA double-strand breaks, which may remain unrepaired or result in the initiation of DNA recombinational repair mechanisms.

The first objective was to investigate the induction of DNA double-strand breaks following *in utero* treatment to 200 mg/kg and 400 mg/kg benzene i.p. using the phosphorylated histone γ-H2A.X as a marker. Using immunoblotting, treatment with benzene did not increase the formation of γ-H2A.X in bone marrow cells of adult C57Bl/6N male mice and in maternal bone marrow, fetal liver, and post-natal bone marrow cells following *in utero* exposure to 200 mg/kg or 400 mg/kg benzene throughout gestational days 7 to 15.

Secondly, the study investigated the induction of micronuclei following *in utero* exposure to benzene. Acute exposure to 400 mg/kg benzene resulted in a statistically significant increase in the percentage of micronucleated cells in adult male bone marrow cells. *In utero* exposure to 400 mg/kg benzene throughout gestational days 7 to 15 also caused a statistically significant increase in the percentage of micronucleated cells in maternal bone marrow and post-natal bone marrow cells. Fetal liver cells also demonstrated a statistically significant increase in the percentage of micronucleated cells following 200 mg/kg and 400 mg/kg benzene.

The third objective was to investigate the initiation of DNA recombination following *in utero* exposure to benzene using the pKZ1 mutagenesis mouse model as a surrogate marker for non-homologous end joining activity. Adult pKZ1 mouse tissue yielded no recombination events; however, post-natal bone marrow cells did contain detectable recombination frequencies.

In utero benzene exposure did cause an increasing trend in recombination events, and upon analysis of only the samples containing detectable levels of recombination, *in utero* exposure to 400 mg/kg of benzene caused a statistically significant increase in recombination frequency within this group.

These results demonstrate that benzene does not increase the formation of γ-H2A.X after acute and *in utero* exposure, however, the induction of micronuclei following acute and *in utero* benzene exposure confirmed that benzene is a genotoxic agent causing chromosomal breaks. *In utero* benzene exposure increased the frequency of DNA recombination in bone marrow from post-natal day 9 pups exhibiting detectable levels of recombination. Further investigations into different types of DNA damage and repair pathways are warranted to fully elucidate the role of genotoxic mechanisms in the etiology of benzene-induced childhood leukemias.

Co-Authorship

This research was conducted by the candidate Annette Anling Lau, under the guidance and supervision of Dr. Louise M. Winn.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AGM	aorta-gonad-mesonephros
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ANOVA	analysis of variance
APS	ammonium persulfate
ATM	ataxia telangiectasia mutated
BCR-Abl	breakpoint cluster region-Abelson
BFU-e	burst forming unit- erythroblast
BZ	benzene
CD	cluster of differentiation
CFU-e	colony forming unit- erythroblast
CFU-gm	colony forming unit- granulocyte macrophage
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CYP	cytochrome P450
CYP 2E1	cytochrome P450 family 2, subfamily E, polypeptide 1
de novo	"from the beginning"
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetracetic acid
EPA	Environmental Protection Agency
ERK1/2	extracellular signal-regulated kinase 1/2
et al.	*et alia* (and others)
GD	gestational day
γ-H2A.X	histone H2A.X phosphorylated at serine 139
H2A.X	histone H2 family A, member X
HPRT	hypoxanthine guanine phosphoribosyl transferase
kDa	kilodaltons
IARC	International Agency for Research on Cancer
in utero	"in the womb"

in vitro	"in glass"- outside the living body
in vivo	"in a living thing"- inside the living body
inv	inversion
i.p.	intraperitoneal
lacI	lac repressor gene
lacZ	β-galactosidase gene
mEH	microsomal epoxide hydrolase
MLL	mixed lineage leukemia gene
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
mM	millimolar
MN	micronucleus
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NIH	National Institutes of Health
OD	optical density
NHEJ	non-homologous end joining
NQO1	NADPH quinone oxidoreductase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	post-natal day
ppb	parts per billion
ppm	parts per million
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
S9	supernatant fraction generated by centrifuging at 9000 x gravity for 20 minutes
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
t	translocation
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
V(D)J	Variable Diversity Joining
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1

Introduction

1.1 STATEMENT OF THE RESEARCH PROBLEM

Leukemia remains the most prevalent childhood cancer, accounting for approximately a third of all new cancer cases diagnosed in people under 14 years of age[1]. Unfortunately, the etiology of these cancers remains largely unknown; however, due to its early onset it is postulated that these childhood leukemias may be initiated *in utero*. Early exposure to environmental carcinogens is hypothesized to play a role in the development of childhood cancers[2-4], and it is therefore highly relevant to investigate whether *in utero* exposure to these environmental agents has the potential to initiate cancer that develops in early life. One such agent is benzene, a known human leukemogen found ubiquitously in the environment. Sources of benzene include cigarette smoke, vehicular exhaust, and industrial emissions[5]. Mechanistic studies have demonstrated that metabolites of benzene have the ability to target the bone marrow and inflict macromolecular damage resulting in hematotoxicity, alterations in bone marrow cell populations, and ultimately leukemia[6-9].

Epidemiological studies suggest an association between *in utero* exposure to benzene and subsequent development of leukemia[10-14]. Animal models have also demonstrated that benzene and its metabolites can be found in the fetus and can cause lower birth weights, delay ossification, and cause chromosomal abnormalities[15-18]. However, it has yet to be determined whether *in utero*

exposure to benzene confers leukogenic damage to the developing fetus through genotoxic mechanisms.

This thesis investigated the role of benzene-initiated double-strand DNA breaks in the hematopoietic tissue of fetal and post-natal mice. Specifically, this thesis examined the induction of DNA double-strand breaks, the persistence of fragmented DNA, and the initiation of aberrant DNA recombination as a consequence of DNA double-strand breaks initiated by acute and *in utero* exposure to benzene.

1.2 HEMATOPOIESIS AND LEUKEMIA

1.2.1 Normal hematopoiesis

Hematopoiesis is the process of blood cell production, and in adult mammals the hematopoietic processes are primarily localized in the bone marrow. The various cellular components of blood are generated from a small population of hematopoietic stem cells, which have the potential to differentiate into two hematopoietic lineages: myeloid and lymphoid (figure 1.1). Myeloid progenitor cells give rise to mature myeloid cells, including red blood cells, platelets, neutrophils, and monocytes. Lymphoid progenitor cells give rise to mature lymphoid cells, including natural killer cells, T cells, and B cells. Hematopoietic stem cells are relatively few in number, heterogeneous in nature, multipotent, capable of self-renewal, and are present in organs other than the bone marrow, such as the peripheral blood, umbilical cord blood, liver, and spleen[19]. The processes that govern hematopoiesis are complex and involve both intrinsic and extrinsic factors[20,21]. In particular, there is a close interaction between a hematopoietic stem cell and its microenvironment, or niche, which allows for regulation and control of stem cell fate[22-25].

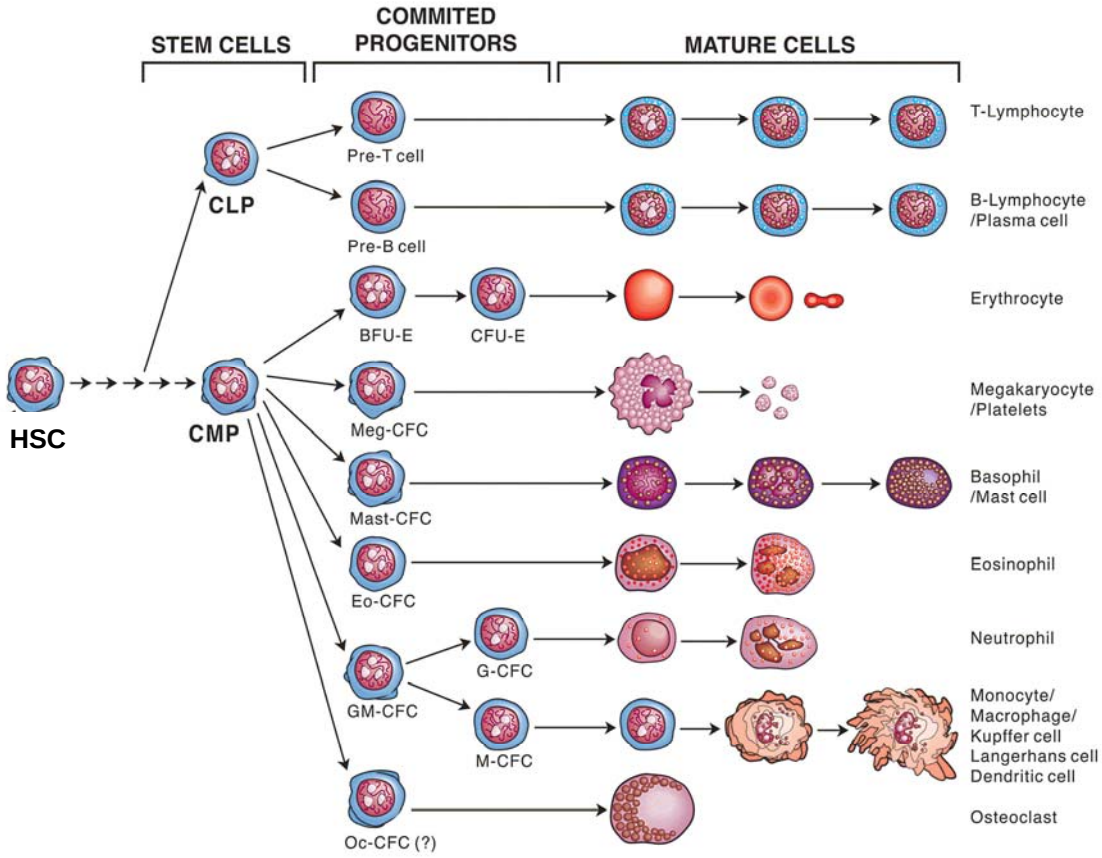


Figure 1.1 Schematic of hematopoietic lineages generated from a hematopoietic stem cell.

CLP: common lymphoid progenitor; CMP: common myeloid progenitor; BFU-E: blast-forming unit-erythroid; CFU-E: colony-forming unit-erythroid; HSC: hematopoietic stem cell; Meg-CFC: megakaryocyte colony-forming cells; Mast-CFC: mast cell colony-forming cells; Eo-CFC: eosinophil colony-forming cells; GM-CFC: granulocyte-macrophage colony-forming cells; G-CFC: granulocyte colony-forming cells; M-CFC: macrophage colony-forming cells; Oc-CFC: osteoclast colony-forming cells. Adapted from Metcalf, 2005[26].

Supporting stromal cells[27], integrins[28,29], chemokines[30,31], and other signaling molecules[23,32-34] have integral roles in constituting the bone marrow niche.

1.2.2 Developmental hematopoiesis

The ontogeny of hematopoiesis is highly conserved in all mammals, and involves the migration of hematopoietic stem cells from different organs and a change in cell population composition. Two types of hematopoietic processes are identified in the embryo: primitive and definitive. Primitive hematopoiesis yields highly proliferative, transient nucleated hematopoietic cells that are ideal for oxygen delivery in the embryo. Definitive hematopoiesis yields hematopoietic stem cells that will ultimately contribute to the adult hematopoietic system. The extra-embryonic yolk sac is the first site of murine primitive hematopoiesis, where hematopoietic cells can be detected on gestational day 8[35-38]. These progenitor cells can enter the primitive circulation at day 8.5[39], though the fully functional circulatory system does not develop until gestational day 10[40]. In the mouse, definitive hematopoietic stem cells are first detected in the dorsal aorta on gestational day 10.5[41] and in the aorta-gonad-mesonephros (AGM) region around gestational day 10.5-11[35,41]. These cells are presumed to enter the circulation or directly migrate through tissues to the subsequent sites of hematopoiesis[35]. The fetal liver is populated with hematopoietic cells originating from the AGM, yolk sac, and placenta starting late in gestational day 9, however the liver does not contain hematopoietic stem cell activity until gestational day 11[38,42,43]. Shortly after colonization of the liver with hematopoietic stem cells, the liver becomes the primary site of fetal hematopoiesis[38,44-46]. Hematopoietic stem cells can also be found in the thymus, spleen, and bone marrow during gestation[38,39]. While the source of hematopoietic stem cells in the thymus and spleen are thought to have originated in the fetal liver[47], it is unclear

whether hematopoietic stem cells in the bone marrow originate from the fetal liver, yolk sac, or are generated *de novo*[38]. Bone marrow hematopoietic processes begin approximately on gestational day 16 to 18 and gradually develops into the main hematopoietic organ shortly after birth[48,49].

In human embryonic hematopoiesis, hematopoietic stem cells can be first detected within the first four to six weeks after conception[50]. Definitive hematopoietic stem cells are first detected in the aorta[51], and the liver becomes colonized with hematopoietic stem cells from week 5 to week 20 of gestation[49,52]. Thymic hematopoiesis occurs between gestational weeks 7 to 9[53]. Bone marrow hematopoiesis begins on gestational week 10 to 11 and becomes the major hematopoietic organ following birth[49,50,54].

There is evidence suggesting that fetal hematopoietic stem cells have unique characteristics and are distinct from adult hematopoietic stem cells. Fetal hematopoietic stem cells exhibit faster rates of cell cycling[55] and may respond differently to certain cytokines[56]. Murine fetal blood cells are also more susceptible to certain clastogenic agents than maternal bone marrow, suggesting that the hematopoietic system is quite vulnerable during gestation[57-59]. This becomes particularly important when toxicant insult occurs *in utero*, which disrupts normal developmental hematopoietic processes resulting in long term deleterious consequences.

