INVESTIGATING THE MECHANISMS OF TOXICITY OF BENZENE AND ITS METABOLITE BENZOQUINONE AND THE ROLE OF SULFORAPHANE AS A POTENTIAL PROTECTIVE AGENT IN CD-1 MOUSE DEVELOPMENT

by

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Abstract

Benzene is an environmental pollutant and a known human leukemogen. It is also suspected to be associated with \textit{in utero}-initiated leukemia. However, the mechanisms of benzene-mediated toxicity and carcinogenicity in both adults and fetuses are not well understood. Two main research hypotheses were tested in this thesis work. The first was that exposure to the benzene metabolite, benzoquinone, leads to increased levels of reactive oxygen species (ROS) and DNA damage in cultured mouse fetal liver cells, and that epigenetic changes in CD-1 mouse fetal livers would result from \textit{in vivo} exposure to benzene. The second was that exposure to the phytochemical, sulforaphane (SFN), would induce detoxification enzymes in CD-1 mouse fetal livers \textit{in vitro} and \textit{in vivo}, and that this would confer protection against benzene-induced cellular damage. Our data demonstrated that exposure to benzoquinone increased ROS levels, increased DNA damage, and altered DNA repair gene expression in cultured CD-1 mouse fetal liver cells. Additionally, although we found that SFN induced various metabolizing enzymes involved in the detoxification of benzene metabolites, SFN did not protect against the deleterious effects of benzene found in this study. In a separate study, we examined whether SFN could induce fetal and/or maternal enzymes involved in benzene detoxification \textit{in vivo} in CD-1 mice and how this compared to non-pregnant CD-1 mice. While we found that chronic SFN exposure induced both gene expression and activity of liver detoxification enzymes of both pregnant and non-pregnant mice, SFN had no effect on expression levels or activity of these genes in fetal livers exposed \textit{in utero}. Lastly, we investigated the effect of benzene exposure on DNA methylation and histone modifications \textit{in vivo}. Benzene exposure decreased global DNA methylation in maternal bone marrow only, having no effect on any other epigenetic modifications measured in these studies. Taken together, the data presented in this thesis only partially supported our hypotheses; nevertheless, they provide valuable information for future research directions pertaining to benzene-associated transplacental carcinogenesis.
Co-Authorship

The research conducted in this thesis was performed by the candidate, Nicola A. Philbrook, under the supervision of Dr. Louise M. Winn.
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List of Abbreviations

°C: degrees celsius
8-OHdG: 8-hydroxy-2’-deoxyguanosine
γH2AX: phosphorylation of histone H2A
variant X
μg: microgram
μl: microlitre
μM: micromolar
A: adenine
ABP: 4-aminobiphenyl
AGM: aorta-gonad-mesonephros
AhR: aryl hydrocarbon receptor
ALL: acute lymphocytic leukemia
AML: acute myelogenous leukemia
ANOVA: analysis of variance
AP-1: activator protein 1
ARE: antioxidant response element
BER: base excision repair
BRCA1: breast cancer 1, early onset
BRCA2: breast cancer 2, early onset
BSA: bovine serum albumin
C: cytosine
CDK4: cyclin-dependent kinase 4
CDK6: cyclin-dependent kinase 6
CDKN2B: cyclin-dependent kinase inhibitor 2B
CHO: Chinese Hamster Ovary
CLL: chronic lymphocytic leukemia
CM-DHFDA: 5,6-chloromethyl 2,7-dichlorodihydrofluorescein diacetate
CML: chronic myelogenous leukemia
CLP: common lymphoid progenitor
CMP: common myeloid progenitor
CpG: cytosine phosphate guanine
CYP: cytochrome P450
DCF: dichlorodihydrofluorescein
DCPIP: dichlorophenol-indophenol
DMBA: 9,10-dimethyl-1,2-benzanthracene
DNA: deoxyribonucleic acid
DNA-PK: DNA-dependent protein kinase
DNMT: DNA methyltransferase
DSB: double stranded break
F(ab’)_2: fragment antigen binding (cleaved)
g: gravity
G: guanine
GCLC: glutamate cysteine ligase catalytic subunit
GD: gestational day
GSH: glutathione
h: hours
H3: histone 3
H3K9: histone 3 lysine 9
H3K27: histone 3 lysine 27
H3K56: histone 3 lysine 56
H4: histone 4
HAT: histone acetyltransferase
HD3: chicken erythroblast cells
HDAC: histone deacetylase
HIF-1: hypoxia inducible factor 1
HL-60: human promyelocytic leukemia cells
HO-1: heme oxygenase 1
Hprt: hypoxanthine guanine phosphoribosyltransferase
HR: homologous recombination
HSC: hematopoietic stem cell
IL: interleukin
IMDM: Iscove’s modified Dulbecco’s medium
IP: intraperitoneal
KEAP1: Kelch-like ECH-associated protein 1
K_M: Michaelis constant
l: litre
m: meter
MAGE1: melanoma antigen gene 1
MAP: mitogen activated protein
mg: milligram
mg/kg: milligrams/kilogram
MLL: mixed-lineage leukemia
mM: millimolar
MPO: myeloperoxidase
MTS2: multiple tumour suppressor 2
MYH: mutY homolog
NAC: N-acetylcysteine:
NFkB: nuclear factor kappa-light-chain enhancer of activated B cells
ng: nanogram
nm: nanometer
NHEJ: non-homologous end-joining
NHL: Non-Hodgkin’s lymphoma
NNK: nicotine-derived nitrosamine ketone
NQO1: NAD(P)H quinone oxidoreductase 1
NRF2: nuclear factor (erythroid-derived 2)-like
OGG1: 8-oxoguanine glycosylase 1
PARP-1: poly (ADP) ribose polymerase 1
PBS: phosphate buffered saline
PEG: polyethylene glycol
PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PI: propidium iodide
ppb: parts per billion
ppm: parts per million
PND: post-natal day
PTEN: phosphatase and tensin homolog
qRT-PCR: quantitative real time polymerase chain reaction
ROS: reactive oxygen species
rpm: revolutions per minute
SEM: standard error of the mean
SFN: sulforaphane
sMAF: small musculoaponeurotic fibrosarcoma
SNP: single nucleotide polymorphism
SOD: superoxide dismutase
S-PMA: s-phenylmercapturic acid
SULT: sulfotransferase
T: thymine
TBST: tris buffered saline with tween-20
TGB: 1,2,4-trihydroxy-5-glutathionyl benzene
TGFβ: Tumour growth factor beta
TSA: trichostatin A
U: units
UGT: uridine diphosphate-glucuronosyl transferase
XRCC4: X-ray repair cross-complementing protein 4
Chapter 1

General Introduction

1.1 Benzene

The chemical compound, benzene, is composed of 6 carbon atoms in a cyclical structure, with the chemical formula \( \text{C}_6\text{H}_6 \). Under normal conditions, benzene is a colourless, sweet smelling liquid that is highly flammable. It is a ubiquitous pollutant since it is emitted into the environment as a consequence of both human-made processes and natural processes, including forest fires and volcanic eruptions. While benzene was historically used in the production of numerous products, including plastics, dyes, pesticides, and as an additive to gasoline [1], it is now primarily utilized in the manufacturing of organic chemicals, and is an intermediate in reactions used to produce numerous chemicals, including ethylbenzene, which is used in the manufacturing of styrene [2]. Benzene is also found in cigarette smoke and in natural gas [1].

In humans, exposure to benzene is typically through the air because of benzene’s high vapour pressure at room temperature [3]. There are two main types of human exposure to benzene: occupational exposure and environmental exposure. Worldwide exposure to benzene is at its highest in industrial occupational settings or among workers who are chronically exposed to vehicle exhaust fumes, including gas station attendants, taxi drivers, and street workers [4]. Because of its negative human health effects, exposure to benzene in the workplace is limited in most countries. In Canada, the Occupational Exposure Limit for benzene for a 40 hour work-week is 0.5 parts per million (ppm), with a short-term exposure limit (15 minutes or less) ranging from 2.5 - 25 ppm depending on the province or territory [5]. It is estimated that 375000
Canadian workers are exposed to benzene yearly; with approximately 10% of workers being exposed to moderate or high exposure levels [5].

Levels of benzene in ambient air in Canada were measured from 1989 – 1998, and it was found that in typical urban locations without immediate industrial exposure, benzene concentrations were between 1.8 and 3.6 µg/m³ (0.56 to 1.13 parts per billion; ppb). In rural areas, benzene concentrations averaged 0.5 µg/m³ (0.16 ppb), whereas ambient air concentrations of benzene near roads or industrial sites were as high as 13.1 µg/m³ (4.1 ppb) [6]. One study conducted in Ottawa, Ontario demonstrated that benzene levels in homes were generally higher than benzene levels outside of these homes, with the primary source being emissions from benzene-containing indoor products, including paint, wax, or glues [7]. Exposure to benzene may also occur via ingestion [8] or dermal absorption [9], although these routes of exposure represent minor pathways.

Additionally, one activity that drastically increases a person’s exposure to benzene is cigarette smoking. A study conducted by the Environmental Protection Agency in the USA determined that benzene concentrations in expired air of smokers was almost 10 times that of non-smokers. Mean breath benzene concentrations in smokers were measured at concentrations as high as 15 µg/m³ (4.7 ppb), with a single cigarette increasing breath concentrations up to 90 µg/m³ (28.2 ppb) [10]. The concentration of benzene in blood of non-smokers averages around 176 ng/l, whereas for smokers it averages at 365 ng/l [11].

Benzene levels and exposure are monitored and restricted worldwide since benzene has been determined to be both a hematotoxicant beginning at concentrations of 1 ppm chronic exposure [12], and a leukemogen beginning at concentrations of 1 to 2.5 ppm chronic exposure [13, 14]. It has been classified by the International Agency for Research on Cancer as a Group 1
human carcinogen, meaning there is evidence that benzene causes cancer in humans; however, its potential as a transplacental carcinogen has not yet been determined [15]. It is considered a “non-threshold toxicant”, which suggests there is no safe exposure limit of benzene [16]. In order to discuss the detrimental health effects of benzene (section 1.3), it is necessary to first discuss the process of hematopoiesis and its associated disorders, including leukemia and childhood leukemia, as well as their prevalence and proposed etiologies.

1.2 Hematopoiesis and Associated Disorders

1.2.1 Hematopoiesis

Hematopoiesis is the primary target of benzene exposure in humans. Hematopoiesis is the process of blood cells differentiating from immature progenitor cells to fully functional mature blood cells. The process of hematopoiesis can be seen schematically in Figure 1.1. All hematopoietic cells arise from a hematopoietic stem cell (HSC). This HSC can either self-renew, producing an identical daughter cell, or it can differentiate to form either a common myeloid progenitor cell (CMP) or a common lymphoid progenitor cell (CLP), each of which eventually give rise to fully differentiated myeloid cells or lymphoid cells, respectively. The CMP will give rise to myeloid cells, including basophils, mast cells, dendritic cells, macrophages, neutrophils, and eosinophils, all of which are critical to innate immune function. It will also lead to development of mature erythrocytes and platelets, important for hemostasis. The CLP, on the other hand, will give rise to various types of T-cells and B-cells, involved primarily in adaptive, or learned, immunity, including the production of antibodies.

Adult and fetal hematopoieses are fundamentally distinct processes differentiated by the specific aim of each process. Fetal hematopoiesis has to simultaneously produce fully
Figure 1.1: Schematic of hematopoiesis.

Hematopoietic stem cells (HSCs) can self renew, or give rise to a common myeloid progenitor (CMP) cell or a common lymphoid progenitor (CLP) cell. Each of these immature cells further differentiate to form mature, fully functioning blood cells with distinct roles in hemostasis and the immune system. Black writing indicates stem cells, blue writing indicates immature cells, green writing indicates mature cells. Adapted from [17, 18].
differentiated blood cells that allow the developing fetus to thrive while at the same time producing an increasing pool of HSCs [19]. Therefore, fetal HSCs are characterized by many symmetrical cell divisions and cell cycling, whereas adult HSCs are largely quiescent, replicating only to maintain a certain level of HSCs in the bone marrow [20]. Additionally, adult hematopoiesis occurs solely in the bone marrow where a very distinct environment is established to support this process, whereas the process of fetal hematopoiesis occurs in differing locations as organogenesis occurs before settling in the bone marrow for the duration of an individual’s life [19, 21]. Lastly, fetal hematopoiesis displays both primitive and definitive hematopoiesis, distinguished by the types of cells that arise from them, whereas adult hematopoiesis displays solely definitive hematopoiesis.

In the developing mouse, the yolk sac is the first site in which hematopoietic precursor cells can be found, with primitive erythropoiesis beginning around gestational day (GD) 7 [22]. Erythroid cells belonging to the primitive lineage give rise to primitive red blood cells (RBCs), which are large in size, express both adult and embryonic globins, and retain their nuclei until after being released into the circulation [22, 23]. The first definitive myeloerythroid progenitor cells are also found in the yolk sac beginning around GD9, with these cells giving rise to both lymphoid and myeloid lineages, including RBCs that are enucleate, smaller, and only express adult globins [19]. These are the RBCs typical of adult circulation. While the yolk sac contains the first hematopoietic precursors, its microenvironment is not supportive of complete blood cell differentiation [19]. Beginning on GD10, the aorta-gonadal-mesonephros region (AGM) is populated with self-renewing HSCs, which are thought to be produced in this tissue [24]. Simultaneously, a larger, more substantial pool of HSCs is found in the placenta, beginning on GD10.5 and expanding until GD13.5 [25]. Following these initial stages of hematopoiesis, the
fetal liver becomes the primary hematopoietic organ during development. The fetal liver is seeded with myeloerythroid progenitor cells beginning on GD9.5 until GD11.5 from cells assumed to be derived from the yolk sac [19]. The fetal population of HSCs continues to expand until GD15 [26]. Interestingly, with increasing gestational age, different cell types undergo more prolific differentiation. Specifically, erythropoiesis appears to be initially predominant in the fetal liver, whereas a couple of days later, differentiation of lymphoid and myeloid cell lines appears to become more abundant [19]. As circulation increases, hematopoietic cells can be found in other tissues, including the spleen on GD14.5, followed by the bone marrow [27]. The bone marrow, which is the primary site of hematopoiesis after birth, becomes populated with HSCs beginning around GD17, near the end of mouse gestation [27].

Developmental hematopoiesis in the mouse and the human is quite similar, making mice a useful surrogate for human embryonic hematopoiesis [28]. Although timing is different, the pattern of movement and blood cell types seen during the different phases of hematopoiesis are comparable between the two species [28, 29]. One primary difference, however, is that the bone marrow becomes a functional site of hematopoiesis much earlier during gestation in humans, with HSCs appearing in the bone marrow beginning in week 10, still in the first half of a 40 week gestational period [29].

1.2.2 Blood Disorders

Benzene exposure has been linked to a variety of hematological disorders. Blood disorders can be divided into those that are characterized by decreased levels of blood cells, or cytopenias, and those that are characterized by increased levels of blood cells. A few different types of cytopenias have been noted following exposure to benzene, namely leukopenia, thrombocytopenia, and anemia (discussed in section 1.3.1). Leukopenia refers to a decrease in
the number of circulating white blood cells, which can result in problems with infection [19]. Thrombocytopenia refers to a decrease in the number of circulating platelets, which can result in increased bleeding [19]. Anemia refers to a decrease in the number of circulating red blood cells [19]. Aplastic anemia is characterized by a dysfunction of the bone marrow, resulting in a broad reduction in numbers of many mature blood cell types, as well as HSCs [19]. Myelodysplastic syndrome presents similarly to aplastic anemia, however it is not accompanied by a reduction in the HSC of the bone marrow, but more so that the replication of HSCs is impaired. A diagnosis of either aplastic anemia or myelodysplastic syndrome is associated with an increased risk of developing leukemia [21].

Disorders characterized by an increased number or over-proliferation of blood cells may be carcinogenic or non-carcinogenic. Myeloproliferative disorders are defined by an excess of myeloid cells in the bone marrow, and often result in leukemia. Leukemia is a type of cancer that is associated with an over-proliferation of immature or mature white blood cells in the bone marrow and/or peripheral blood. Lymphomas, both non-Hodgkin’s (NHL) and Hodgkin’s, are also characterized by an over-proliferation of these cells, but instead occurs in discrete tissues of the lymphatic system. Acute leukemias are aggressive forms of leukemia that show a rapid proliferation of immature blood cells in the bone marrow. Acute leukemias can arise in lymphoid or myeloid cells, with acute lymphocytic leukemia (ALL) being characterized by an abundance of immature B (pre-B) or T (pre-T) cells in the bone marrow. Acute myelogenous leukemia (AML) is an accumulation of immature myeloid cells in the bone marrow. Alternatively, chronic leukemias can also arise in both of these cell lineages, resulting in a diagnosis of chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML), respectively, but both are much less aggressive and more slowly replicating [21]. The distinction between these many
types of hematopoietic cancers is blurred, with lymphomas and leukemias often overlapping in characteristics. Recently, the World Health Organization reclassified lymphomas, placing CLL and multiple myeloma into the family of NHLs due to molecular and pathologic similarities between these diseases [30].

AML and ALL are the most common leukemias diagnosed in children [21]. While childhood leukemias are found in the same cellular subtypes as adult leukemias, the behavior of all cancer in children is often different, such that these cancers grow more rapidly and spread more quickly than what is characteristically observed in adults [31, 32]. Additionally, some childhood cancers have distinct characteristics that are less prevalent in the adult version of the same disease. For example, in infant leukemias (<1 year old), 80% of diagnoses are defined by a genetic abnormality on chromosome band 11q23, which consists of a breakage of the mixed-lineage leukemia (MLL) gene, and subsequent fusion with a number of possible genes [33, 34]. While these abnormalities exist in adult leukemias as well, they are only found in approximately 15% of adult AML and ALL diagnoses combined [35].

1.2.2.1 Statistics on Leukemia

It is estimated that approximately 191300 people were diagnosed with cancer in Canada in 2014 [31]. Specifically, the lifetime probability of developing leukemia in Canada is 1.8% for men and 1.4% for women, accounting for 3.4% and 2.7% of new cancer diagnoses amongst men and women, respectively. This results in an estimated 5900 new cases of leukemia in Canadian adults in 2014 [31]. While CLL is the most commonly diagnosed form of leukemia, AML is the most commonly diagnosed acute leukemia in both Canada and the USA, accounting for 30% of all leukemia diagnoses amongst adults [31, 36]. ALL, CML, and other types of leukemia account for approximately 11-13% of leukemia diagnoses in the same population [36]. There are
numerous risk factors associated with increasing one’s likelihood of acquiring leukemia, including various genetic abnormalities and syndromes, exposure to radiation, as well as exposure to various chemicals, including benzene (to be discussed in more detail in section 1.3.1; reviewed in [32]).

Childhood cancer diagnoses are less frequent than a diagnosis of cancer in adults, with an estimated 4600 new total cancer diagnoses in Canada in 2014 [31]. However, leukemia is the most commonly diagnosed cancer among children, representing 32% of all childhood cancer diagnoses and 26% of deaths by childhood cancer between the years 2005 to 2010. As of 2014, there does not appear to be a substantial difference between the numbers of diagnoses of leukemia in boys and girls in Canada [31], although earlier studies suggested there is a higher frequency of ALL diagnoses in boys [37]. In contrast to adults, ALL is much more commonly diagnosed in this age group compared with AML, accounting for approximately 78% of acute leukemia diagnoses in children [31]. Interestingly, AML accounts for a much bigger proportion of infant leukemias only, with the incidence of AML and ALL being approximately equal in this age group. Since 1985, the incidence of diagnoses of leukemia in children has remained fairly level in Canada [31], with some fluctuating rates within this time period. For example, from 1992 – 1999, there was an increasing trend in incidence of childhood leukemia, followed by a decreasing trend from 1999-2002, followed again by an increasing trend from 2002 – 2006 [38]. However other countries, such as the USA and parts of Europe, have experienced a more steady increase in childhood leukemia incidence by 1% per decade for the past number of decades [39-41]. It is therefore hypothesized that the causes of childhood cancer are not diminishing in our environment [42]. There are a few known risk factors associated with childhood leukemia, including exposure to ionizing radiation [43], and a number of genetic syndromes including
Down’s [44], neurofibromatosis [45], and Fanconi’s anemia [46]. However, these explanations only account for approximately 10% of cases [42]. This reveals the possibility that childhood leukemia could largely be a result of in utero exposure to various environmental toxicants, including benzene [47, 48]. This may be particularly true considering the known association between benzene exposure and adult hematopoietic cancers.