1.2.3 Leukemias

The bone marrow is a particularly susceptible organ due to its high rate of cellular proliferation and its requirement for tight regulation of the microenvironment. Sensitivity to xenobiotic (foreign chemical) insult is demonstrated by the prevalence of disorders and

malignancies that manifest following exposures to ionizing radiation[60,61], cytotoxic therapy[62-64], viral infections[65,66], tobacco smoke[67,68], and benzene[69,70]. Leukemia is cancer of the blood and bone marrow, and is characterized by uncontrolled proliferation of immature blood cells which crowd the bone marrow and impede the development of healthy cells. There are four main types of leukemia which are differentiated by the subpopulation of blood cells affected (myeloid or lymphoid) and the state of the malignant cell maturity (acute or chronic). Acute leukemias are characterized by over-proliferation of blast cells and disease progression is generally rapid if left untreated. Chronic leukemias are characterized by over-proliferation of more mature progenitor cells and progression is slower. Leukemias can be further divided by specific lineages, morphology, chromosomal abnormalities, prognosis, and therapy-related etiology[71,72].

1.2.4 Childhood leukemias

Childhood cancers are considered rare; however, the appearance of malignancies in early life is the leading cause of death in children over one month of age[1]. Childhood cancers are distinct from adult cancers as they are more aggressive, metastatic, invasive, and include a higher proportion of hematopoietic malignancies[1,73,74]. Treatments for childhood cancers differ from that of adults due to these physiological differences, as well as the psychological vulnerabilities of individuals in this age group[75].

In children less than 14 years old, leukemia accounts for a third of all cancer diagnoses, with the most common subtype stemming from lymphoid origins[1] (figure 1.2). Despite trends suggesting an increase in incidence[76-78], childhood cancer treatment has advanced greatly over the years and the five year survival rate in children diagnosed with leukemia is approximately 85%[1].

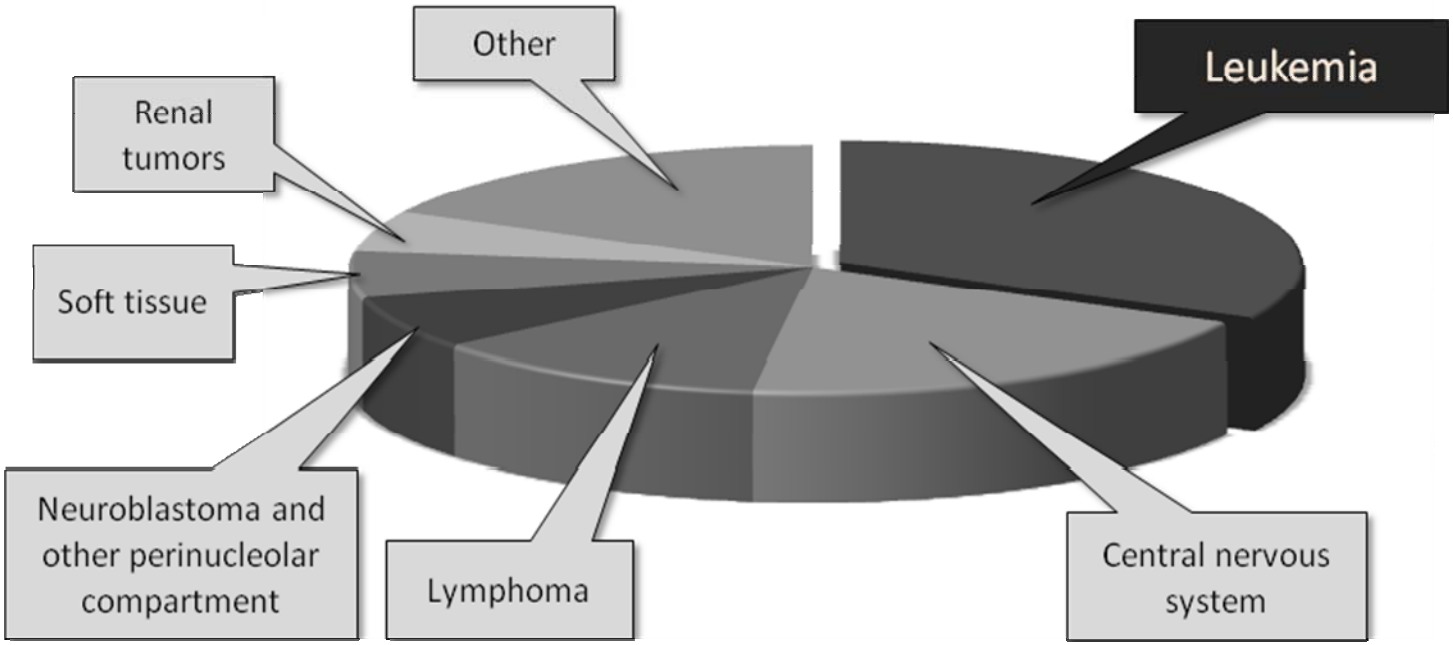


Figure 1.2 Distribution of new cancer cases in children aged 0-14 years old by diagnostic group.

Leukemia remains the most prevalent childhood cancer accounting for approximately a third of new cancer cases. Data from Canadian Cancer Statistics, 2008[1].

The etiology of childhood cancers remains poorly understood, however there is evidence to suggest that genetic alterations that are implicated in the etiology of certain leukemias can be acquired *in utero*[79,80]. In addition to known inherited genetic defects[81,82] and polymorphisms[83-86], there is speculation that early life exposure to carcinogens may be required to initiate childhood cancers even in the presence of these genetic risk factors[87,88]. It is important to note that fetal responses to carcinogens may differ from adult responses due to the differences in metabolic and detoxifying capabilities, critical susceptibility periods, proliferation and differentiation rates, and anatomical location of hematopoietic tissue[57,73,89-91]. Currently, *in utero* exposures to ionizing radiation (reviewed in Fucic *et al,* 2002[92]) and diethylstilbestrol[93] have been definitively linked to cancer outcome. Ongoing studies are investigating the role of environmental exposures to air pollution[94-96], cigarette smoke[97,98], pesticides[2,99], and dietary intake of topoisomerase II inhibitors[100,101] as potential risk factors for the development of childhood cancers.

1.3 BENZENE

1.3.1 Human exposure to benzene

Benzene is a volatile pollutant found ubiquitously in the environment. It is used as an industrial organic solvent and precursor chemical in the production of drugs, rubbers, plastics, and dyes[102]. Benzene is also a by-product of combustion and can be found in automobile exhaust, cigarette smoke, and industrial emissions[5]. Various consumer products such as glue, paint, and waxes may also contain levels of benzene that contribute to elevated indoor levels[103,104]. The

majority of human exposure occurs through the inhalation of benzene fumes from in-transit vehicular emissions[105,106], indoor sources such as cigarette smoke[107,108] and from occupational settings[109,110], though exposure through accidental chemical spills is also a relevant concern[111]. Benzene partitions mainly into air[112]; however, oral and transdermal absorption of benzene are also noted routes of exposure[113]. Occupational exposure to benzene is tightly regulated; the Ontario Occupational Health and Safety Act has set the maximal allowable average concentration of benzene in air at 0.5 ppm[114], and the United States Occupational Health and Safety Administration has set the allowable level at 1 ppm for an 8 hour workday for a 40 hour work week[115]. Benzene concentration in gasoline is regulated at 1% volume[116], and benzene content in drinking water is set at 5 ppb by the United States Environmental Protection Agency (EPA)[117]. Benzene does not bioaccumulate in foodstuffs to any appreciable extent.

An average non-smoker may take in a total of 200-500 μg of benzene per day[106,118], and a smoker is estimated to intake 2-3 times more, hypothetically up to 800 μg of benzene per day from cigarette exposure alone[106]. Animal studies have demonstrated that the parent compound is mostly stored in fat, and metabolites can be found in higher concentrations in the bone marrow than the blood[119]. The bioactive dose of benzene accumulates in the bone marrow and liver, with peak levels attained at 12 hrs and 1 hr post-exposure respectively[120].

1.3.2 Adverse effects of benzene exposure

The need for tight occupational regulations became apparent with the clinical symptoms observed in workers using benzene occupationally[121-123]. In humans, acute benzene toxicity produces neurotoxic effects that may manifest symptoms such as headache, nausea, vertigo,

respiratory effects, and in severe cases coma and death[106,124]. Chronic exposure to benzene causes hematopoietic disorders and bone marrow depression, and may result in anemia, pancytopenia, eosinophilia, thrombocytopenia, and leucopenia[6-9]. These effects have been reproduced in animal models of chronic benzene toxicity[125-127].

Benzene is an immunotoxicant and exposure can result in deficits in humoral and cellular acquired immunity[128-130], activation of autoimmunity[131], and inhibition of interleukin-2 production[132]. The ability of benzene to induce reproductive effects has not been well-documented. Animal studies have demonstrated that benzene can increase the frequency of chromosomal aberrations in sperm[133], and this was supported by a Chinese epidemiological study reporting an increase in the incidence of DNA damage in the sperm of benzene-exposed workers[134]. Benzene can also cause inhibition of oviduct functioning *ex vivo*[135].

Among the toxicities of benzene, one of the more detrimental health effects is the increased risk of developing cancer. Benzene is considered a high ranking environmental carcinogen and leukemogen[136] and chronic exposure to benzene is most strongly associated with the development of leukemias.

1.3.3 Benzene and leukemia

Benzene has been identified as a known human carcinogen by the International Agency of Research on Cancer (IARC)[137] and the Environmental Protection Agency[113], and is most strongly associated with acute myeloid leukemia. The Canadian Cancer Society and the American Cancer Society both list benzene exposure as a known risk factor for the development of acute myeloid leukemia. Epidemiological studies have also suggested that chronic benzene

exposure is associated with the development of acute lymphocytic leukemia, acute erythrocytic leukemia, acute myelomonocytic leukemia, acute promyelocytic leukemia, acute undifferentiated leukemia, hairy cell leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and multiple myeloma[7,69,110,138-144].

The carcinogenicity of benzene is not limited to the bone marrow; other organs reported to be targets in humans include the lung, bladder, stomach, and prostate[110,124,145-148]. In animal models, increases in tumorigenesis following benzene exposure has been reported in the lung, nasal and oral cavity, fore-stomach, liver, skin, zymbal gland, mammary gland, ovary, and uterus[149-151].

1.3.4 Benzene and childhood leukemia

Benzene has the ability to cross the placenta and enter the embryo/fetus, where it can be bioactivated into metabolites that can cause cytotoxicity and DNA damage[90,152,153]. Because leukemia remains the most prevalent childhood cancer, much interest has been focused on determining whether exposure to benzene during gestation plays a causal role in the development of leukemia in early life. Animal models have demonstrated that *in utero* exposure to benzene can result in a number of teratogenic effects, including decreased crown-rump length, decreased body weight, skeletal malformations, delayed bone ossification, and brain defects in rodents[15,16,18]. Transplacental genotoxicity[90,152,153] and alterations in bone marrow populations[154,155] have been demonstrated in mouse models, however, these have yet to be associated with a leukemia outcome.

Several epidemiological studies have attempted to elucidate the role of parental exposure to sources of benzene and the subsequent development of leukemia in the offspring; however, the

evidence remains inconclusive. A case-control epidemiological study in China associated childhood acute myelogenous leukemia to maternal occupational exposure to benzene and gasoline during pregnancy[10], and subsequent studies in North America also noted a positive association between parental occupational solvent exposure and the development of childhood cancers in Canada and the United States[11-14]. However, parental occupational exposure to benzene was also found to demonstrate little[156] or no increased risk of childhood leukemia[157-160] in other studies conducted in the United Kingdom, Finland, and in Massachusetts, United States. Exposure to benzene through vehicular exhaust and air pollution has also been investigated. Studies examining the incidence of childhood cancer in the United Kingdom and France suggest that residential proximity to a main road or petrol station may increase the risk of developing childhood leukemia[159,161,162]. This finding was challenged when studies in Denmark and California, United States revealed that living near high automobile traffic areas resulted in no increased risk of developing leukemia [94,163]. Critical reviews of these aforementioned epidemiological studies discerned several shortcomings. The main limitation is the difficulty in accurately assessing benzene exposure as there are often no biomarkers or air measurements made and exposure is to a mixture of compounds. Moreover, some studies fail to classify the types of leukemia in their disease outcome, and therefore associations with certain subclasses of leukemia may be masked. Epidemiological studies are further hindered due to the fact that childhood cancers are rare and there is a long latency between *in utero* benzene exposure and the development of leukemia. Therefore, this increases the cost and decreases the practicality of a long follow-up period[124,164-166].

1.4 POTENTIAL MECHANISMS BEHIND BENZENE-MEDIATED LEUKEMOGENESIS

1.4.1 Benzene metabolism and detoxification

It is generally accepted that benzene must be bioactivated by cytochrome P450 (CYP) 2E1 in order to exert its toxic effects (figure 1.3). Biotransformation of benzene into an epoxide by CYP 2E1 mainly occurs in the liver, but may also occur to a lesser extent in the lung and bone marrow[167,168]. Benzene epoxide can spontaneously form phenol or be conjugated with glutathione to produce a less toxic or nontoxic derivative. Metabolic intermediates can be further biotransformed into other reactive metabolites by enzymes including CYP 2E1[169], CYP 2B1[170], myeloperoxidase (MPO)[171], and microsomal epoxide hydrolase (mEH)[172]. Hydroquinone and muconic acid are the major metabolites found in the plasma, liver, and bone marrow of mice following acute exposure to benzene[173]; however, the polyphenolic metabolites and quinones, especially hydroquinone, catechol, 1,4-benzoquinone, and 1,2,4-benzene triol are proposed to be the most toxic[70,174]. These metabolites can accumulate in target tissue and exert damage potentially through synergistic mechanisms[175]. Interestingly, the kinetics of benzene exposure differ for each organ, with the bone marrow accumulating more benzene metabolites, such as hydroquinone or catechol[119], over a longer period of time than the liver[176]. Following bioactivation, detoxification of the metabolites can occur through a number of pathways. Glutathione conjugation, sulfation, and glucuronide conjugation have been shown to detoxify phenol, catechol, and hydroquinone[177-179].