1.3 Benzene-mediated Toxicity and Carcinogenicity

1.3.1 Epidemiological Evidence of Benzene Toxicity and Carcinogenicity

1.3.1.1 Toxicity and Carcinogenicity of Benzene in Adults

Long-term benzene exposure is most well known for its association with AML. However, chronic exposure to benzene has also been long known to be associated with numerous other aforementioned hematological abnormalities. Benzene’s association with cytopenias, including pancytopenia, or aplastic anemia, was noted as early as the late 1800s and since has been confirmed in studies investigating hematological disruptions of individuals occupationally exposed to benzene prior to the establishment of occupational exposure limits [49-51]. Lower level benzene exposures have also been linked to hematological disorders, such as the increased incidence of myelodysplastic disorder observed in individuals exposed to > 3 ppm benzene [52]. Additionally, exposure of individuals to benzene levels ranging from 0.06 ppm to 122 ppm was positively correlated with decreases in various myeloid-derived blood cells [53]. Similarly, another study examined the effects of benzene exposure in workers exposed to <1 ppm benzene and found a decrease in circulating myeloid-derived cells and platelet levels, as well as a dose-dependent decrease in progenitor cell colony formation [12]. As indicated above, benzene exposure has also been associated with more severe cytopenias, such as aplastic anemia. The incidence of aplastic anemia as a result of benzene exposure is dose dependent, with an incidence
of 1 in 100 people with exposure levels of 100 ppm or greater, followed by an incidence of 1 in $10^4$ and 1 in $10^5$ people in those exposed to 10 – 100 ppm and 1 – 10 ppm, respectively [54]. Even in the low exposure group, these rates are increased compared to the baseline incidence rate of aplastic anemia in North America, which is approximately 1 – 2 in $10^6$ people [54].

AML was first identified in shoemakers exposed to benzene-containing adhesives in the late 1970s in Turkey [55, 56], where a case report indicated the development of AML in 3 of 4 shoemakers who previously displayed thrombocytopenia and aplastic anemia [55]. These reports were followed by a more extensive study demonstrating that the incidence of AML in this population was more than double that of the general population [56]. These findings were confirmed in Italy and China also amongst shoemakers [57, 58], and in the United States amongst rubber manufacturers [59]. Exposure levels in each of these environments were very high, in the range of 100 to 650 ppm across these populations [60, 61]. Since the establishment of benzene as a leukemogen in these flagship studies, benzene’s association with AML has received a lot of attention. An increased incidence of AML has been found consistently amongst occupational benzene exposures at levels ranging from 100 to 200 ppm-years [62, 63]. All of these studies suggest that the risk of developing leukemia following benzene exposure would only be increased at levels much higher than what is currently permitted in occupational settings in most developed countries. However, benzene exposure at much lower levels (8 ppm-years or less) has also been associated with increased risk of leukemia as demonstrated in an Australian petroleum industry cohort [64]. Similarly, a 3.2 times greater risk for acute non-lymphoblastic leukemia was seen in China amongst workers exposed to 10 ppm or less of benzene, and that risk jumped to 7.1 times greater in workers exposed to over 25 ppm benzene [13]. In addition to leukemia, an increased risk of myelodysplastic syndrome was noted in petroleum workers exposed to 2.93 ppm-years
and less [52]. It is important to note that 33% of myelodysplastic syndrome diagnoses convert into AML [65]. Taken together, these studies suggest that individuals exposed to much lower levels of benzene than originally found to be harmful may still be at increased risk for hematological disorders, including AML. This may be explained by the finding that benzene metabolism begins to saturate at exposure levels of less than 1 ppm; thus linear extrapolation from higher exposure levels may be an underestimation of risk [66-68]. In contrast, studies investigating incidences of leukemia and other hematological disorders amid gasoline attendants have produced limited evidence of a correlation in this group [69-71]. However, one study did find an increased risk of leukemia amongst gasoline attendants and utilities workers exposed to higher-than-expected levels of benzene for their occupation (ie: >16 ppm compared to the average exposure of <2 ppm) [72]. Thus, benzene exposure still remains a potential occupational hazard.

Non-occupational exposures to benzene were associated with increased incidence of hematopoietic cancer. One study found that car ownership was correlated to increased incidence of not only AML, but also lymphoproliferative disorders [73]. Similarly, increased car density per square kilometer was associated with increased incidence of AML in young adults in a study conducted in Sweden [74]. An increased risk of developing NHL was found in individuals living in close proximity to industrial factories, with those living within 3.2 km of the factory at an increased risk compared to those living further away [75]. This finding was confirmed for other industrial sites that release benzene into the atmosphere and surface water [76].

The link between cigarette smoking and prevalence of leukemia has also been extensively studied; however, there is conflicting evidence as to this association. A number of studies have found an association between cigarette smoking and increased incidence of various types of
leukemia and lymphoma, most notably AML [77-79], CLL [80, 81], CML [81], and NHL [82]. Other studies suggest that there is little evidence for an association between cigarette smoking and the development of both leukemia and NHL [83, 84]. Trying to tackle this discrepancy, more recent studies investigating particular subtypes of AML and NHL have found little association between AML and NHL as a whole and cigarette smoking, whereas they found an increased correlation between particular AML [85] and NHL [86] subtypes and smoking. This suggests that the absence of stratification of hematopoietic cancer by subtype in previous studies may be masking any observed correlations.

As a whole, these data strongly suggest an association between benzene exposure and increased risk for various hematopoietic disorders, most notably leukemia, in adults. In addition to adult leukemia, numerous other studies discussed in the subsequent section have evaluated whether a similar risk is associated with in utero and early childhood benzene exposure and the development of childhood leukemia.

1.3.1.2 Carcinogenicity of Benzene in Children

The link between in utero benzene exposure and increased incidence of childhood cancers is less clear than its association in adults. A number of early studies demonstrated that occupational exposure of the pregnant mother to benzene was associated with an increased risk of both ALL and AML (or other non-lymphocytic acute leukemia) in their offspring [87-89]. Additionally, some studies demonstrated an association between maternal smoking and childhood leukemia, including both AML [90] and ALL [91, 92], as well as lymphoma [91, 92]. However, while a recent meta-analysis found an association between maternal occupational exposure to solvents, paints, and petroleum products and childhood ALL in their children, no association was found between maternal smoking during pregnancy and ALL in these children [93]. Similarly, a
number of other studies have found little association between maternal smoking during pregnancy and infant or childhood AML or ALL [94-97]. Interestingly in one of these studies, maternal exposure to second hand smoke was associated with increased incidence of childhood leukemia [95]. Additionally, a meta-analysis in 2011 demonstrated that maternal smoking was significantly associated with increased incidence of NHL in their offspring [98]. There is a stronger association between paternal preconception smoking and incidence of childhood leukemia [91, 94, 99]. This association also stands for paternal preconception exposure to solvents, paints, and employment in industries associated with motor vehicles [100]. Childhood leukemia and childhood cancer fatalities are associated with higher density traffic exposure and residential exposure to benzene release “hotspots” in many studies [101-106], with the majority of these studies demonstrating a direct exposure of the children to these environmental pollutants. Due to this contradictory evidence in the literature, it remains unclear whether in utero exposure to benzene is associated with increased childhood cancer cases; however, there is a possible association between the two that requires further study. Furthermore, one study found an association between benzene exposure and increased risk of spontaneous abortion amongst pregnant petrochemical workers [107], suggesting there may be an underestimation in the literature as to the extent of effect of in utero benzene exposure on child health.

1.3.2 Toxicity and Carcinogenicity of Benzene Exposure in Animal Models

1.3.2.1 Toxicity and Carcinogenicity in Adult Animal Models

When laboratory animals are exposed to benzene, hematotoxicity, as well as cancers involving other organ systems, have been noted. Exposure of both mice and rats to benzene leads to decreased hematopoietic cellularity, although mice appear to be more sensitive to the hematotoxic effects of benzene [108]. Chronic exposure of mice by inhalation to doses of
benzene greater than 100 ppm results in decreased numbers of bone marrow and progenitor cells, as well as circulating levels of red and white blood cells [109, 110]. In addition to high-dose exposure, one early study demonstrated a reduction in granulocyte precursor cells in mice exposed to benzene doses as low as 0.7 mg/kg for 6 days [111].

In addition to acute hematotoxicity, benzene was first established as a multipotential carcinogen in a study that was conducted in 1976 by Maltoni and his colleagues. In this study, it was shown for the first time that long-term administration of benzene by inhalation or ingestion resulted in tumours in the Zymbal gland, oral and nasal cavities, forestomach, skin, mammary glands, liver, and lungs of Wistar rats, Sprague Dawley rats, Swiss mice, and RF/J mice [112]. Similar studies conducted in the following years confirmed these types of cancer and also found increased occurrences of lymphoma in rodents [113-115]. Additionally, increased incidences of leukemia occur in C57B1/6 mice exposed to 300 ppm benzene for 16 weeks; 5 days per week [116, 117]. Benzene exposure in mice is also a model of benzene-associated aplastic anemia, albeit the doses given to these animals are extremely high at 1940 mg/kg subcutaneous injection, 3 days per week for a total of 20 doses [118].

1.3.2.2 Transplacental Carcinogenicity and Developmental Effects Caused by Benzene in Animal Models

Similar to adult animal models, benzene is both a hematotoxicant and a carcinogen to offspring exposed during in utero development in mice. Exposure of murine fetuses via maternal inhalation of 5, 10, or 20 ppm benzene from GDs 6 to 15 resulted in decreased numbers of circulating erythroid and granulocytic precursor cells in the offspring on post-natal day (PND) 2, which was sustained until 6 weeks of age in the highest dose group [119, 120]. In our laboratory, exposure of CD-1 fetal mice to benzene during gestation via intraperitoneal (IP) injection resulted
in an initial increase in erythroid progenitor cells when measured on GD16, followed by a
significant decrease in levels of erythroid progenitors by PND2 when compared to vehicle-treated
control offspring [121]. Under the same dosing protocol, progeny exposed to benzene during
gestation also had significantly decreased myeloid bone marrow cell (CD11b+) levels one year
after birth [122]. Taken together, it is apparent from these data that fetal mice are also sensitive
to the hematotoxic effects of benzene.

Additionally, benzene was shown by our laboratory to be a transplacental carcinogen in
mice [122]. Exposure of pregnant CD-1 mouse dams, but not C57Bl/6 dams, to 200 mg/kg
benzene on GDs 8, 10, 12, and 14 resulted in increased hematopoietic and hepatic tumours in
female and male offspring, respectively, one year after birth. Increased tumourigenesis was not
observed at the higher dose of 400 mg/kg benzene on GDs 8, 10, 12, and 14 in either strain.
Correspondingly, maternal benzene levels in C57Bl/6 mice were lower than those in CD-1 dams,
and it was proposed that the susceptibility to benzene carcinogenesis in CD-1 offspring may be
due to strain differences in fetal metabolism, as different benzene metabolites were found at
higher concentrations in fetal livers of each strain. Liver tumours found in male offspring were
primarily adenomas, as well as focal nodular hyperplasias and carcinomas were seen. In females,
tumours consisted mainly of hyperplasias, myeloproliferative disorders, and myeloid/lymphoid
neoplasias [122].