The role of metabolism in benzene toxicity is of paramount importance as demonstrated through the use of knockout mouse models and epidemiological associations between certain

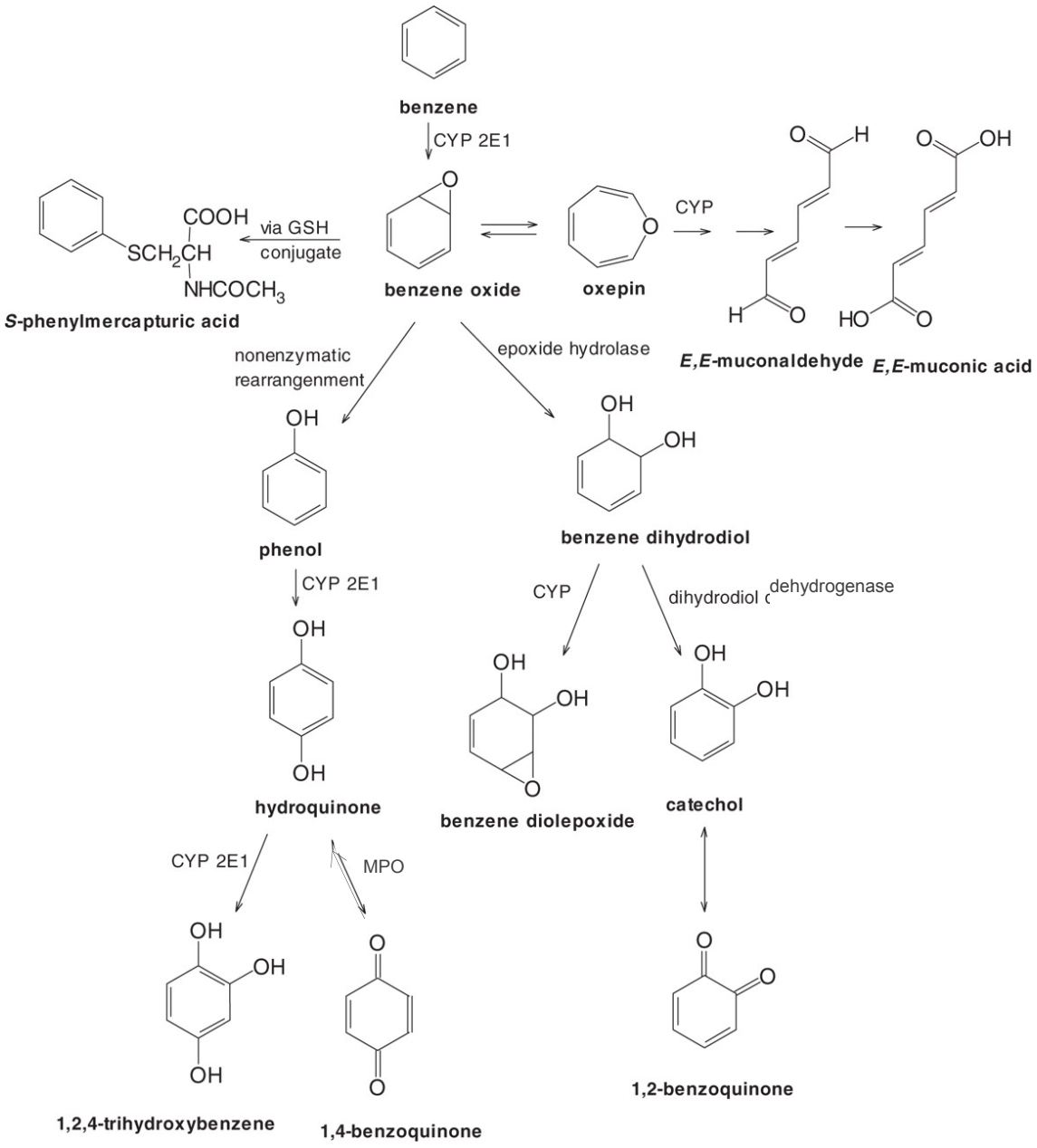


Figure 1.3 Simplified overview of benzene metabolism.

Benzene must be bioactivated in order to exert its toxic effects. It is generally accepted that benzene is metabolized by cytochrome P450 2E1 mainly in the liver to benzene epoxide. Other enzymes such as epoxide hydrolase and myeloperoxidase (MPO) can further bioactivate these metabolites in other organs such as the bone marrow. Adapted from Kim *et al,* 2006[180].

polymorphisms and leukemia outcome after benzene exposure. For example, CYP 2E1 knockout mice are resistant to benzene toxicity when compared to wild-type mice[181]. In addition, CYP 2E1 polymorphisms have been associated with increased risk of developing leukemia in children[182] and adults[84,183]. Consumption of ethanol, which upregulates CYP 2E1 expression, exacerbates benzene hematotoxicity both in male adult mice as well as male fetal mice[184,185]. The gender differences observed in benzene toxicity are also attributed to disparities in metabolic capabilities between males and females[186]. Various other polymorphisms in benzene metabolizing enzymes have also been explored, including NADP(H) quinine oxidoreductase, microsomal epoxide hydrolase, glutathione-S-transferases, and myeloperoxidase[187-191]. These genes also play a role in benzene toxicity.

Benzene metabolites can be found in the fetal liver following benzene inhalation in animal models, suggesting that placental or fetal bioactivation of benzene is possible[17]. CYP 2E1 is expressed in mouse fetal tissue, with the appearance of mRNA transcripts at around gestational day 17[192]. Human expression of CYP 2E1 during fetal development is more variable, with detection of CYP 2E1 protein generally appearing in the second or third trimester[193].

1.4.2 Oxidative stress

Following metabolism, benzene has the ability to induce oxidative stress via redox cycling and the generation of reactive oxygen species[194-196] and reactive nitrogen species[197,198]. These reactive products can then go on to generate oxidative damage, and benzene administration *in vitro* and *in vivo* has been shown to increase lipid peroxidation and the oxidative DNA lesion 8-hydroxy-2-deoxyguanosine[195,199-201]. Reactive oxygen species are also partially responsible for benzene-induced cytochrome P450 destruction[202,203]. Benzene exposure can deplete various

antioxidative enzyme levels, and evidence of this has been found through epidemiological studies[204] as well as studies conducted in our lab demonstrating that *in utero* administration of benzene rapidly lowers reduced to oxidized glutathione ratios in mouse embryos[205]. There is also evidence that co-treatment with either the antioxidative enzymes catalase[206], superoxide dismutase[205], or antioxidants found in green tea[204] attenuate benzene toxicity, further demonstrating the role of oxidative stress.

1.4.3 Alterations in cell signaling, growth, and apoptosis

Several studies have demonstrated that benzene has effects on multiple cell signaling pathways. Benzene and its metabolites have been shown to activate pro-inflammatory cytokines and inhibit anti-inflammatory cytokines *in vitro* [207-209]. Benzoquinone, a potent metabolite, has been shown to have effects on the ERK1/2 signaling pathway[210]. In hematopoietic stem cells, *in vivo* benzene exposure induces changes in cell cycle regulators, including p53, gadd45a, and bax[211-213]. In lung epithelial cells, benzene can also alter pro- and anti-apoptotic signals, with a shift towards pro-apoptogenic events[214]. Studies conducted in our lab have also demonstrated that benzene metabolites can activate the c-Myb signaling pathway, possibly leading to inhibition of differentiation of hematopoietic precursor cells[205,215]. In addition to having effects on signaling molecules, benzene metabolites can also interfere with gap junction intercellular communication[216], which has been implicated to have a role in tumorigenesis and in hematopoietic regulation.

Benzene exposure also has an effect on cell growth and apoptosis. *In vitro* exposure to benzene metabolites cause an inhibition of nuclear DNA synthesis in bone marrow cells[217] and can also inhibit mRNA synthesis[218,219]. Furthermore, hematopoietic stem cells exposed to

benzene also highly upregulate wig1, a protein inhibiting cell growth[212]. Furthermore, various benzene metabolites can induce apoptosis in HL60 cells and human bone marrow $CD34^+$ progenitor cells *in vitro*[220], but hydroquinone has been shown to inhibit apoptosis in myeloblasts[221]. Induction of apoptosis by benzene metabolites is proposed to be partly mediated by deficiencies of essential cytokines[222]. In terms of cell populations, benzene is known to decrease the levels of circulating B and T cells[126,223] and can also inhibit lymphocyte proliferation[129]. These changes in cell population may be due to attempted repair or removal of damaged hematopoietic cells. There is also evidence that fetal hematopoietic progenitor cells may be more susceptible to the cytotoxic effect of certain mixtures of benzene metabolites compared to the adult counterparts[224].

1.4.4 Epigenetic mechanisms

Changes in methylation and acetylation patterns following benzene exposure have not been fully investigated. A study conducted by Bollati and colleagues (2007) examined DNA methylation patterns in occupationally-exposed subjects. The paper reported benzene-induced changes in methylation patterns of *LINE-1, AluI, p15,* and *MAGE-1* genes[225]. Further investigation into epigenetic mechanisms of benzene-induced leukemias is warranted.

1.4.5 DNA damage

Benzene exposure has been implicated in a wide range of genotoxic damage in different model systems. Benzene and its metabolites have been shown to cause chromosomal aberrations, sister chromatid exchanges, DNA-protein cross-links, and induced DNA strand breakage *in vitro*[226-228] and *in vivo*[229-234]; there is also evidence to suggest that even low benzene exposure may

confer genotoxicity[230,235,236]. Transgenic models with *lacI* reporter DNA constructs have also demonstrated positive mutagenic results[237,238]; however *in vitro* bacterial and mammalian mutation assays have produced conflicting results for certain metabolites of benzene[239-241], that are most likely attributed to the metabolic inadequacies of the S9 fractions generated with these systems[242]. Benzene exposure also increases the transcription of H2A.X[212], a histone subfamily most recognized for its role in DNA double-strand break signaling.

Mechanisms behind benzene-induced genotoxicity are suggested to include several pathways including oxidative DNA damage, inhibition of topoisomerase II, disabling of the mitotic apparatus, or through adduct formation. Benzene exposure is known to result in an increase in reactive oxygen species and increases in the DNA lesion 8-hydroxy-2-deoxyguanosine have been detected[199,201,243]. Topoisomerase II is an enzyme responsible for relieving torsional strain on the double-stranded DNA helix. Inhibition of this enzyme can result in deleterious effects including sister chromatid exchange, non-homologous recombination, gene deletion and gene rearrangements[244]. Metabolites of benzene can inhibit topoisomerase II *in vitro*[245-248] and *in vivo*[249]. Benzene-induced aneuploidy has been linked with disruption of the mitotic spindle formation and function[250,251], the consequence of which is an abnormal number of chromosomes and the potential for malignant transformation. DNA adduct formation has been shown to form *in vitro* following exposure to hydroquinone[252]; however, whole animal models have suggested that adduct formation only occurs at high levels of benzene exposure and is not an important mechanism behind benzene-initiated carcinogenesis[253-255].

1.4.6 Aberrant DNA repair and recombination

After a genotoxic insult, DNA repair mechanisms are activated depending on the type of damage. Single nucleotide damage can be repaired through direct reversal, nucleotide excision repair, or base excision repair. Double-strand DNA breaks can be repaired through two main mechanisms: homologous recombination and non-homologous end joining (NHEJ). All DNA repair pathways involve errors, and erroneous repair or deficits in repair pathways have been implicated as major causative factors of genomic instability and malignant cell transformation[256].

In addition to the structural damage to DNA, benzene can also interfere with the integrity of the genetic information by increasing the likelihood of erroneous repair. Interestingly, the damage exerted by one metabolite of benzene may be repaired by a different mechanism than that of another metabolite of benzene. A study conducted by Gaskell and colleagues (2005) reported that DNA damage caused by hydroquinone was repaired via base excision repair, while the damage caused by 1,4-benzoquinone was repaired via nucleotide excision repair[257]. Benzene exposure *in vivo* has also been shown to change RNA transcripts of several DNA repair enzymes including xpc and Ku80, which have been shown to be gender-specific[258]. Impairment of DNA repair capacities was investigated in benzene-exposed workers and a significantly lower capacity to repair radiation-induced damage was observed in exposed individuals[259].

Aberrant induction of DNA double-strand repair mechanisms have not been investigated to a great extent, although benzene has been reported to induce intrachromosomal DNA recombination in yeast[260] and homologous recombination in mammalian cells[261]. It becomes relevant to study these mechanisms since many of the common leukemias exhibit distinct genetic markers that are acquired through recombination.

1.5 DOUBLE-STRAND DNA BREAKS AND DNA RECOMBINATION IN LEUKEMIA

1.5.1 Double-strand DNA breaks and repair

DNA double-strand breaks are the most toxic DNA lesion. They can be induced by ionizing radiation[262], radiomimetic drugs[263,264], topoisomerase II inhibitors[244,265,266], reactive oxygen species (reviewed in Cadet *et al*, 2003[267]), or can be introduced intentionally with endogenous nucleases involved in DNA replication[268] or recombination[269,270]. There are two repair mechanisms involved in double-strand DNA repair: homologous recombination and NHEJ. Briefly, homologous recombination involves the use of a homologous chromosome template to repair the strands of DNA containing the break (reviewed in Helleday, 2003[271]; Li and Heyer, 2008[272]). In NHEJ, the broken ends of the DNA are processed and ligated back together (reviewed in Burma *et al*, 2006[273] and Lieber, 2008[274]). Neither DNA repair mechanisms are error-free, and errors in double-strand DNA repair may introduce mutations and chromosomal translocations. As mentioned previously, DNA double-strand breaks can be introduced intentionally by the cell. There are several circumstances during which intentional double-strand breaks are generated: topoisomerase-mediated breaks to relieve torsional strain during replication[265,266]; recombinase-mediated breaks to initiate cross-over during miosis[269,270,275]; V(D)J signal-mediated breaks to initiate immunoglobin type switching in lymphocytes[276-279]; and DNA fragmentation during apoptosis[280]. Exposure to chemicals that interfere with these processes can also result in an increased frequency of DNA double-strand breaks as in the case with topoisomerase II inhibitors[281,282].

1.5.2 Chromosomal translocations and leukemia

Chromosomal translocations are a hallmark of leukemias and play a role in their etiology. These leukemic translocations are acquired through erroneous repair of two double-strand breaks mediated through NHEJ[283-285] or V(D)J recombination machinery[286]. The adverse consequences of these translocations include the juxtaposition of highly active promoters to oncogenes, or the generation of fusion proteins with novel functions[287]. For example, the Philadelphia chromosome is a well-documented chromosomal translocation found in more than 90% of chronic myelogenous leukemia cases that results from a translocation between chromosome 9 and 22 (designated *t*(9;22)) (reviewed in Kurzrock *et al*, 2003[288] and Koretzki, 2007[289]). The resulting fusion protein BCR-Abl is constitutively active due to the acquired ability to auto-phosphorylate, resulting in continual activation of Abl-mediated cellular growth signal transduction cascades. Other functional chromosomal translocations implicated in the etiology of leukemias include *t*(1;19), *t*(12;21), *t*(4;11), *t*(15;17), *t*(8;21), and *inv*(16); however, there are many additional non-random translocations that are associated with specific subtypes of leukemia (table 1.1)[287].

1.5.3 DNA double-strand breaks and DNA recombination in childhood leukemia

Increased DNA damage to the developing embryo/fetus, either through exposure to genotoxicants such as ionizing radiation or topoisomerase II inhibitors (reviewed in Lightfoot, 2005[91]; and Godschalk, 2008[290]) or deficiencies in DNA repair enzymes (reviewed in Hales, 2005[291] and Papaefthymiou, 2008[81]), have been correlated with several teratogenic effects including childhood leukemia. The frequency of chromosomal translocations are quite high in childhood leukemia, with the *mixed lineage leukemia* (*MLL*) translocations alone present in 80% of infant acute lymphocytic leukemia, and 65% of infant acute myeloid leukemia[292,293]. The

Table 1.1 Examples of chromosomal translocations frequently found in leukemia.

Chromosomal translocations and inversions are a hallmark of many leukemias and are associated with specific subtypes. Adapted from Zhang and Rowley, 2006[287].

Translocation	Gene Involved	Leukemia Associated
***MLL* associated translocations**		
t(4;11)(p12;q23)	*AF4p12/MLL*	t-ALL
t(6;11)(q21;q23)	*AF6q21/MLL*	AML
t(9;11)(p22;q23)	*AF9/MLL*	AML/ALL
t(10;11)(p12;q23)	*AF10/MLL*	AML
t(10;11)(p12;q14)	*AF10/CALM1*	AML, t-ALL
t(11;19)(q23;p13.1)	*MLL*/*ELL*	AML
t(11;19)(q23;p13.3)	*MLL*/*ENL*	AML/ALL
***TEL*/*ETV6-associated* translocations/inversion**		
t(3;21)(q26;q22)	*EVI1*/*MDS1*/*EAP*/*AML1*	t-AML/CML
t(8;21)(q22;q22)	*ETO*/*AML1*	AML
t(12;21)(p12;q22)	*TEL*/*AML1*	ALL
inv(16)/t(16;16)(p13;q22)	*MYH11*/*CBFB*	AML
t(5;12)(q33;p13)	*PDGFRB*/*TEL*	CMML
***RARA* associated translocations**		
t(15;17)(q22;q21)	*PML*/*RARA*	APL
***E2A* associated translocations**		
t(1;19)(q23;p13)	*PBX1*/*E2A*	ALL
Tyrosine kinase associated translocations		
t(5;12)(q33;p13)	*PDGFRB*/*TEL*	CMML
t(9;22)(q34;q11)	*ABL*/*BCR*	CML, ALL
***NUP98*/*NUP214* associated translocations**		
t(6;9)(p23;q34)	*DEK*/*NUP214*(*CAN*)	AML
Immunoglobulin (IG) or TCR gene related translocations		
t(8;14)(q24;q32)	*IGH*/*c-MYC*	ALL
t(14;19)(q32;p13)	*IGH*/*BCL-3*	CLL

AML: acute myeloid leukemia; ALL: acute lymphocytic leukemia; CML: chronic myeloid leukemia; CLL: chronic lymphocytic leukemia; CMML: chronic myelomonocytic leukemia; t-AML: therapy-related acute myeloid leukemia; t-ALL: therapy-related acute lymphocytic leukemia.

t(8;21) translocation appears in 12% of childhood acute myeloid leukemia cases[294,295] and the *t*(15;17) translocation appears in 11%[294]. There is a large body of evidence that suggests that these functional translocations can arise *in utero*[2,79,80,87,296,297]. Chromosomal rearrangements have been found in blood spots obtained from neonates that were later diagnosed with leukemia[2,296,298,299] and from twins that have concordant leukemia[79,300-302]. It is hypothesized that these translocations may be acquired through environmental exposures to genotoxicants. Benzene is an environmental leukemogen and investigation into the role of *in utero* induction of DNA double-strand breaks and DNA recombination by benzene is required to determine if it is a mechanism behind benzene-induced childhood leukemias.

1.6 RESEARCH HYPOTHESIS AND OBJECTIVES

The etiology of childhood leukemias remain largely unknown, however it is proposed that *in utero* exposure to environmental carcinogens plays a role. Benzene is a ubiquitous genotoxic agent and chronic exposure has been associated with an increased risk of developing leukemia in adults. Toxicant induction of DNA double-strand breaks during gestation may be a mechanism by which leukemia is initiated in childhood, as erroneous DNA repair during a critical period may result in recombination events and characteristic translocations that are implicated in the etiology of leukemia (figure 1.4).

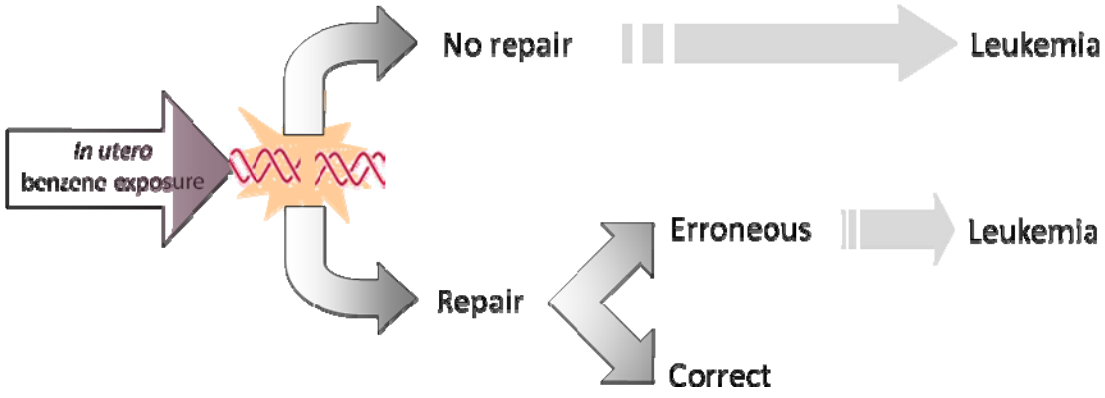


Figure 1.4 Schematic of the hypothesized mechanism of benzene-induced DNA double-strand breaks in initiating childhood leukemia.

Benzene is a genotoxic agent known to cause DNA double-strand breaks in hematopoietic cells. These breaks can remain unrepaired and persist, possibly leading to malignant transformation of a cell into a leukemic state. The break can also undergo repair, however DNA double-strand break repair mechanisms are not completely error-free, and erroneous repair of these breaks may lead to chromosomal aberrations that may also lead to malignant transformation of a cell. Induction of DNA double-strand breaks by *in utero* benzene exposure may be a mechanism by which leukemia is initiated in childhood.

1.6.1 Hypothesis

In utero exposure to benzene induces DNA double-strand breaks in fetal hematopoietic tissue that lead to aberrant DNA recombination.

1.6.2 Objectives

Objective 1: To determine if subacute *in utero* exposure to benzene during a vulnerable period of hematopoietic development induces DNA double-strand breaks in hematopoietic tissue of fetal mice and whether this damage persists in early post-natal life.

Objective 2: To determine if subacute *in utero* exposure to benzene during a vulnerable period of hematopoietic development causes permanent chromosomal breaks in hematopoietic tissue of fetal mice that persist in early post-natal life.

Objective 3: To determine if subacute *in utero* exposure to benzene during a vulnerable period of hematopoietic development causes increases in DNA recombination in response to genotoxic damage in hematopoietic tissue of fetal mice and whether this increase in recombination frequency can be detected in early post-natal life.

Chapter 2
Materials and Methods

2.1 ANIMALS AND BREEDING

2.1.1 C57Bl/6N mice

C57Bl/6N mice (Taconic Farms, United States) were purchased at 7-9 weeks of age and housed in a temperature controlled room with a 12 hour light:dark cycle. Standard rodent chow (Purina Rodent Chow, Ralston Purina International, Strathroy, Canada) and tap water were given *ad libitum*. Mice were allowed to acclimate for 1 week. All practices were in accordance with the guidelines of the Canadian Council on Animal Care and experimental procedures were approved by the Queen's University Animal Care Committee.

2.1.2 Transgenic pKZ1 mice

For the recombination assay, pKZ1 mating pairs were generously donated by Dr. Pamela Sykes from Flinders University, Australia. The pKZ1 mutagenesis mouse model has been described in previous studies as a sensitive tool for detecting somatic intrachromosomal recombination events and is a surrogate marker for non-homologous end joining enzyme activity[303-307]. Briefly, pKZ1 mice possess a DNA construct (figure 2.1) with an *E. coli lacZ* (β-galactosidase) reporter transgene in an inverse orientation to a chicken β-actin enhancer/promoter complex. If somatic intrachromosomal recombination is induced, the *lacZ* gene reorients using

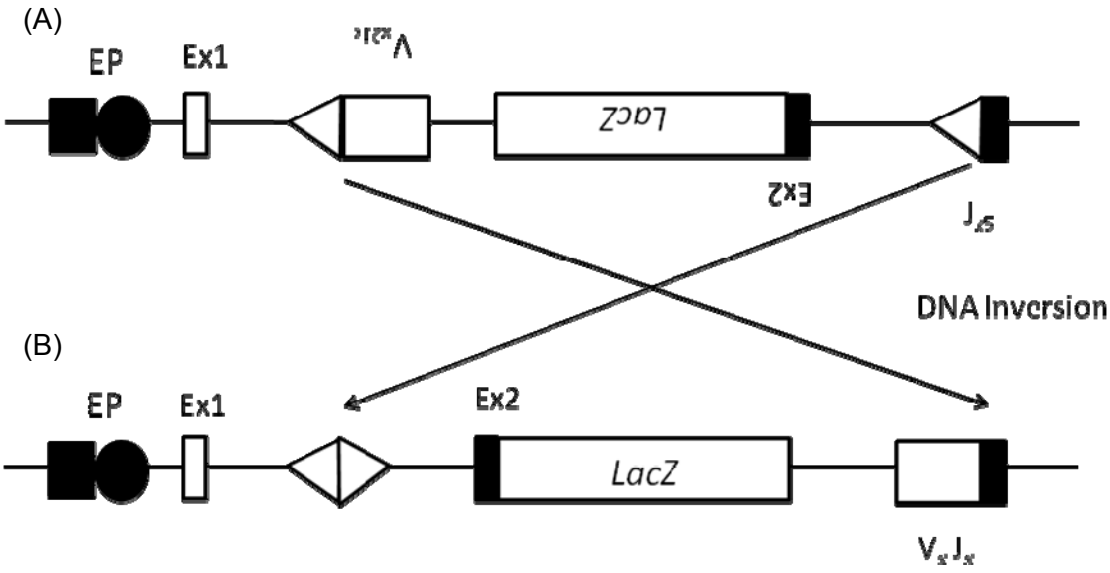


Figure 2.1 Schematic of the pKZ1 transgenic DNA reporter construct.

Briefly, the pKZ1 DNA construct contains an *E. coli lacZ* reporter transgene in inverse orientation to a chicken β-actin enhancer/promoter (EP) complex (A). If DNA recombination is induced, the *lacZ* gene reorients using V(D)J recombination signals $V_{\kappa 21c}$ and $J_{\kappa 5}$ to the correct transcriptional orientation with respect to the promoter and a functional gene product can be detected using the chromogenic substrate stain X-gal (B). Adapted from Matsuoka *et al*, 1991[308].

V(D)J recombination signals to the correct transcriptional orientation with respect to the promoter and a functional gene product can be detected using the chromogenic substrate stain X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

2.1.3 Genotyping transgenic mice

Experimental pKZ1 mice were genotyped at time of sacrifice and breeding pKZ1 mice were genotyped at 21 days of age. Tail snips were taken and sheared with surgical scissors and added to 200 µL of 5% w/v Chelex Resin (Bio-Rad, Hercules, United States) and 70 mg proteinase K (Sigma-Aldrich, St. Louis, United States) and incubated overnight at 55°C. Samples were boiled the next morning for 5 minutes and DNA content was determined by spectrophotometry. Samples were screened for the pKZ1 transgenic construct using polymerase chain reaction (PCR). All PCR reagents were purchased from Promega (Madison, United States) unless otherwise specified. Approximately 100 ng of DNA was added to a PCR vial containing 2 µL of 5x GoTaq Flexi buffer, 0.8 µL of 25 mM $MgCl_2$, 0.3 µL of 10 mM dNTP, 0.06 µL of primer ZL1675 (5'-ATGAAAGCTGGCTACAGGAAGGCC-3') (Cortec, Kingston, Canada), 0.06 µL of primer ZR1970 (5'-GGCAACATGGAAATCGCTGATTTG-3') (Cortec, Kingston, Canada), 2 µL of nuclease-free H_2O, and 0.6 µL of Flexi GoTaq. Samples were cycled at 94ºC for 3 minutes; then 30 cycles of 94ºC for 1 minute, 63.1ºC for 1 minute, 72ºC for 1 minute; then 72ºC for 7 minutes. PCR products were electrophoresed on a 1% agarose gel (Fisher Scientific, New Jersey, United States) prepared in 1x TAE buffer (Sigma-Aldrich, St. Louis, United States) containing 3% ethidium bromide (ICN Biomedicals, Ohio, United States). The gel was visualized under a UV light. Sample genotyping was done in duplicate.