In addition to investigating the hematotoxic and carcinogenic effects of benzene
following in utero exposure, the teratogenic effects of gestational benzene exposure has also been
studied. In rats, maternal exposure to 2200 ppm benzene from GD 6 to 15 via inhalation was
found to be associated with decreased fetal weights and crown-rump lengths, as well as increased
number of fetuses with delayed ossification [123]. Similarly, skeletal abnormalities and
decreased body weights were detected in GD20 rats gestationally exposed to 500 ppm benzene via maternal inhalation [124]. In other laboratory animals, maternal exposure to 308 ppm benzene throughout the same gestational time period also led to delayed ossification in mouse fetuses as well as increased spontaneous abortions in rabbits [125]. However, in all three of these studies, benzene exposure had an impact on maternal weight gain during pregnancy [123-125], suggesting that the developmental effects of benzene in these studies may have been confounded by maternal toxicity. Correspondingly, studies that exposed pregnant dams to levels of benzene that did not produce maternal toxicity in rodents did not demonstrate any negative effects of in utero benzene exposure on fetal weight or ossification [126]. Interestingly, one study investigating the effects of maternal benzene exposure on fetal neurodevelopment found that a one-time dose of 0.1 mg/kg benzene via subcutaneous injection on GD15 resulted in long-lasting alterations of cognition and motor behavior in rats [127], suggesting that the developmental effects of benzene may be more subtle than what traditional teratology studies uncover.

1.4 Benzene Metabolism

1.4.1 Introduction to Benzene Metabolism

Benzene rapidly distributes throughout the body following inhalation exposure in rats, with higher concentrations found in fat and bone marrow than in plasma, suggesting that benzene may accumulate in these two tissues [128]. In both rodents and humans, benzene elimination follows a two-compartment model. In rats, the first half-life is 45 minutes and in humans it is 1 hour, whereas the second half-life is 13 hours in rats and 24 hours in humans [128, 129]. Benzene is also known to be able to cross the placenta and has been found in cord blood, with concentrations close to or exceeding the concentrations found in maternal blood [130].
Benzene’s toxicity and carcinogenicity is unequivocally dependent on its metabolism, which can be seen schematically in Figure 1.2. Benzene undergoes extensive metabolism in the body of both humans and rodents, producing a number of metabolites with varying levels of reactivity and toxicity. In the liver, benzene is hydroxylated primarily by cytochrome P450 (CYP) 2E1 to produce benzene oxide, which exists in equilibrium with benzene oxepin [131]. While benzene can be metabolized by other CYP isoforms, CYP2E1 had a ten times lower \( K_M \) value for benzene than other CYPs, suggesting it is the most likely pathway for benzene’s initial oxidation \textit{in vivo} [132]. In the presence of epoxide hydrolase, benzene oxide can be converted to benzene dihydrodiol, which can be further oxidized to form catechol. From catechol, 1,2-benzoquinone can be produced through metabolism by myeloperoxidase (MPO). Non-enzymatically, benzene oxide can also convert into phenol, which can be oxidized to form hydroquinone first, and then 1,2,4-trihydroxybenzene. Alternatively, hydroquinone can be metabolized by MPO to form 1,4-benzoquinone. Oxepin, on the other hand, can be metabolized by CYP enzymes to produce \( t,t \)-muconaldehyde which can is converted into the readily excretable \( t,t \)-muconic acid, again by oxidation.

Benzene metabolites are also metabolized by a number of other enzymes, mainly contributing to its detoxification. In the presence of glutathione-s-transferase (GST), benzene oxide can be converted to S-phenylmercapturic acid (S-PMA), an easily excreted, less toxic metabolite of benzene [133]. Evidence has also suggested that glutathione (GSH) conjugates of benzene can lead to the production of at least one reactive metabolite, 2,3,5-tris(glutathion-S-yl)hydroquinone, which has been shown to cause erythrotoxicity in the bone marrow of both
Figure 1.2: An overview of benzene metabolism.

CYP: cytochrome P450; GST: glutathione S-transferase, MPO: myeloperoxidase; NQO1: NAD(P)H quinone oxidoreductase 1; t,t: trans, trans. Enzymatic reactions are written in red font and structures are written in black font. Adapted from [134].
mice and rats [135]. Both 1,2- and 1,4-benzoquinone can be converted back to catechol and hydroquinone, respectively, by the enzyme NAD(P)H quinone oxidoreductase 1 (NQO1), both of which are more easily conjugated to form less toxic metabolites [136]. Additionally, phenol, catechol, hydroquinone, and 1,2,4-trihydroxybenzene can be conjugated by sulfotransferases (SULT) and UDP-glucuronosyltransferases (UGT) to form sulfate and glucuronic acid conjugates, respectively, ultimately detoxifying benzene and allowing it to be eliminated from the body [137, 138].

The location of benzene’s metabolizing enzymes is also thought to contribute to its toxicity. CYP2E1 is primarily found in the liver, and its location within the liver is concentrated mainly to the hepatic triad area close to the central vein [138]. Because of its location, one hypothesis proposes that benzene is hydroxylated on its way out of the liver and into systemic circulation, suggesting that benzene may bypass conjugation into less toxic metabolites in the liver and is instead bioactivated just before exiting the liver, sending toxic metabolites to the bone marrow [138]. CYP2E1 is also appreciably expressed in bone marrow of mice, rabbits, and humans, although not to the same extent as its expression in liver [139]. Additionally, mature and progenitor myeloid cells in the bone marrow possess high MPO activity levels, contributing to the toxification of benzene, primarily by catalyzing the conversion of hydroquinone to 1,4-benzoquinone [140], which is thought to be one of the most reactive metabolites of benzene (discussed in sections 1.4.3 – 1.4.5). Therefore, it is hypothesized that bone marrow is the target of benzene toxicity in humans at least partially due to this enzymatic location.

The elucidation of benzene’s metabolism was conducted in adult animal models. However, the interest of our laboratory is the carcinogenicity of benzene in offspring exposed in
uterine. Therefore, a discussion of differences in crucial drug metabolizing enzymes during pregnancy, as well as the extent of fetal metabolic capabilities, is warranted.

1.4.2 Maternal and Fetal Expression of Drug Metabolizing Enzymes During Pregnancy

Pregnancy brings about numerous physiological and biochemical changes to the female body so that it can adapt to the needs of the developing fetus. In addition to changes such as increased blood volume and glomerular filtration rates that alter the pharmacokinetics of xenobiotics during pregnancy, maternal expression levels of numerous xenobiotic-metabolizing enzymes exhibit altered activity during pregnancy. Some of these alterations include increased activity of CYP2A6, 2C9, 2D6, and 3A4, as well as decreased activity of CYP1A2 and 2C19 (reviewed in [141]). Additionally, as the placenta develops, numerous CYPs are expressed in this transient organ at varying times during gestation [142].

To make this discussion more succinct, the focus will be on enzymes known to be critically involved in the metabolism of benzene. CYP2E1 protein expression in the liver of pregnant rats is similar to non-pregnant rats until mid-gestation, following which decreased expression is observed during late pregnancy [143]. While it does not appear that protein levels of CYP2E1 were investigated in maternal liver during pregnancy in humans, CYP2E1 mRNA and protein levels were detected in human placenta throughout gestation, however efforts to confirm enzyme activity in these studies was not successful [142, 144, 145]. Numerous Phase II metabolizing enzymes are also expressed in the human placenta. Various isoforms of UGT have increased activity levels during pregnancy (reviewed in [141]), as well as have mRNA expression and, to some extent, activity in the human placenta [146-148]. Most isoforms of GST are also expressed in the placenta, although GSTP1 is the most highly expressed in both maternal and fetal interfaces [149]. SULT isoforms are also expressed in the placenta throughout gestation,
although it is suggested that their primary function is to conjugate endogenous compounds, such as steroid hormones [150]. To the best of my knowledge, the expression of these enzymes in the maternal liver during pregnancy, as well as the expression of NQO1 in either the placenta or maternal liver have not been studied. As a whole, these shifts in expression levels of pertinent enzymes in the maternal liver, coupled with the potential for placental metabolism of benzene may ultimately alter the metabolism of benzene in the body and consequently could have an effect on benzene’s transplacental toxicity.

Additionally, the expression and activities of various enzymes in the fetal liver could also further alter the metabolism of benzene and the extent of fetal toxicity. Expression levels of CYP enzymes have been fairly extensively studied in the human embryo and fetus, with numerous reviews being published on the subject [151-154]. In humans, CYP3A7 is the most highly expressed CYP in the embryonic, fetal, and neonatal liver, whereas it has fairly low expression levels in adult liver [155, 156]. CYP3A4, 2E1, 2D6, and 2C all have low fetal expression and activity, with increasing expression late in gestation, during infancy, and peaking in adult life for the majority of these enzymes (reviewed in [151]). Large variability exists in expression levels for many of these CYP isoforms at all stages of human development, including adulthood. For example, CYP2E1 activity levels in neonates can vary up to 80-fold [151], which has the potential to have substantial consequences on metabolism of toxicants, including benzene. Despite this variability, CYP2E1 was found to be the most abundant CYP in late-term human fetal livers [157].

Relevant to benzene’s metabolism, numerous Phase II metabolizing enzymes show differential expression patterns throughout human development as well. Certain SULT isoforms have been demonstrated to be fairly active during fetal development, including SULT2A1 [158],
1A3, and 1A1 [159]. Protein expression levels of these enzymes not only exist in the developing liver, but also in endocrine organs, highlighting the importance of these enzymes in hormonal regulation during this stage of development. SULTs are some of the most highly expressed Phase II metabolizing enzymes in the developing fetus, suggesting that they may play an important role in xenobiotic detoxification at this stage of development [151]. Another relatively highly expressed enzyme in the fetal liver is epoxide hydrolase, although activity levels in the fetal liver are still 30 – 40% that of the adult liver [160, 161]. All other Phase II metabolizing enzymes are expressed at much lower levels in comparison to epoxide hydrolase and SULTs. GST activity is detectable in human embryonic livers early in gestation, with different isoforms showing different patterns of activity. For example, GSTA and GSTM are detected at very low levels in the fetal liver, reaching adult protein expression levels around 2 years after birth. Conversely, GSTP is detected at its highest levels during the first trimester; with decreasing protein expression levels throughout the second and third trimesters, and is hardly detectable in the adult liver [162, 163]. Similarly, while there are many isoforms of UGTs, activity levels and expression patterns of these enzymes appear to be isoform and substrate specific [151]. Generally, however, UGT activity becomes measurable late in the second or early in the third trimester at levels 80% less than adult activity levels [152]. NQO1 fetal mRNA expression and activity is less studied than other drug metabolizing enzymes, however it does appear to be expressed in the fetal liver, whereas its expression in adult human liver is difficult to detect [136, 164].