2.1.4 Breeding

Mice were bred at a female to male ratio of 3:1 overnight and the presence of a vaginal plug the next morning designated gestational day 1 (GD1). For the recombination assay, heterozygous pKZ1 transgenic mice were back-crossed with non-transgenic C57Bl/6N mice. Dams included in post-natal studies underwent spontaneous delivery on gestational day 20.

2.2 TREATMENT

Benzene (Sigma-Aldrich, St. Louis, United States) was diluted in Mazola corn oil to a final injection volume of 0.1 mL/g. Vehicle control animals were given an equivalent volume of corn oil. For the acute studies, adult male mice were treated with one intraperitoneal (i.p.) injection of 0 mg/kg, 200 mg/kg, or 400 mg/kg of benzene. For the recombination assay, additional adult male mice were treated with 0 mg/kg, 200 mg/kg, or 400 mg/kg of benzene daily for 3 days. One dose of 40 mg/kg cyclophosphamide dissolved in saline was also administered to a positive control group of adult male mice for the recombination assay. For the *in utero* studies, timed-pregnant females were treated with daily intraperitoneal injections of 0 mg/kg, 200 mg/kg, or 400 mg/kg of benzene from gestational day 7 to 15. Upon time of sacrifice, male adult mice were sacrificed by cervical dislocation, dams were sacrificed by CO_2 asphyxiation, and post-natal pups were sedated with CO_2 followed by decapitation.

2.3 TISSUE COLLECTION

Spleen, liver, and brain tissue obtained from adult male pKZ1 mice were embedded in Tissue-Tek O.C.T. embedding compound (Electron Microscopy Sciences, Hornby, Canada) on dry ice and stored at -80°C until time of slicing. Tissue was sliced at 5 μm with a cryostat (Reichert-Jung Cryocut). Bone marrow from C57Bl/6N and pKZ1 males, dams, and post-natal day 9 pups were collected by flushing both femurs with a 25 gauge needle and syringe containing 0.3 mL of saline (or lysis buffer for samples undergoing immunoblotting). Fetal livers were extracted from gestational day 16 mice, sheared with surgical scissors, and aspirated with a needle and syringe to produce a single cell suspension. All tissue samples were coded and scored blind to treatment. Samples undergoing immunoblotting or the recombination assay were frozen at -80°C until time of analysis.

2.4 FORMATION OF γ-H2A.X

2.4.1 Nuclear protein extraction

Bone marrow samples were flushed with lysis buffer containing 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 μM chemostatin, 1 μM leupeptin, 1 μM antipain, 1 μM pepstatin, 1 mM phenylmethanesulphonylfluoride, 1mM benzamidine, and HALT phosphatase inhibitor cocktail (Fisher Scientific, Ottawa, Canada). Fetal liver samples were extracted, placed in lysis buffer, sheared with surgical scissors, and aspirated with a syringe and needle. Samples were kept on ice for 5 minutes, then centrifuged at 21 000 x *g* for 5 minutes. The pellet was

resuspended in lysis buffer containing 0.5% igepal (Sigma-Aldrich, St. Louis, United States) and sonicated for 20 seconds. Protein concentration was determined using a Lowry-based assay (Bio-Rad DCF protein determination kit, Hercules, United States). SDS loading dye buffer (5% β-mercaptoethanol, 0.25 M Tris base, 12.5 mM EDTA, 0.01% bromophenol blue, 35% glycerol, and 10% SDS) was added and samples were boiled for 5 minutes.

2.4.2 SDS-PAGE and immunoblotting

Samples were electrophoresed on a 15% acrylamide gel (Bio-Rad, Hercules, United States) and transferred onto a PVDF membrane (Millipore, Massachusetts, United States). The membrane was cut in half at the 25 kDa band and probed for either β-actin (42 kDa) or γ-H2A.X (15 kDa). For β-actin, membranes were blocked with 3% non-fat milk for 30 minutes, then incubated overnight with anti-β-actin primary antibody (Sigma-Aldrich, St. Louis, United States). Membranes were then incubated with sheep anti-mouse secondary antibody for 90 minutes and visualized with an enhanced chemiluminescence kit (PerkinElmer, Boston, United States). For γ-H2A.X, membranes were blocked with 3% bovine serum albumin for 30 minutes, and then incubated overnight with anti-γ-H2A.X primary antibody (Millipore, Massachusetts, United States). Membranes were then incubated with donkey anti-rabbit secondary antibody (Amersham, United Kingdom) for 90 minutes and visualized with an enhanced chemiluminescence kit. Samples were performed in triplicate and underwent densitometric analysis using Image J software (NIH).

2.5 MICRONUCLEUS ASSAY

The micronucleus assay was adapted from the protocol outlined by Krishna and Hayashi (2000)[309]. Bone marrow and fetal liver samples were smeared on silane-treated glass slides (Fisher Scientific, Ottawa, Canada) and allowed to dry at 37°C. Slides were fixed with 100% methanol (Sigma-Aldrich, St. Louis, United States) and allowed to dry. 0.2 M acridine orange solution (Sigma-Aldrich, St. Louis, United States) was prepared in 1/15 M Sorensen's phosphate buffer. One drop of acridine orange solution was placed on each slide, a glass coverslip was placed on top, and excess solution was blotted off. Slides were observed under a fluorescent microscope (Reichert Scientific Instruments) equipped with a 515-530 nm barrier filter within 2 hours of staining. The number of micronucleated cells and the number of total cells in the field were counted manually. At least 2000 cells were scored from 3 random fields and the percentage of micronucleated cells was calculated by dividing the number of cells exhibiting a micronucleus by the total number of cells x 100%.

2.6 RECOMBINATION ASSAY

The methods were adapted from the protocol described by Sykes *et al* (1998)[305]. Briefly, tissue slices or bone marrow cells smeared on silane-treated glass slides were fixed with 0.25% gluteraldehyde (Sigma-Aldrich, St. Louis, United States) for 7 minutes. The X-gal stain (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Invitrogen, Burlington, Canada) was prepared in buffer containing 0.1 M phosphate buffer, 5 μM potassium ferricyanide, 5 μM potassium

ferrocyanide, 2 mM MgCl, 1 mg/mL of X-gal (from 40 mg/mL fresh stock dissolved in DMSO). Slides were covered in the X-gal stain and incubated overnight at 37°C. The next morning, the slides were counterstained with 0.25% aqueous neutral red solution (Sigma-Aldrich, St. Louis, United States), followed by two washes of 100% ethanol and xylene (Fisher Scientific Co, Ottawa, Canada). The slides were mounted with DPX Mountant for microscopy (Electron Microscopy Sciences, Hatfield, United States) and a glass coverslip was placed on top. Slides were scored blind for the presence of positive X-gal staining. The frequency of recombination was calculated by dividing the number of positive-staining cells by the total number of cells in the field. Brain slices were used as a positive control for staining.

2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 4 software. For immunoblots, samples were run in triplicate and the relative optical density measures for γ-H2A.X were averaged and normalized to average β-actin values. A two-way analysis of variance (ANOVA) was used to compare treatment groups in the male acute study. A Bonferroni post-hoc test was used to compare each treatment group. For the *in utero* studies, a one-way analysis of variance (ANOVA) was used to compare treatment groups for the dams and the offspring. A Dunnett's post-hoc test was used to compare the exposed groups to the vehicle control. For the micronucleus assay, data were analyzed with a Kruskal-Wallis non-parametric test followed by a Dunn's multiple comparison test. For the recombination assay, a Mann-Whitney U-test was used. Statistical significance was designated if $p < 0.05$.

Chapter 3
Results

3.1 γ-H2A.X FORMATION

Acute exposure to 400 mg/kg benzene did not alter the formation of γ-H2A.X in bone marrow cells of adult male mice 1, 3, 6, and 24 hours after exposure (figure 3.1). Exposure to 200 mg/kg and 400 mg/kg of benzene during gestational days 7 to 15 did not significantly alter formation of γ-H2A.X in the bone marrow cells of the dams, gestational day 16 fetal liver cells, or post-natal day 9 bone marrow cells compared to the vehicle control (figures 3.2 and 3.3).

3.2 MICRONUCLEUS ASSAY

Acute exposure to 400 mg/kg benzene resulted in a statistically significant increase in the percentage of micronucleated bone marrow cells in adult male mice compared to vehicle controls 24 hours after exposure (figure 3.4). In dams, exposure to 400 mg/kg benzene resulted in a statistically significant increase in the percentage of micronucleated bone marrow cells on gestational day 16 compared to vehicle controls (figure 3.5A). Fetal liver cells also exhibited statistically significant increases in the percentage of micronucleated cells on gestational day 16 following exposure to 200 mg/kg and 400 mg/kg benzene (figure 3.5B). On post-natal day 9,

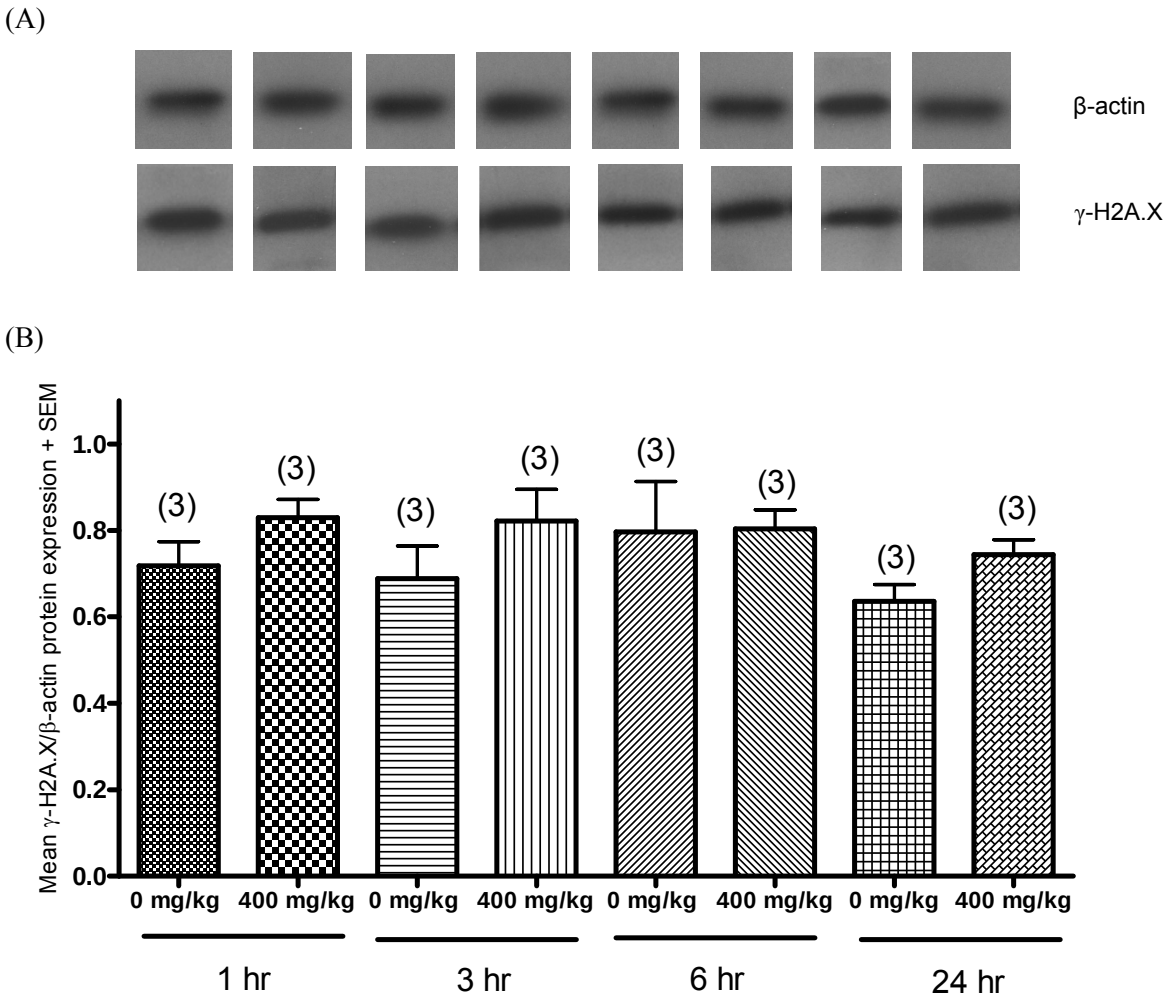


Figure 3.1 γ-H2A.X formation in bone marrow cells of adult male mice acutely exposed to 400 mg/kg benzene.

Adult male mice were treated with a single dose of 400 mg/kg benzene or vehicle control via an i.p. injection and bone marrow cells were harvested 1, 3, 6, and 24 hours after treatment. Formation of γ-H2A.X was determined by immunoblot. (A) Representative immunoblots. (B) Values represent mean γ-H2A.X optical density values normalized to β-actin values + standard error of the mean (SEM). No significant changes in the formation of γ-H2A.X were detected ($p > 0.05$ for all).