In the mouse, a study investigating expression of 10 different CYP orthologs on GDs 7, 11, 15, and 17 found that all but CYP1A2 exhibited specific patterns of mRNA expression during development [157]. For example, CYP2S1 was expressed throughout murine gestation, whereas CYP2R1 and CYP1A1 were only expressed on GD7, and CYP2E1 was only expressed on GD17
Studies have also demonstrated that SULT mRNA expression is fairly high during murine fetal development, and dependent on the isoform [165]. SULT2A1/2 and 1A3 had the highest expression in the adult liver, and although detected in the fetal liver, the expression levels were very low until after birth, after which activity levels rose in adulthood for all except SULT1A3 in male mice [165]. Only SULT1C1 had higher expression during fetal development than after birth [165]. Similar to humans, overall expression and activity levels of both GST and UGT are low early in gestation and increase towards the end of gestation, and are isoform dependent in the mouse [166, 167].

Benzene metabolism is extensive, and potentially even more so with the changing enzyme disposition that occurs during pregnancy. With the study of benzene’s metabolism, it has also become evident that this process is very important to the toxicity and carcinogenicity exerted by benzene.

1.4.3 Importance of Benzene Metabolism to Its Toxicity

Two studies conducted in the 1970s highlighted the importance of benzene’s metabolism in its toxicity, stimulating a large body of research that is still active; to elucidate the full metabolism of benzene and its corresponding toxicity. One of these studies, conducted by Andrews and colleagues, demonstrated that co-treatment of mice with toluene and benzene resulted in decreased levels of benzene metabolites in bone marrow and urine, as well as decreased hematotoxicity, both effects due to toluene competitively inhibiting CYP metabolism of benzene [168]. Using a different approach, Sammett and colleagues demonstrated that benzene metabolism and hematotoxicity were decreased in rats following a partial hepatectomy [169], emphasizing the importance of liver biotransformation in the toxification of benzene. Similarly, cytopenias induced by inhaled benzene in C57Bl/6 mice were exacerbated by co-
administration with ingested ethanol, due to ethanol’s induction of CYP2E1 activity [170]. Correspondingly, mice that lack the Cyp2e1 gene experienced greatly reduced cytotoxicity and genotoxicity of hematopoietic cells and organs compared to wildtype mice [171].

Polymorphisms of other key enzymes have also been studied to determine whether metabolism and/or toxicity of benzene are altered with different polymorphisms. Studies investigating various polymorphisms of NQO1, MPO, and CYP2E1 in humans, and their consequences on excreted metabolites and/or hematotoxicity or genotoxicity of benzene have either seen an effect [12, 172, 173] or have seen no effect [173-175]. In a meta-analysis investigating the effects of numerous polymorphisms on benzene’s toxicity, the only genes that demonstrated consistent alterations in benzene metabolism and/or measures of toxicity were GSTM1 and GSTT1 [173]. In general, null GSTT1 and GSTM1 phenotypes resulted in a reduction in the production of the urinary benzene metabolite, S-PMA [172, 176, 177], as well as an increase in chromosomal aberrations [178], although not every study identified an association [173]. The authors of this study emphasize the importance of measuring many polymorphisms together in one study and in each subject to identify the cumulative effect of an individual’s polymorphisms in all relevant genes on benzene’s metabolism and toxicity [173].

The metabolism of benzene via various enzymes in different tissues within the body results in the production of reactive metabolites that lead to benzene-mediated hematotoxicity and carcinogenicity. However, the mechanisms of how benzene metabolites lead to these endpoints are not yet fully elucidated. The mechanisms involved in benzene’s toxicity are proposed to involve the generation of reactive oxygen species (ROS), genotoxicity, and alterations in the epigenome. These potential mechanisms of toxicity will be discussed in the following section.
1.5 Proposed Mechanisms of Benzene-Mediated Toxicity

1.5.1 Benzene and Reactive Oxygen Species

One of the most consistent findings when investigating benzene’s toxicity is the increased levels of cellular ROS following benzene exposure. ROS are molecules containing oxygen that are in a more reactive state than molecular oxygen, meaning that they have been reduced to some extent [179]. Common ROS include the superoxide radical anion (\( \text{O}_2^- \)), the hydroxyl radical (\( \text{OH}^- \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), nitric oxide (\( \text{NO}^- \)), and hypochlorous acid (\( \text{HOCl} \)), all of which are produced as a consequence of normal cell functioning [179]. Some of these ROS have specific roles in the cell, for example, \( \text{H}_2\text{O}_2 \) functions as a signalling molecule, whereas other ROS appear to only be detrimental, particularly if produced in excess [179]. There are a number of endogenous pathways that are involved in ROS detoxification, including enzymes such as superoxide dismutase (SOD) and catalase, which detoxify the superoxide radical anion and \( \text{H}_2\text{O}_2 \), respectively. Additionally, there are dietary components that may act either directly or indirectly as antioxidants, including \( \beta \)-carotene, vitamin E, vitamin C, and more newly identified compounds, such as sulforaphane (discussed further in section 1.6).

While ROS production is a normal consequence of cell functioning, uncontrolled levels of ROS have the potential to lead to oxidative damage [180, 181]. Types of damage incurred by increased ROS levels include: lipid peroxidation, protein oxidation, oxidative DNA damage, altered cell signalling, and altered epigenetics. Lipid peroxidation is a type of oxidative macromolecular damage, which may affect the structure of either lipid membranes or lipid proteins. Proteins can also be oxidized, which can affect protein structure or enzyme functioning. ROS can also result in a number of different oxidative DNA lesions, which may be mutagenic if not repaired (discussed in section 1.5.2). When unrepaired, oxidative DNA damage can contribute
to the initiation of cancer [182]. Additionally, increased levels of ROS may alter and activate various signalling pathways, the most well-characterized of which are nuclear factor erythroid 2-related factor 2 (NRF2) [183], mitogen-activated protein (MAP) kinase/activator protein (AP-1) [184], nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) [185], and hypoxia induced transcription factor 1α (HIF-1) [186]. Signalling molecules involved in each of these pathways have been shown to have aberrant expression in carcinogenesis, suggesting another link between increased ROS and cancer. ROS has also been linked to epigenetic changes. Explained in more detail in section 1.5.3, DNA methylation is a type of epigenetic mark that is typically at lower genome-wide levels in cancer [187]. Increased levels of ROS have been associated with changes in patterns of DNA methylation by, for example, altering activity levels of enzymes involved in methylating DNA [188]. Alternatively, oxidative DNA damage can inhibit the ability of DNA methyltransferase (DNMT) to interact with DNA, indirectly reducing methylation levels (discussed in more detail in section 1.5.4) [189].

Therefore, there are a number of ways in which ROS are damaging to various cellular components, which may ultimately lead to cancer. Exposure to benzene and/or benzene metabolites has repeatedly been demonstrated to lead to increased ROS production in numerous cell types and animal models. Benzoquinone, 1,2,4-trihydroxybenzene, phenol, and t,t-muconaldehyde were all shown, with decreasing potency, to lead to an increase in ROS levels in exposed human lymphoblast (HL-60) cells, which was abolished by pretreatment with catalase, among other antioxidants [190]. Similarly, exposure of chicken erythroblast (HD3) cells to benzoquinone or hydroquinone led to a significant increase in ROS levels, as measured by levels of 2,7-dichlorodihydrofluorescein (DCF) fluorescence following exposure to 5,6-chloromethyl 2,7-dichlorodihydrofluorescein diacetate (CM-DCFDA) [121, 191, 192]. Benzoquinone was also
demonstrated to rapidly lead to an increase in ROS production in cultured primary GD14 C57Bl/6 fetal liver cells, with increased DCF fluorescence detected after 15 minutes of benzoquinone exposure [193]. Our laboratory has also detected increased ROS production in exposed fetuses following *in utero* exposure to benzene. Following exposure to benzene throughout gestation, GD14 C57Bl/6 and CD-1 mouse fetuses had significantly higher DCF fluorescence in their livers than did vehicle-exposed control fetuses [194, 195]. Similarly, exposure of pregnant CD-1 mice to benzene during gestation resulted in decreased reduced to oxidized glutathione ratio (GSH:GSSG), which is a sensitive marker of cellular redox status [196]. The importance of ROS production in benzene-mediated toxicity was demonstrated in mice that overexpress human thioredoxin, an antioxidative enzyme. These mice demonstrated reduced chromosomal damage as well as decreased development of thymic lymphoma following benzene exposure [197]. Additionally, benzene exposure has also been linked to oxidative DNA damage, which will be discussed further in section 1.5.2.

The full mechanism by which benzene produces ROS remains to be determined, but there are a number of proposed hypotheses (Figure 1.3). While it was once assumed that ROS production by benzene occurred via redox cycling of 1,4-benzoquinone to hydroquinone, it has since been determined that this cycling is inhibited at physiological pHs and only possible at pHs close to or above the $\text{PK}_a$ of hydroquinone, which is 9.85 [198]. Alternatively, one hypothesis suggests that the production of ROS by benzene may be iron dependent. Benzene metabolites, namely hydroquinone, catechol, and 1,2,4-benzenetriol have been demonstrated to induce the release of iron from ferritin [199]. Release of iron from ferritin makes it available to catalyze the generation of ROS via the Fenton reaction [200], thus iron dysregulation could lead to an increase
Figure 1.3: Proposed mechanisms by which benzene is thought to produce reactive oxygen species (ROS).

A: Benzene results in an increased release of iron (Fe) from ferritin, resulting in iron dysregulation and an increase in ROS production via cycling of the Haber-Weiss Reaction and the Fenton Reaction. B: Benzene metabolite, phenol, is metabolized by myeloperoxidase (MPO) to produce a phenoxy radical, which reacts with glutathione (GSH) to produce a thyl radical (GS•). Reactions of the thyl radical with GSH and oxygen (O₂) produces superoxide radical anions (O₂•⁻).

C: Benzene metabolite, 1,4-benzoquinone, reacts with hydrogen peroxide (H₂O₂) to produce 2,3-epoxy-p-benzoquinone. This molecule is conjugated by GSH to produce 1,2,4-trihydroxy-5-glutathionyl-benzene (TGB). TGB auto-oxidizes to form a hydroxysemiquinone radical and superoxide radical anion. Adapted from [17].
in cellular ROS (Figure 1.3A). The other two hypotheses involve GSH. The first hypothesis states that MPO catalyzes a reaction that converts phenol into a phenoxy radical, which is then reduced by GSH to produce a thyl radical (GS’), restoring phenol. Meanwhile, the thyl radical interacts with another thiol molecule to produce a disulfide and a superoxide anion (Figure 1.3B) [201]. The second hypothesis involves the interaction of 1,4-benzoquinone and H$_2$O$_2$ to produce 2,3-epoxy-p-benzoquinone. After conjugation with GSH, this results in the formation of 1,2,4-trihydroxy-5-glutathionyl benzene (TGB). This molecule can auto-oxidize in the presence of a flavoprotein to form a hydroxysemiquinone radical and superoxide radical anion. The hydroxysemiquinone can then cycle back to produce the original molecule, TGB (Figure 1.3C) [202, 203].

Regardless of the mechanism of ROS production by benzene, it is evident that increased ROS levels are a consequence of benzene exposure and that there is, therefore, the potential for macromolecular damage including DNA damage.

1.5.2 Benzene-Mediated Genotoxicity and Chromosomal Aberrations

Several studies have shown that exposure to benzene results in an increase in oxidative DNA damage, as measured by increased levels of 8-hydroxy-2’-deoxyguanosine (8-OHdG), which is an oxidized derivative of deoxyguanosine and an effective measure of oxidized DNA. *In vitro*, exposure of HL-60 cells and cultured primary lymphocytes to 1,2,4-benzenetriol led to an increase in levels of 8-OHdG [204]. Increased levels of oxidative DNA damage has also been detected in the bone marrow of mice following exposure to benzene *in vivo* [205, 206]. Oxidative DNA damage has also been measured following *in utero* exposure in animal models, where exposure of pregnant mice to environmental cigarette smoke resulted in increased levels of 8-OHdG in fetal livers [207]. However, in a study using cultured GD14 fetal liver cells extracted
from C57Bl/6 mice, no increase in 8-OHdG was seen following exposure to the benzene metabolite benzoquinone [193]. Oxidative DNA damage, when unrepaired, can be mutagenic and therefore, carcinogenic, primarily leading to GC to TA transversions [208]. In humans, urinary 8-OHdG levels were investigated in gas station attendants and a significant correlation between urinary 8-OHdG concentrations and benzene levels was found [209].

Exposure to benzene or its metabolites has also been associated with other types of DNA damage. A common and particularly lethal type of DNA damage is a DNA double stranded break (DSB), which is often measured by immunodetection of phosphorylated H2AX (γH2AX); an early indicator of DNA damage. In vitro, exposure of DNA fragments (cell-free system), HL-60 cells, mouse bone marrow cells, or human lymphocytes to various metabolites of benzene led to an increase in DNA single and/or double strand breaks [193, 210-214]. Additionally, exposure of mice to benzene via IP injection led to increased micronucleus formation in bone marrow of adult males [215]. In the same study, in utero exposure to benzene daily from GD 7 to 15 resulted in increased micronucleus formation in the livers of GD16 fetuses and in the bone marrow of PND9 pups; however, no changes in γH2AX levels were detected [215]. In many of these experiments, treatment with an antioxidant or antioxidative enzyme prevented the incurred damage. For example, the increase in γH2AX in HL-60 cells following exposure to benzoquinone and hydroquinone was attenuated by treatment with the antioxidant N-acetylcysteine (NAC) [211]. Similarly, DNA breaks induced by benzoquinone and 1,2,4-trihydroxybenzene were inhibited by GSH pretreatment, but not by treatment with catalase [214]. Contradictorily, NAC pretreatment did not protect against the DNA damage observed in fetal livers following in utero exposure to environmental cigarette smoke [207].
Benzene metabolites also cause DNA adducts. For example, there are four major adducts possible following benzoquinone exposure, and one following hydroquinone exposure [216]. The benzoquinone adducts, in particular, have been demonstrated to be highly mutagenic in both a cell-free environment [217], as well as in both mouse and human cell lines [218]. Interestingly, benzoquinone-induced mutations occurred at similar frequencies in these mouse and human cells, however, induced different types of mutations. Specifically, while there were fewer tandem mutations in humans, there was an increased frequency of GC to CG transversions compared to mouse cells [218]. The ultimate relevance of these differences, however, remains unknown.

Benzene exposure is also associated with chromosomal aberrations, instability and translocations when investigated in humans. Low-level benzene exposure was correlated to various chromosomal aberrations, namely chromatid deletions and gaps, in peripheral blood lymphocytes of individuals occupationally exposed to benzene [219]. Benzene exposure at low levels has also been associated with increased incidences of monosomy and hyperdiploidy of chromosomes 8 and 21, as well as increased translocations between the two [215, 216]. Gene polymorphisms in certain Phase II metabolizing enzymes known to be associated with benzene metabolism, including GSTT1 and GSTM1 null phenotypes, were also associated with increased chromosomal aberrations in this study [178]. The benzene metabolites, hydroquinone and benzoquinone, were also shown to induce monosomy and long arm deletions of chromosomes 5 and 7 in cultured human lymphocytes [220], which have similarly been shown to be prevalent in certain types of hematopoietic cancers [221, 222].

Benzene exposure is therefore associated with various types of DNA and chromosomal damage. However, the cell is equipped with numerous mechanisms to repair this damage as discussed in the next section.
1.5.3 DNA Repair and Benzene

Depending on the type of DNA damage, different repair pathways are initiated to restore the integrity of the DNA. The three most pertinent repair pathways pertaining to the research conducted in this thesis, as well as the types of damage mentioned above, are base excision repair (BER), homologous recombination (HR), and non-homologous end-joining (NHEJ), all of which are summarized in Figure 1.4.

BER is the primary pathway for repair of oxidative DNA damage, including 8-OHdG. The goal of this pathway is to remove the damaged base, and in the case of 8-OHdG, replace it with a non-oxidized guanine nucleotide. In BER, glycosylases such as 8-oxoguanine DNA glycosylase (OGG1) removes the oxidized guanine, producing an apurinic site. This site is then repaired by a sequence of enzymatic actions, including but not limited to those executed by AP endonuclease, DNA polymerase, and DNA ligase, which cleave the AP site, insert nucleotides into the empty site, and attach the newly repaired DNA to the rest of the strand, respectively (reviewed in [223]). Deficiencies in the BER pathway have been found to be associated with cancer. For example, mice that are deficient in OGG1 and mutY homolog (MYH), a protein involved in preventing 8-OHdG incorporation into DNA, have increased incidence of lung cancer, ovarian cancer, and lymphoma [224]. To-date, few studies have investigated the role of BER in benzene-mediated toxicity.

HR and NHEJ are the two primary pathways by which DSBs are repaired. While the goals of these two pathways are the same, the steps involved in the repair of DSBs are quite different. HR is characterized by repairing a break in both strands of the DNA by using the undamaged sister chromatid as a template for the damaged region of DNA. NHEJ, on the other hand, is characterized by direct ligation of the broken ends of DNA back together without the
Figure 1.4: An overview of the DNA repair pathways, base excision repair (BER), non-homologous end-joining (NHEJ), and homologous recombination (HR).

A: BER is the primary mechanism by which oxidative DNA damage, induced by reactive oxygen species (ROS), is repaired. 8-oxoguanine glycosylase (OGG1) plays an important role in identifying and removing the oxidized base (O), initiating repair. Otherwise, a mutation may result. B: NHEJ is one pathway by which double stranded breaks (DSBs) are repaired. This pathway involves end-processing of broken DNA ends by Artemis and ligation of broken ends by DNA ligase, which is facilitated by X-ray repair cross-complementing protein 4 (XRCC4), resulting in repaired DNA. C: HR is another pathway by which DSBs are repaired. This pathway involves end-processing by RAD proteins, and the use of the sister chromatid as a template for DNA repair. Adapted from [223, 225, 226].
need for a homologous template. Some of the main enzymes involved in catalyzing this pathway include DNA-dependent protein Kinase (DNA-PK), X-ray repair cross-complementing protein 4 (XRCC4), Artemis, and DNA ligase [227, 228]. Briefly, in NHEJ, DNA-PK phosphorylates Artemis, which allows for DNA strands to be processed at the location of the break. This processing allows DNA to be repaired by DNA ligase, which is facilitated by XRCC4 [229].

Typically, NHEJ is said to be an “error-prone” DNA repair pathway, compared to the relatively “error-free” HR pathway, although both are capable of producing mutations as a consequence of their repair methods. HR is the primary mechanism of repairing DSBs that occur at replication forks, whereas either pathway can repair most other DSBs. Because of the requirement of a homologous template for HR, HR is traditionally thought to be limited to the S/G2 phase of the cell cycle, making NHEJ the likely candidate for the predominant repair pathway for DSBs in eukaryotic cells [230]. However, recent research suggests that there may be overlap in the mechanisms and proteins involved in these two pathways [231]. Additionally, the dominant form of DSB repair during fetal development changes over time, with HR being the dominant form in early embryos, which is replaced by NHEJ beginning on GD14 [232].

Like BER, altered activity of either HR or NHEJ has been associated with various cancers. For example, a meta-analysis conducted in 2012 found a significant correlation between single nucleotide polymorphisms (SNPs) of XRCC4 and cancer risk in humans, including prostate, lung, and bladder cancers [233]. Whereas mice deficient in critical NHEJ constituents have increased chromosomal instability and increased incidence of lymphoma [234], interestingly, myeloid leukemia cells have also been found to have increased NHEJ activity accompanied by increased chromosomal instability [235]. These seemingly contradictory studies may suggest that while lack of NHEJ ability can lead to tumourigenesis, increases in erroneous
repair conducted by NHEJ may be as detrimental. Additionally, it is well characterized that silencing mutations of the genes of the DNA repair proteins BRCA1 and BRCA2 are associated with primarily breast and ovarian cancers [236]. BRCA1 and BRCA2 are tumour suppressor genes involved in DSB repair. The BRCA1 protein is quickly phosphorylated upon detection of a DSB, and relocates to the region of the break, and together, BRCA1 and BRCA2 play a role in maintaining genomic stability by complexing together to initiate DNA repair [237].