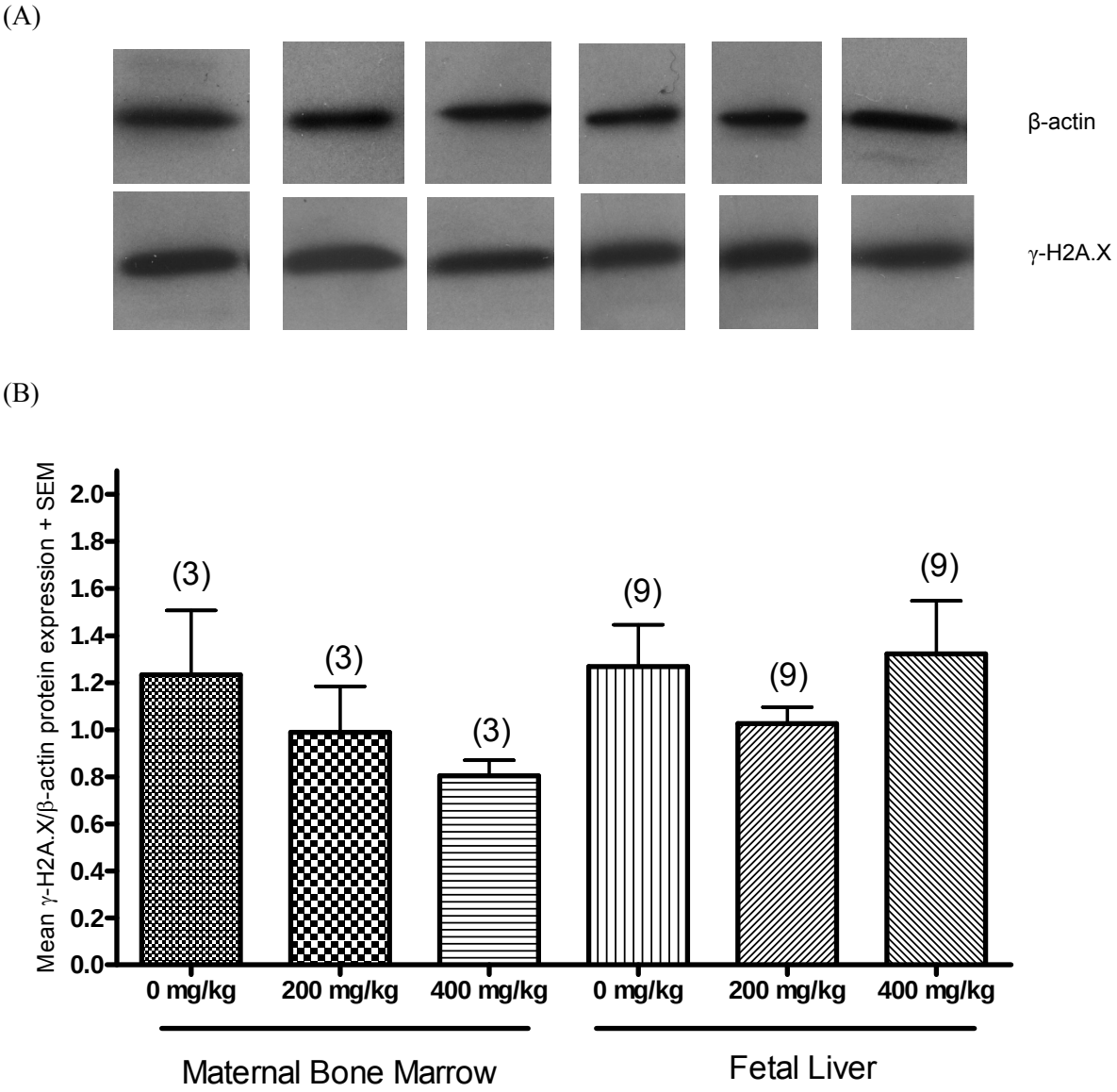
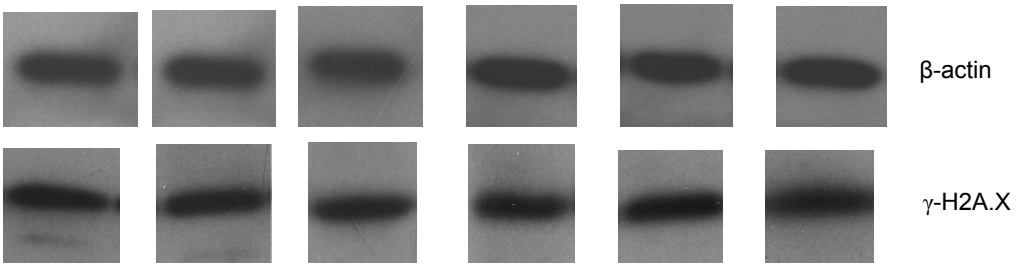


Figure 3.2 γ-H2A.X formation in maternal bone marrow and fetal liver cells on gestational day 16 following subacute *in utero* benzene exposure.

Timed-pregnant dams were treated on gestational days 7 to 15 with either 0 mg/kg, 200 mg/kg, or 400 mg/kg benzene. Maternal bone marrow cells and fetal liver cells were harvested on gestational day 16. Formation of γ-H2A.X was determined by immunoblot. (A) Representative immunoblots. (B) Values represent mean γ-H2A.X optical density values normalized to β-actin values + SEM. No significant changes in the formation of γ-H2A.X was detected ($p > 0.05$ for maternal bone marrow and for fetal liver).

(A)



(B)

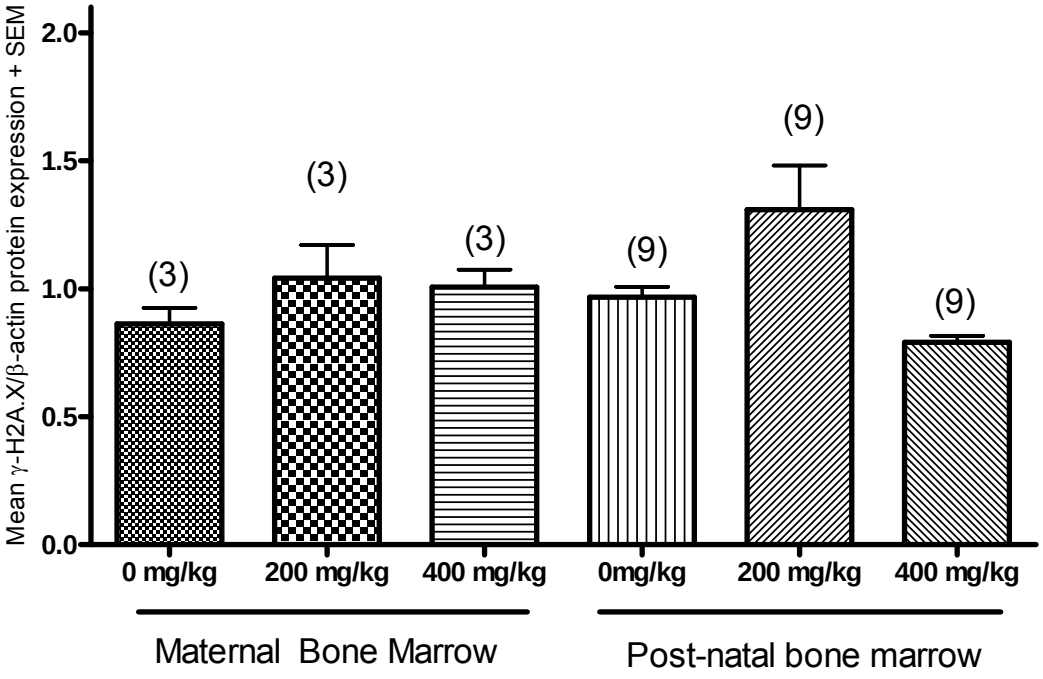


Figure 3.3 γ-H2A.X formation in maternal bone marrow and offspring bone marrow cells on post-natal day 9 following subacute *in utero* benzene exposure.

Timed-pregnant dams were treated on gestational days 7 to 15 with either 0 mg/kg, 200 mg/kg, or 400 mg/kg benzene. Maternal bone marrow cells and offspring bone marrow cells were harvested on post-natal day 9. Formation of γ-H2A.X was determined by immunoblot (B) Values represent mean γ-H2A.X optical density values normalized to β-actin values + SEM. No significant changes in the formation of γ-H2A.X was detected ($p > 0.05$ for maternal bone marrow and post-natal bone marrow).

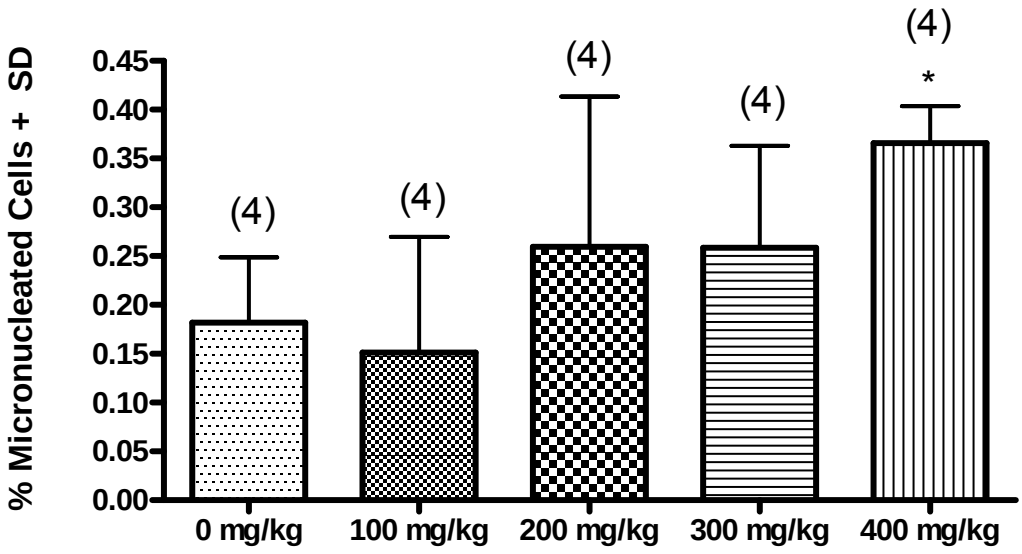
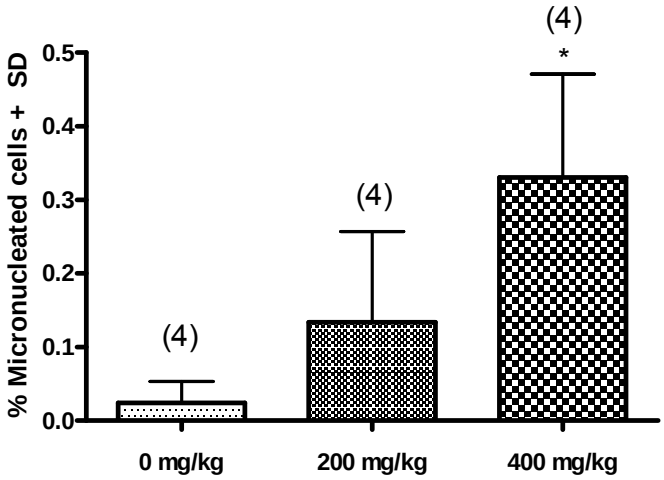


Figure 3.4 Percentage of micronucleated cells in male adult mouse bone marrow cells 24 hours after acute exposure to benzene.

Male adult mice were treated with a single i.p. injection of 0 mg/kg, 100 mg/kg, 200 mg/kg, 300 mg/kg, or 400 mg/kg benzene. A statistically significant increase in the percentage of micronucleated cells was detected following exposure to 400 mg/kg benzene (* indicates $p < 0.05$ compared to 0 mg/kg group).

(A) Maternal bone marrow cells



(B) Fetal liver cells

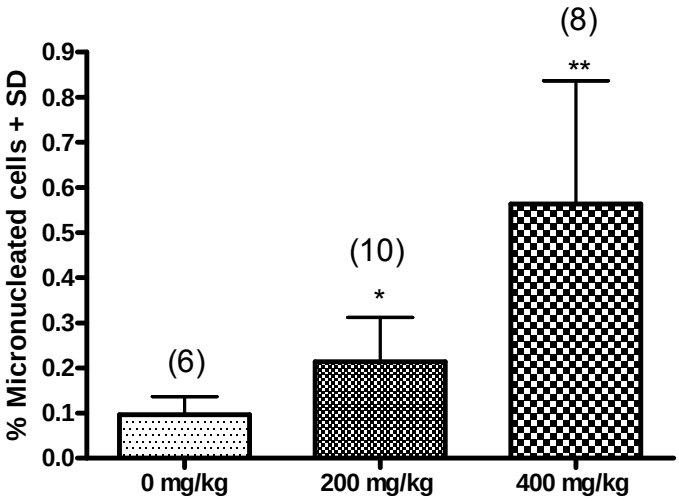


Figure 3.5 Percentage of micronucleated cells in maternal bone marrow cells and fetal liver cells on gestational day 16 following *in utero* exposure to benzene.