Though not yet thoroughly investigated, there are suggestions that benzene exposure may affect DNA repair pathways. For example, in our laboratory, benzene metabolite exposure has led to an increase in recombination events in both Chinese Hamster Ovary (CHO) cells as well as PKZ1 transgenic mouse GD14 fetal liver cells, both of which contain reporter constructs that are used as surrogate measures of HR and NHEJ, respectively. In each of these studies, increases in activity of DNA repair pathways were ameliorated by pretreatment with catalase [193, 238]. Whether or not benzene had a direct effect on these pathways, or whether the catalase treatment reduced benzene-induced DNA damage thus indirectly preventing the observed increase in DNA repair remains to be determined. Additionally, benzoquinone exposure has been found to significantly reduce the expression of Parp-1 in mouse bone marrow cells [239]. PARP-1 is an enzyme involved in numerous pathways in the cell. In the context of DNA repair, however, it binds to the damage site, modifying a variety of proteins involved in DNA repair pathways [240]. Lastly, SNPs of a number of different DNA repair genes were found to be associated with hematotoxicity in a population of people occupationally exposed to benzene, as measured by reduction in white blood cell counts [241, 242]. Therefore, together these data suggest that investigating the influence of benzene exposure on DNA repair and vice versa may be fruitful in uncovering the mechanisms of benzene-mediated transplacental carcinogenicity.
1.5.4 Benzene and Epigenetic Changes

In recent decades, it has become more apparent that epigenetic alterations can be associated with certain xenobiotic exposures. Epigenetic modifications are the heritable changes to DNA that can occur that do not modify the actual gene sequence. DNA methylation and histone modifications, primarily methylation and acetylation, are the most widely studied types of epigenetic modifications that may happen as a result of xenobiotic exposure. The best-characterized modification is DNA methylation that occurs at CpG islands, which are stretches of DNA in the promoter region of genes that have an increased abundance of CpG dinucleotides. CpG island methylation plays an important role in the regulation of gene expression in both normal and transformed cells, however these DNA methylation patterns may become aberrant in cancer cells [243]. Global DNA hypomethylation, as well as gene-specific hypo- and hypermethylation, are associated with numerous types of cancer, including some forms of leukemia [244-246]. DNA hypomethylation is generally associated with increased gene expression via increased transcription, whereas hypermethylation is generally associated with transcriptional repression [247]. Methylation of DNA can also occur at non-CpG islands; however, the function of this modification is not well characterized [248].

Several types of histone modifications are also associated with transcriptional activation or repression, depending on the modification, and these two types of epigenetic modifications are linked. Methylated CpG islands of DNA can interact with histone modifying enzymes, such as histone deacetylases, which are enzymes responsible for removing acetyl groups from histone residues [249, 250]. As a consequence, increased histone acetylation is associated with increased transcription, and deacetylation is associated with decreased transcription. Histones may also be modified by methylation at specific residues, which correspond to either transcriptional activation
or repression, depending on the alteration [251]. Histones may also undergo a variety of other modification types including phosphorylation, ubiquitination, and ADP-ribosylation, however these modifications are not as well characterized (reviewed in [252]).

While numerous differences in epigenetic modifications have been noted in various types of cancer compared to normal tissue, it is unclear as to whether these modifications are involved in initiating carcinogenesis or whether they are a consequence of carcinogenesis. An increasing number of carcinogens have been shown to result in epigenetic alterations, however, suggesting that these modifications may play a role in cancer initiation. For example, tobacco smoke has been shown to result in DNA hypomethylation, gene-specific promoter DNA hypermethylation, and alterations in acetylation and methylation of histones [253]. Similarly, benzene or benzene metabolite exposure has recently been demonstrated to be associated with genome-wide DNA hypomethylation in cultured human lymphoblast and liver cell lines, as well as in the blood of exposed subjects [254-256]. Benzene exposure was also associated with gene specific DNA hypermethylation of the tumour suppressor gene, \( P15 \), and hypomethylation of \( MAGE1 \), a gene encoding a tumour-specific cell antigen of unknown function [257], in the blood of these same individuals [254]. While not yet extensively studied, these studies suggest that alterations in DNA methylation patterns may be associated with benzene exposure, which begs the question of whether histone modifications are also altered. To-date, neither type of modification has been studied following \textit{in utero} exposure to benzene.

1.6 Sulforaphane as a Potential Protective Agent

Sulforaphane (1-isothiocyanate-(4R)-(methylsulfinyl)butane; SFN; structure in Figure 1.5) is a phytochemical classified as an isothiocyanate, extracted from cruciferous vegetables such as broccoli. Phytochemicals are the non-nutritive compounds in plants that possess anti-
mutagenic and anti-carcinogenic activity, and therefore interest in these compounds, including SFN, has been increasing in recent years [258]. SFN is passively absorbed from the gastrointestinal tract and undergoes metabolism very quickly. SFN appears to be metabolized primarily via the mercapturic acid pathway, initially forming a GSH conjugate. A number of other modifications of this conjugate can arise via various enzymatic reactions, including the formation of SFN-NAC, which is the primary metabolite detected in urine [259]. SFN, its GSH conjugate, and SFN-NAC all have potential anti-cancer activity [260-262]. SFN is rapidly eliminated from the body of both rodents and humans, with SFN and its metabolite levels being virtually undetectable after 24 hours [259, 263, 264]. SFN metabolites distribute widely in a dose-dependent manner to all major tissue types in mice [265], and have been measured in the neonatal plasma of mice exposed to SFN in utero [266].

SFN has been demonstrated in numerous studies to protect against the development of cancer in animal models. SFN has successfully reduced the incidence of UV-induced skin cancer[267] and benzo[a]pyrene-induced forestomach tumours [268] in mice, as well as prevented colon [269] and 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced mammary tumours [270] in rats. There is also epidemiological evidence that supports the reduction in cancer incidence by consumption of SFN-containing cruciferous vegetables, including prostate [271, 272], breast [273, 274], lung [275-277], gastric [278], and colon cancers [279].

SFN’s ease of absorption, wide distribution, and retained activity of its metabolites all contribute to its potential as an anti-cancer agent; however, its plethora of biological effects is what has piqued the interest of researchers and clinicians alike.
1.6.1 Sulforaphane’s Mechanisms of Action

While interest in SFN is largely due to its anti-carcinogenic properties, SFN is also being investigated for other purposes, including neuroprotection and cardioprotection. One of the main reasons for SFN’s versatility is because it appears to act on a wide range of molecular targets. With cancer in particular, SFN has been demonstrated to target all three stages of carcinogenesis: tumour initiation, tumour promotion, and tumour progression [280]. The process of carcinogenesis is multifactorial and complex, and is beyond the scope of this thesis. Very briefly, the initiation phase is characterized by genetic mutations resulting in the activation of oncogenes, or tumour-promoting genes, and the down-regulation of tumour-suppressor genes. Mutations in these key genes results in the second stage, which is characterized by uncontrolled cellular proliferation with decreased apoptotic cell death. With increased proliferation, malignant tumour cells can invade other tissue types, which is characteristic of the progression phase. At the same time, malignant cells can acquire more mutations, which can result in increased malignancy (reviewed in [281]). Six physiological commonalities underlie these three phases of carcinogenesis, which are termed “The Hallmarks of Cancer”: independent cellular growth, decreased responsiveness to external growth factors, evasion of programmed cell death (apoptosis), the ability for infinite replications, persistent angiogenesis, and metastatic abilities [282].

SFN has been demonstrated to prevent many of these malignant changes, prohibiting advancement through these three stages of carcinogenesis, as well as preventing numerous hallmarks of cancer in animal studies. Most relevant to the research conducted in this thesis, cancer initiation has been modulated by SFN treatment due to the ability of SFN to alter the activity of bioactivating and detoxifying enzymes involved in the metabolism of carcinogens.
SFN has been shown to inhibit CYP2E1 and CYP3A4 activity [283, 284], which in the case of benzene, may result in decreased production of toxic metabolites. Alternatively, SFN induces a number of detoxifying enzymes in both animal models as well in humans, including GST, UGT, and NQO1 [260, 262, 285-289], which may increase the detoxification of benzene and its metabolites.

Evidence demonstrates that SFN induces these enzymes via the activation of NRF2. NRF2 is a basic leucine-zipper transcription factor that is the primary activator of antioxidant-response elements, or ARE, which are found in promoter regions of many enzymes involved in benzene detoxification. Under resting conditions, NRF2 is sequestered and targeted for proteosomal degradation in the cytoplasm by Kelch-like ECH-associated protein 1, or KEAP1. NRF2 can be activated by a number of different endogenous signals, such as ROS or reactive nitrogen species, and exogenous signals, including SFN. When SFN interacts with the NRF2-KEAP1 complex, NRF2 dissociates from KEAP1 and translocates into the cytoplasm. Binding of NRF2 and associated transcription factors, such as small MAF (sMAF) proteins, to the ARE stimulates transcription of a number of genes involved in the protecting the cell from excessive stress. Included in these genes are those involved in detoxification of electrophiles or free radicals, regulation of glutathione levels, inhibition of inflammatory responses, and molecular transporters (reviewed in [290]). Additionally, NRF2 interacts with a number of different signalling pathways, many of which are also involved in the response of the cell to stress. In addition to regulation by KEAP1, various kinases can phosphorylate NRF2, resulting in increased stability of the NRF2 transcript, modifying its activity. NRF2 also appears to interact with the aryl hydrocarbon receptor (AhR), another transcription factor involved in activation of
Figure 1.5: Schematic of NRF2 signalling.

Under normal conditions, nuclear factor erythroid 2-related factor 2 (NRF2) is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (KEAP1) and targeted for ubiquitination. When exposed to sulforaphane (SFN), NRF2 dissociates from KEAP1, allowing translocation of NRF2 into the nucleus. Once in the nucleus, it interacts with small MAF proteins (sMAF) and other transcription factors to activate the antioxidant response element (ARE) in the promoter regions of numerous genes, facilitating transcription. Adapted from [290].
enzymes involved in xenobiotic metabolism. While once thought to be independent of each other, induction of NQO1 by substrate binding to the AhR is dependent on NRF2 activation [291] (Figure 1.5). NRF2 also interacts NFKB, with increased NFKB activity being found in mouse embryonic fibroblasts deficient in Nrf2, although this interaction is not well elucidated [292].

To-date, the ability of SFN to induce expected enzymes involved in benzene’s detoxification during pregnancy and in the developing fetus has yet to be studied. Additionally, its ability to prevent benzene and/or benzene metabolite toxicity has not been investigated.

1.7 Hypothesis and Objectives

There are two overall hypotheses for this thesis. The first is that benzene, or its metabolite benzoquinone, leads to an increase in ROS-mediated genetic or epigenetic changes that contribute to its developmental toxicity/carcinogenicity. The second is that SFN treatment will induce enzymes involved in benzene detoxification in CD-1 mouse fetal livers, either in vivo or in vitro, and any observed cellular changes induced by benzene or its metabolite benzoquinone will be prevented by pretreatment with SFN. The following objectives are addressed in the forthcoming chapters:

1a. To determine whether benzoquinone exposure elevates ROS levels and/or leads to DNA damage in cultured CD-1 mouse GD14 fetal liver cells in vitro.