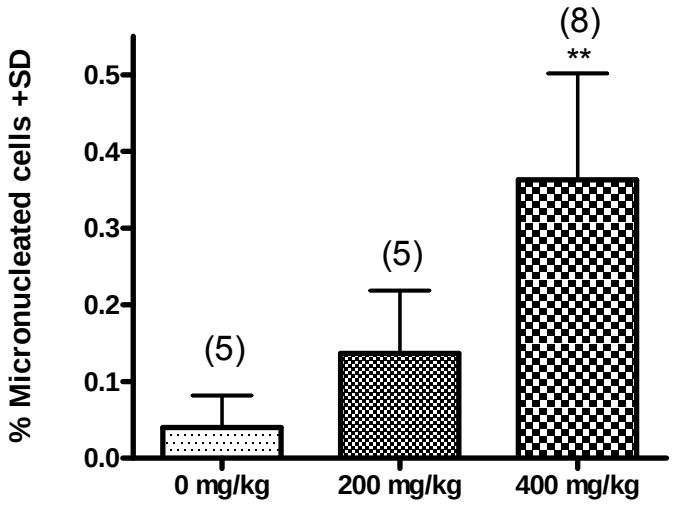
Timed-pregnant dams were treated on gestational days 7 to 15 with either 0 mg/kg, 200 mg/kg, or 400 mg/kg benzene. Percentage of micronucleated cells in (A) maternal bone marrow cells and (B) fetal liver cells on gestational day 16. A statistically significant increase in the percentage of micronucleated cells was detected in maternal bone marrow cells following 400 mg/kg benzene exposure and in fetal liver cells following exposure to 200 mg/kg and 400 mg/kg benzene compared to unexposed controls (* indicates $p < 0.05$; ** indicates $p < 0.01$).

maternal and offspring bone marrow micronucleus frequency remained significantly elevated in the 400 mg/kg benzene group compared to vehicle controls (figure 3.6A and B).

3.3 RECOMBINATION ASSAY

Positive X-gal staining was detected in pKZ1 transgenic mouse brain (figure 3.7), which is used as a positive control for staining. Adult male mice were treated with a daily i.p. injection of 0 mg/kg, 200 mg/kg, or 400 mg/kg benzene for one or three days. Twenty-four hours after the last exposure, no detectable recombination events were found in the spleen, liver, or bone marrow. Following one or three days of treatment with 40 mg/kg of cyclophosphamide, no detectable recombination events were found in the spleen, liver, or bone marrow of adult mice. For the *in utero* studies, no recombination events were detected in maternal bone marrow on gestational day 16 or post-natal day 9. In addition, no recombination events were detected in fetal liver. However, a low frequency of recombination events was detected in post-natal day 9 bone marrow (figure 3.8). Although there was an increasing trend, there was no statistically significant difference in the frequency of recombination in post-natal bone marrow cells following *in utero* benzene exposure. Due to the infrequent occurrence of positive-staining samples, further analysis of only samples containing detectable levels of recombination events was performed and revealed that *in utero* exposure to 400 mg/kg benzene caused a statistically significant increase in the frequency of recombination within this group.

(A) Maternal bone marrow



(B) Post-natal offspring bone marrow

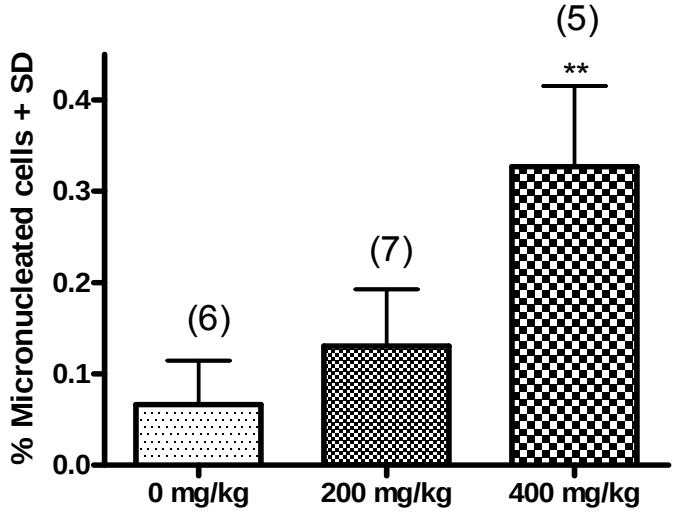


Figure 3.6 Percentage of micronucleated cells in maternal bone marrow cells and offspring bone marrow cells on post-natal day 9 following *in utero* exposure to benzene.

Timed-pregnant dams were treated on gestational days 7 to 15 with either 0 mg/kg, 200 mg/kg, or 400 mg/kg benzene. Percentage of micronucleated cells in (A) maternal bone marrow cells and (B) offspring bone marrow cells on post-natal day 9. A statistically significant increase in the percentage of micronucleated cells was detected in maternal and post-natal offspring bone marrow cells following 400 mg/kg benzene exposure compared to unexposed control groups (** indicates $p < 0.01$).

(A) pKZ1 brain slices (B) pKZ1 post-natal bone marrow cells

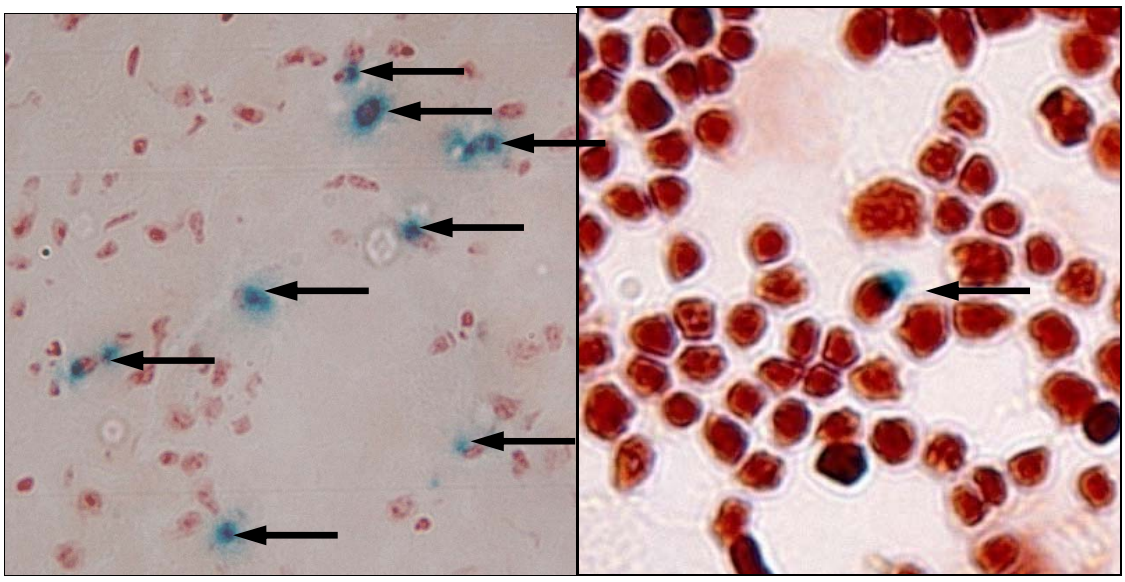


Figure 3.7 Positive-staining recombination events in pKZ1 transgenic mouse brain tissue slices and post-natal offspring bone marrow.

pKZ1 transgenic mice possess a DNA reporter construct designed to detect somatic intrachromosomal recombination events. If recombination occurs, the *E. coli lacZ* gene will reorient to its correct orientation with respect to a β-actin enhancer/promoter complex and a functional gene product can be detected through X-gal staining. (A) pKZ1 transgenic mouse brain slices. (B) pKZ1 transgenic mouse post-natal day 9 bone marrow cells. Cells that have undergone recombination and are expressing the functional gene product are identified by the blue stain (indicated by arrows).

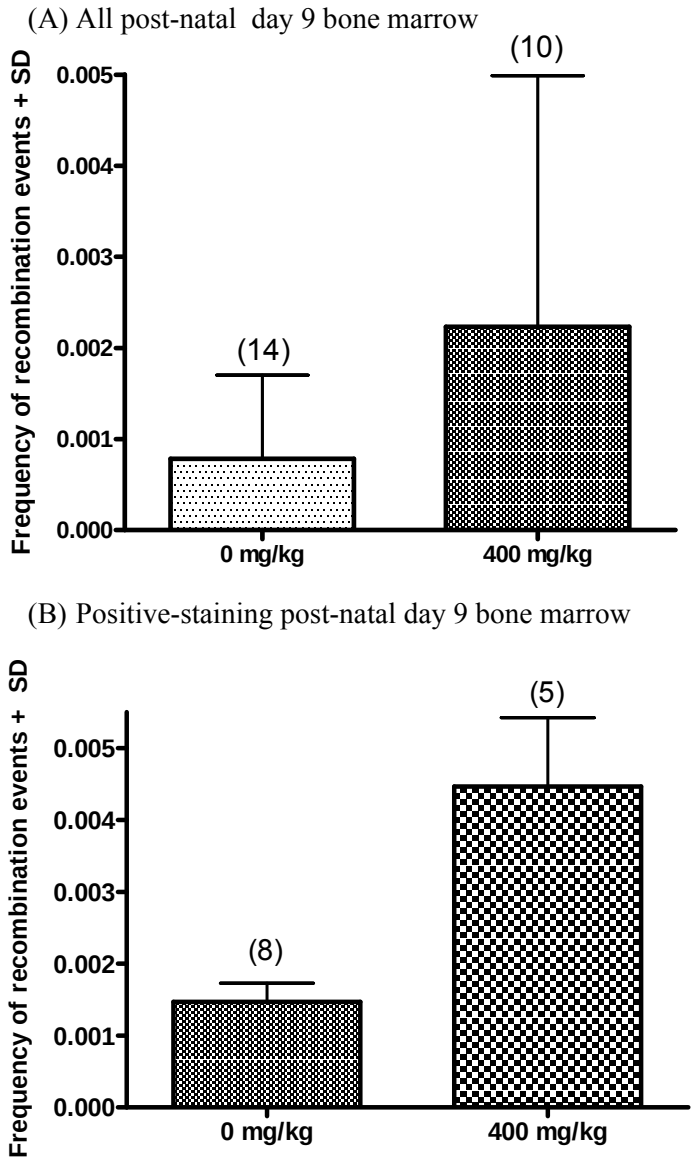


Figure 3.8 Frequency of recombination events in pKZ1 post-natal day 9 offspring bone marrow cells following *in utero* exposure to benzene.

Timed-pregnant dams were treated with 0 mg/kg or 400 mg/kg benzene on gestational days 7 to 15. Bone marrow cells were collected from the offspring on post-natal day 9 and stained for recombination events using X-gal. (A) The frequency of recombination events in all pKZ1 post-natal day 9 offspring. (B) The frequency of recombination events within positive-staining pKZ1 post-natal day 9 offspring. When comparing offspring with detectable staining events, there was a statistically significant increase in the frequency of recombination events following *in utero* exposure to 400 mg/kg benzene (** indicates $p < 0.001$).

Chapter 4
Discussion

In the present study, mice were exposed to acute or *in utero* doses of benzene and hematopoietic tissue was harvested and assayed for levels of γ-H2A.X as a marker of DNA double-strand breaks, the percentage of micronucleated cells, and the frequency of DNA recombination utilizing a transgenic mouse model.

4.1 γ-H2A.X FORMATION IS NOT ALTERED FOLLOWING ACUTE EXPOSURE AND SUBACUTE *IN UTERO* EXPOSURE TO BENZENE

The phosphorylation of H2A.X at serine 139 (subsequently referred to as γ-H2A.X) is one of the earliest signaling events following a DNA double-strand break[310]. γ-H2A.X formation occurs within minutes of a double-strand break and may last from 6 hours[311] to 24 hours[312] following induction. γ-H2A.X plays a role in recruiting and concentrating DNA repair enzymes into the site of damage[265,313-315]. Exposure to benzene and its metabolites has been demonstrated to induce single- and double-strand DNA breaks through the Comet assay; however, the formation of γ-H2A.X following benzene exposure in mice has yet to be examined in the literature.

In the present study, adult male mice were exposed to a single i.p. dose of 400 mg/kg benzene and the formation of γ-H2A.X was examined in bone marrow cells at four time points: 1, 3, 6, and 24 hours after exposure. Using an antibody specific for the phosphorylated form of

H2A.X and immunoblotting, no changes in the formation of γ-H2A.X were detected in adult male mice (figure 3.1). The *in utero* studies produced similar results, with subacute *in utero* exposure to benzene yielding no changes in the formation of γ-H2A.X in gestational day 16 fetal liver and post-natal day 9 bone marrow. Interpretation of these results should be made with caution as other studies have demonstrated benzene-induced DNA double-strand breaks using the Comet assay[316] and chromosomal breaks using the micronucleus assay[226,234,317,318]. Benzene metabolites are also identified as topoisomerase II inhibitors[245,246,248], and the inhibition of topoisomerase II is known to stabilize double-strand DNA breaks[244,281,319]. Since γ-H2A.X expression is transient, it is possible that either the time-points selected for the study did not capture the period of DNA double-strand break formation, or the DNA double-strand breaks were rapidly repaired before the cells were harvested, or it is also possible that the use of immunoblotting techniques was not sensitive enough to detect changes in γ-H2A.X levels. Other studies have utilized immunofluorescence to count the number of γ-H2A.X foci generated in a cell[265,313,320], which may be a more sensitive assay as each focus corresponds to a single DNA double-strand break. Thus, further studies investigating benzene-induced γ-H2A.X formation should consider employing confocal microscopy and the counting of γ-H2A.X foci.

4.2 SUBACUTE *IN UTERO* BENZENE EXPOSURE INCREASES THE PERCENTAGE OF MICRONUCLEATED CELLS IN MATERNAL BONE MARROW, FETAL LIVER, AND POST-NATAL BONE MARROW CELLS

Micronuclei are DNA fragments generated from a chromosomal break and they are common indicators of genotoxic damage. Micronucleated cells can undergo one of three fates:

cells can undergo apoptosis, cells can remain cytostatic for a period of time, or cells can survive despite the loss of genetic information and may undergo malignant transformation[321,322]. Assessing micronuclei formation as an endpoint has many advantages: the assay is simple, inexpensive, reproducible, allows efficient screening of thousands of cells, and is applicable to many cell types[323].

Two previous studies have reported micronuclei formation following *in utero* exposure to benzene. Ning and colleagues (1991) reported significant increases in the frequency of micronucleated cells in fetal liver and fetal peripheral blood of Swiss Webster mice following a single i.p. dose of 219 to 874 mg/kg[90]. Xing and colleagues (1992) reported significant increases in micronuclei formation in CD-1 mice maternal bone marrow and fetal liver following two very high doses of 1318 mg/kg benzene on gestational days 14 and 15[152]. The current study is the first to examine micronuclei formation in subacutely exposed fetal mice, and subsequent frequencies in bone marrow cells of post-natal mice.

In the present study, significant increases in the percentage of micronucleated cells were detected in fetal liver cells following *in utero* exposure to 200 mg/kg and 400 mg/kg of benzene throughout gestational days 7 to 15 (figure 3.5). Micronucleus frequency remained significantly elevated in post-natal day 9 bone marrow tissue of mice treated *in utero* with 400 mg/kg of benzene (figure 3.6). Thus, chromosomal breaks acquired from *in utero* exposure can persist in hematopoietic organs of post-natal mice. If these breaks occur in critical genes such as tumor suppressor genes, malignant transformation of a hematopoietic stem cell may lead to leukemogenesis in early life. *In utero* exposure to benzene has been associated with other adverse outcomes in early post-natal life. Disruptions in hematopoietic cell populations have been demonstrated to persist up to 6 weeks after birth in mice exposed to inhalational benzene

throughout gestational days 6 to 15[155]. Therefore, *in utero* exposure to benzene has the potential to cause damage that can persist in early life and further investigation is required to explore the possible mechanisms and endpoints of these changes.

4.3 BENZENE DOES NOT INDUCE INTRACHROMOSOMAL RECOMBINATION IN THE ADULT pKZ1 MOUSE MODEL FOLLOWING ACUTE EXPOSURE.

DNA recombination is a crucial aspect of DNA repair and the maintenance of genomic stability. NHEJ is an error-prone mechanism of DNA double-strand break repair (reviewed in Burma, 2006[273] and Lieber, 2008[324]). DNA repair capabilities during ontogeny is especially important, as there is a high rate of cellular proliferation and an increased susceptibility to chemical insult in the developing embryo/fetus[325,326].

Using the transgenic pKZ1 mutagenesis mouse model, the frequency of somatic intrachromosomal recombination can be measured and used as a surrogate marker of NHEJ activity. The pKZ1 mouse model has been previously described in other papers[305,306,308], which have stated that the sensitivity of this model exceeds that of other transgenic mutagenesis reporter mice. This model is unique in that it allows for the quantification of *in vivo* inversion events from a transgenic reporter construct. In adults, benzene exposure has been associated with DNA recombination events, including the *t*(8;21) translocation[327], and deletions in chromosome 5 and 7[328] both of which are associated with acute myeloid leukemia. Benzene exposure has been reported to increase recombination frequencies in the surrogate markers *HPRT*[329] and *glycophorin A*[330] in humans. To date, only a couple of studies have examined DNA recombination in mouse

models following benzene exposure either using fluorescent *in situ* hybridization[331] or assessing sister chromatid exchange frequency[229].

In the present study, acute exposure to benzene or cyclophosphamide did not produce detectable DNA recombination events in adult male spleen, liver, and bone marrow. Cyclophosphamide is an alkalating agent that generates DNA crosslinks and consequently DNA strand breaks. Doses of cyclophosphamide used in this study were similar to doses used in previous studies reporting increased DNA recombination frequencies in this animal model[305]. Despite a lack of positive staining in the aforementioned adult tissues, positive staining was observed in the transgenic brain (figure 3.7), which has been reported to be a positive control for staining.

Site-specific recombination assays are conservative surrogate markers of DNA recombinational repair. DNA damage induced by benzene may not affect that specific site of recombination and may be repaired by another mechanism, therefore it is possible that benzene-induced DNA recombination may not be detected in this experimental system. It is also possible that benzene-induced DNA damage is not repaired by this pathway and that other repair mechanisms such as homologous recombination are being employed.

4.4 BENZENE MAY INCREASE THE FREQUENCY OF INTRACHROMOSOMAL RECOMBINATION IN BONE MARROW CELLS OF pKZ1 POST-NATAL MICE EXPOSED *IN UTERO*

Fetal mice exposed to 200 mg/kg and 400 mg/kg benzene did not exhibit positive-staining recombination events in liver cells on gestational day 16. However, positive X-gal

staining was observed in post-natal bone marrow cells (figure 3.8). *In utero* benzene exposure increased the frequency of this recombination, however, this effect was not statistically significant. Two studies have examined sister chromatid exchange following benzene exposure and have reported increases in the frequency of sister chromatid exchange in both maternal and fetal cells[152,332]. DNA translocations and inversions are prevalent in childhood leukemias and can be acquired *in utero*[2,79,80,87,296,297], therefore it is important to elucidate the effect of environmental exposures on these events. Currently, no risk factors have been identified for associating childhood exposures with an increased frequency of leukemic translocations (reviewed in McHale and Smith, 2004[333]; and Wiemels, 2008[334]), however, ongoing studies are examining maternal dietary intake of topoisomerase II inhibitors and an increased frequency of *MLL* translocations[165]. Although the frequency of recombination events reported in our study is low, it should be noted that *in utero* benzene exposure caused an increasing trend in this frequency. Due to the infrequent occurrence of positive-staining samples, further analysis of only samples containing detectable levels of recombination events revealed that *in utero* exposure to 400 mg/kg benzene caused a statistically significant increase in the frequency of recombination within this group. It is possible that gender, maternal factors, fetal position within the uterine horn, or genetic factors may contribute to differences in the offspring that do not exhibit detectable levels of recombination compared to offspring that do, however further studies are required to characterize the differences between these two groups.

The concept that a low population of cancer or leukemic stem cells is responsible for propagating the disease is becoming more popular among the scientific community[335-338]. This implies that only a small population of hematopoietic cells needs to be malignantly transformed into leukemic stem cells before leukemia can develop. Therefore, the frequencies reported in our

study may be highly biologically relevant. Although leukemic translocations are prevalent in childhood leukemia, the disease itself is a rare event, and therefore it is expected that studies investigating genetic events leading up to childhood leukemia must utilize sensitive models and include a large number of samples.

4.5 LIMITATIONS

4.5.1 Administration and dose of benzene

The majority of human exposures to benzene are through inhalation, as benzene is volatile and is found in air pollutants such as vehicular emissions and cigarette smoke. The route of exposure in this study was through i.p. injection, which allows for the administration of precise dosages at desired time points. With inhalation exposure paradigms, the rate of respiration differs between species and strain and thus differences in absorption are present. Administration of benzene through i.p. injection is very similar to that of gavage treatment when comparing absorption and excretion percentages[339], and with higher doses of benzene, proportionally more benzene is exhaled unchanged[340]. When comparing inhalational exposure to that of i.p. treatment, more benzene is retained internally through inhalation than that of an i.p. exposure, and it was reported that an inhalational exposure of 50 ppm for 6 hours is equivalent to an i.p. dose of 150 mg/kg when comparing tissue metabolite levels as an end-point[340]. Unfortunately, it is not possible to extrapolate this to the doses used in the present study because excretion rates of unchanged benzene are non-linear at higher doses. In any case, the toxicity observed with high dose animal exposure regimes is an underestimation of the risk of low dose human exposure[341].

4.5.2 Undetectable pKZ1 recombination events in adult tissues other than the brain

Acute and subacute doses of benzene and cyclophosphamide did not produce detectable recombination events in adult tissues, although previous studies have reported background and induced recombination frequencies that should have been detected with the number of cells screened in this study[304-307]. Various attempts at modifying the staining protocol were undertaken in order to reproduce the data reported in previous studies, including increasing the stain incubation time, increasing the amount of X-gal in the solution, and changing the width of the histological slices. Unfortunately, these modifications were unsuccessful in generating positive-staining recombination events in the adult tissues. Progression into *in vitro* exposure paradigms in primary bone marrow cell cultures obtained from pKZ1 mice should be the next step in determining whether benzene-induced DNA recombination can be measured with this experimental model.

4.5.3 Unexplored pathways of *in utero* DNA damage and repair

There are a wide range of DNA lesions and DNA repair mechanisms that can occur in a cell. This study focuses on the most toxic genetic lesion: DNA double-strand breaks. However, benzene can induce other types of DNA damage and induce other repair mechanisms that were not investigated in the assays performed in this study. It should be recognized that this study only examines the induction of a specific type of DNA damage and a specific type of DNA repair process and further investigations examining different DNA lesions and repair pathways must be considered in order to elucidate the full extent of benzene's genotoxic action on the fetus.

4.5.4 Micronuclei persistence and follow-up with disease outcome

Benzene-induced elevations in micronuclei frequency have been demonstrated to last for up to 85 days post-exposure in adult mice[342]. The present study only examined the persistence of micronuclei two weeks after the last *in utero* dose. Post-natal day 9 was chosen as it was the earliest time point in which bone marrow cells could be manually obtained from pups and the bone marrow is a fully functional hematopoietic organ during this period. Examination into later time-points such as murine adolescence or early adulthood may be of additional value and should be considered in future studies. The general purpose of this study was to elucidate a possible mechanism behind toxicant-initiated childhood cancer. Unfortunately, this study does not directly associate the induction of DNA double-strand breaks and the leukemia outcome. Ideally, the maintenance of mice exposed *in utero* to benzene and the association of leukemia development and the frequency of DNA double-strand breaks or DNA recombination would provide a clearer understanding of the role of this mechanism in initiating childhood leukemia.

4.5.5 Possible confounders in animal care conditions

In the latter half of these studies, it was made known that the room in which experimental mice were housed had positive cases of Theiler's murine encephalomyelitis GDVII virus. This virus is associated with the development of a central nervous system demyelinating disease[343]. No paralysis was observed in our mice and none of the mice used in this study were definitively proven to harbor the virus. Although it is unclear whether this viral infection interfered with findings in this study, it was noted that an increased incidence of dystocias was observed in dams in the post-natal study. There were three cases in the 0 mg/kg benzene group and two cases in the 200 mg/kg benzene group. Upon a literature search, there were no studies linking Theiler's virus

to reproductive toxicity and labor difficulty. There was also increased construction activity in the animal care facilities during our experiments. Loud noises and vibrations from drilling may have contributed to different breeding and nesting behaviors although there were no differences in litter sizes observed between groups.

4.6 FUTURE DIRECTIONS

4.6.1 Gender-specific susceptibility to benzene-induced *in utero* genotoxic damage

There have been gender differences reported in benzene toxicity susceptibility. Male mice are generally more susceptible to benzene-induced colony-forming unit-erythroid (CFU-e) progenitor cell cytotoxicity[184,224] and mRNA microarrays have shown that differences in gene expression induced by *in vivo* benzene exposure are gender-specific[258]. Male mice are also more susceptible to genotoxic effects induced by benzene[344]. In humans, micronuclei formation in response to benzene exposure exhibits gender differences, however, this may be attributed to differences in dietary selenium intake[345]. Interestingly, childhood leukemia is more prevalent in the male population[1] and it would be notable to investigate whether differential susceptibility to the genotoxic effects of environmental carcinogens plays a role in this statistic. Gender identification and stratification in the analysis of the assays outlined in this study may elucidate novel relationships in benzene-induced *in utero* genotoxicity.

4.6.2 Hematopoietic cell subtype susceptibility to benzene-induced *in utero* genotoxic damage

To determine if a certain hematopoietic cell subtype is particularly susceptible to benzene-induced *in utero* genotoxic damage, cell surface markers can be utilized in conjunction with markers of DNA damage. The micronucleus assay has been optimized for flow cytometry[346], and thus fetal liver or post-natal bone marrow can be analyzed simultaneously for DNA damage and cluster of differentiation (CD) markers such as $CD34^{+}$, a marker for hematopoietic progenitor and stem cells[347]. Benzene is most strongly associated with acute myeloid leukemia[9,348], and numerous studies have demonstrated benzene's toxic effects on myeloid progenitor cells[154,223,349-351]. Therefore, it would be of interest to see if benzene's genotoxic effects also target cells of the myeloid lineage.

4.6.3 Epigenetic mechanisms behind benzene-initiated childhood leukemias

Epigenetic changes following benzene exposure has not been investigated fully. To date, there has only been one study conducted in Italy examining methylation changes in benzene-exposed workers[225]. Maternal folic acid supplementation has been associated with a decreased risk of the development of acute lymphocytic leukemia in her offspring, suggesting a role of hypomethylation[352]. It would be interesting to explore benzene-induced methylation changes in animal models and determine whether *in utero* exposure elicits these epigenetic effects as well.

4.7 CONCLUSIONS

In conclusion, these findings have shown that acute and *in utero* benzene exposure did not alter γ-H2A.X formation for the exposure paradigms chosen for our studies. However, using the micronucleus assay, an increase in the frequency of chromosomal breaks was detected in adult male mice exposed acutely to 400 mg/kg benzene, and to maternal bone marrow cells, fetal liver cells, and post-natal offspring bone marrow cells following subacute *in utero* exposure to benzene. DNA recombination as a response to DNA double-strand breaks was measured using the pKZ1 mutagenesis mouse model, and no recombination events were detected in adult male spleen, liver, and bone marrow cells. Maternal bone marrow cells and fetal liver cells also yielded no recombination events, however post-natal day 9 bone marrow cells exhibited an increasing trend in the frequency of recombination after *in utero* benzene exposure. Analysis of only positive-staining post-natal offspring samples revealed a statistically significant increase in the frequency of recombination following *in utero* exposure to 400 mg/kg benzene within this group. Additional studies are needed to fully elucidate the relationship between *in utero* benzene exposure and the induction of recombination events in the fetus. Future investigations into gender-specific and cell type-specific differences in susceptibility to benzene genotoxicity are warranted and studies examining other DNA damage and repair pathways are necessary to fully elucidate the role genotoxic of mechanisms in the etiology of benzene-induced childhood leukemias

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