1b. To determine whether pretreatment with SFN can protect against any observed toxicity in cultured CD-1 mouse GD14 fetal liver cells in vitro.

2. To determine whether SFN induces NRF2 regulated genes in CD-1 mouse dams and/or fetuses in vivo.

3. To determine whether epigenetic modifications occur in CD-1 mouse dams and/or fetuses following exposure to benzene in vivo.
Chapter 2

Benzoquinone-mediated Increased DNA Damage and Decreased Ogg1 Gene Expression are not Altered by Sulforaphane Pretreatment in Cultured CD-1 Mouse Fetal Liver Cells.


2.1 Abstract

Benzene is an environmental pollutant known to cause leukemia in adults, and thought to be associated with childhood leukemia. While the mechanisms of benzene-mediated carcinogenicity have not been fully elucidated, increased reactive oxygen species (ROS) levels and DNA damage have been implicated. Sulforaphane (SFN) potently activates nuclear factor erythroid 2-related factor 2 (NRF2), which is thought to contribute to SFN-mediated protection against carcinogenesis. In this study, we exposed cultured CD-1 mouse fetal liver cells to the benzene metabolite, benzoquinone, to determine its potential to cause DNA damage and alter DNA repair. Cells were also exposed to SFN to determine any potential protective effects against benzoquinone-mediated toxicity. Initially, cultured cells were exposed to benzoquinone to confirm that ROS levels were increased and to SFN to confirm NRF2 activation. Subsequently, cells were treated with benzoquinone (with or without SFN pretreatment) and levels of ROS, 8-OHdG (a marker of oxidative DNA damage), γH2AX (a marker of DNA double stranded breaks; DSBs), and expression levels of certain DNA repair genes were measured. Benzoquinone exposure led to a significant increase in ROS, which was not prevented by pretreatment with SFN or the antioxidative enzyme, catalase. DNA damage was increased following benzoquinone exposure, which was not prevented by SFN. Benzoquinone exposure significantly decreased the expression of the critical base excision repair gene, 8-oxoguanine glycosylase (Ogg1), which was
not prevented by SFN. The findings of this study demonstrate that DNA damage and altered DNA repair are a consequence of benzoquinone exposure and that SFN conferred little protection in this model.

2.2 Introduction

Benzene is emitted into the atmosphere by industries and exhaust fumes, resulting in chronic human exposure to this pollutant. Benzene is a known hematotoxicant and adult leukemogen, and epidemiological evidence suggests that benzene exposure is associated with childhood leukemia [89, 105]. Benzene exposure in mice leads to cancer [116], including hepatic and hematopoietic tumours in CD-1 mouse offspring one year after in utero exposure [122]. In order to exert its toxicity, benzene must be metabolized, primarily by cytochrome P4502E1 to benzene oxide, which is further metabolized to a number of reactive metabolites, including benzoquinone, by different enzymes [293].

While the mechanisms of benzene-mediated toxicity are not entirely understood, benzene metabolites, including benzoquinone, have been shown to lead to an increased production of ROS both in vitro [190, 294] and in vivo [194, 196]. While endogenous ROS play critical roles in various cellular activities, increased ROS levels can damage cellular macromolecules, including DNA [295]. The benzene metabolites, hydroquinone and benzoquinone, have been shown to increase levels of oxidative DNA damage, as measured by increased 8-hydroxy-2-deoxyguanosine (8-OHdG), in human myeloid progenitor HL-60 cells [205], cultured murine fetal liver cells [193], and in urine collected from benzene-exposed humans [209]. This is concerning, given that 8-OHdG lesions have the potential to be mutagenic, resulting in GC to TA transversions [208]. Additionally, both hydroquinone and benzoquinone can cause DNA double
stranded breaks (DSBs), as measured by increased phosphorylation of histone H2AX (γH2AX) [211, 214].

It is also hypothesized that altered or erroneous DNA repair pathways may play a role in benzene-mediated carcinogenesis. There are a number of different DNA repair pathways that predominate for different types of DNA damage. For example, base excision repair (BER) is the main pathway by which 8-OHdG is repaired [296]. DSBs, on the other hand, are primarily repaired by two main pathways; non homologous end-joining (NHEJ) or homologous recombination (HR; reviewed by [297]). The primary difference between these two pathways is the use of a homologous template in HR. While NHEJ is more erroneous than HR, NHEJ remains the major repair pathway for DSBs in vertebrates, and is the predominant pathway by which DSBs are repaired in later stages of fetal development [232, 298]. While the goal of each pathway is to repair incurred damage, erroneous DNA repair may result in mutations [299].

There is some evidence that benzene exposure has an effect on DNA repair pathways. For example, benzene metabolite exposure has been demonstrated to increase recombinational events in both Chinese Hamster Ovary (CHO) cells as well as cultured fetal liver cells [193, 238].

An increasing number of studies are examining the potential for prophylactic anti-cancer agents to prevent carcinogenesis in susceptible populations. For example, the use of the phytochemical, sulforaphane (SFN) is currently being investigated for its potential anti-cancer effects in rural China, where exposure levels to carcinogens, such as aflatoxin B1, are high due to contaminated food [263]. SFN is a phytochemical derived from cruciferous vegetables, which has been shown to block various stages of the process of carcinogenesis (reviewed in [300]). The cellular actions of SFN are numerous, including alterations of drug-metabolizing enzyme activity [301], initiation of apoptosis [302], epigenetic alterations (reviewed in: [303]), and disruption of
various signalling pathways \[290, 304\]. However, its activation of the transcription factor, NRF2, and the consequential induction of downstream detoxifying enzymes are thought to be a primary mechanism of SFN-mediated chemoprevention \[290\].

In the present study, we addressed two major objectives. The first was to determine whether benzoquinone caused increased ROS production, DNA damage, and alterations in expression of DNA repair genes in cultured fetal liver cells extracted from CD-1 mice. The second objective was to determine whether pretreatment with SFN could protect against toxicities observed following benzoquinone exposure.

### 2.3 Materials and Methods

#### 2.3.1 Tissue Collection and Cell Culture

Four to six week old CD-1 mice were purchased from Charles River Canada (Montreal, Quebec) and housed in a temperature-controlled room with a 12-h light/dark cycle in the Queen’s University Animal Care Facility. Mice were fed standard rodent chow (Purina Rodent Chow, Ralston Purina International, Strathroy, ON, Canada) and provided tap water \textit{ad libitum}. All animal procedures and handling were performed according to the guidelines of the Queen’s University Animal Care Committee and the Canadian Council on Animal Care. Female mice were bred in a 2:1 ratio with male CD-1 mice overnight. A vaginal plug the next morning was designated gestational day (GD) 1. On the morning of GD14, pregnant CD-1 mice were euthanized via cervical dislocation, and pups were removed. GD14 fetal livers were used since the fetal liver is a primary site of hematopoiesis at this gestational age, with the population of hematopoietic stem cells doubling from GD12 to 15 \[305\].

Fetal livers were carefully extracted, and homogenized using a 23-gauge needle to create a single cell suspension in a small amount of sterile media. Cells from fetal livers were cultured
in supplemented IMDM media, containing 14.5% fetal bovine serum (Sigma-Aldrich, St, Louis, MI), 0.05 µg/ml mouse stem cell factor (Bioshop Canada Inc., Burlington, ON), interleukins 3 and 6 (0.01 µg/ml; Bioshop Canada Inc.), β-mercaptoethanol (1 in 100000 dilution; Sigma-Aldrich), 1X antibiotic/antimycotic (Sigma-Aldrich), and L-glutamine (1.92 mM; Sigma-Aldrich). After being cultured for 48 hours on treated cell culture plates, cells remaining in suspension were utilized for further experimentation.

2.3.2 Benzoquinone, Sulforaphane, and PEG-catalase preparation

*p*-Benzoquinone was purchased from Sigma-Aldrich and dissolved in PBS at a concentration of 25 mM immediately prior to use. Working concentrations of 2.5, 1.5, and 0.5 mM were diluted from the 25 mM stock solution. R-sulforaphane (SFN) was purchased from LKT Laboratories (St. Paul, MN) and diluted to a 5 mM stock solution in PBS (pH 7.4). The 5 mM stock solution of SFN was further diluted to 0.5, 1, and 2.5 mM solutions, aliquoted, and stored at -20°C until needed for experimentation. Polyethylene glycol conjugated catalase (PEG-catalase) was purchased from Sigma-Aldrich and dissolved in PBS before use to make up a stock concentration of 40000 units/ml and was stored for a maximum of 1 week at 4°C. PEG-catalase was used since PEG-conjugation increases cellular enzyme uptake and half-life of this enzyme in cells [306].

2.3.3 DCFDA ROS Detection Assay and Cell Death Detection

To assess whether exposure to benzoquinone led to a change in ROS levels in GD14 fetal liver cells, the fluorescent dye, 5,6-chloromethyl 2,7-dichlorodihydrofluorescein diacetate (CM-DCFDA; Invitrogen) was used. The protocol that was followed was adapted from Eruslanov and Kusmartsev [307]. Briefly, 0.5 million cells/ml were plated in 6 well tissue culture plates for 24 hours, after which, cells were removed from the media, washed once with PBS, and incubated for
0.5 hours in 1 µM CM-DCFDA (Life Technologies, Burlington, ON) in pre-warmed PBS. Cells were then centrifuged at 1200 rpm for 3 minutes, re-suspended in pre-warmed media, and exposed to 0, 5, 15, or 25 µM benzoquinone for 0.5, 1, 2, or 6 hours. Cells were collected, washed three times in PBS, and re-suspended in 400 µl PBS containing 5 µg/ml propidium iodide (PI; Sigma-Aldrich). Fluorescence of DCF in cells was measured at 530 nm and PI at 617 nm using a flow cytometer. PI was used as a measure of cell death in benzoquinone and SFN exposed cells.

2.3.4 NAD(P)H Quinone Oxidoreductase 1 Activity Assay

CD-1 mouse GD14 fetal liver cells were plated at a density of 0.5 million cells/ml in 10 cm tissue culture plates. At the time of plating, cells were exposed to 0, 0.5, 1, 2.5, or 5 µM of SFN (final concentration in media) for 2, 6, or 24 hours. Cells were collected, centrifuged at 1200 rpm for 3 minutes, washed once in PBS, and then assayed for NAD(P)H quinone oxidoreductase 1 (NQO1) activity. At the time of collection, an aliquot of cells was used to determine cell death using the Trypan Blue Exclusion Assay. To assess NQO1 activity levels, a modified protocol from Current Protocols in Toxicology was used [308]. Briefly, cells were homogenized and sonicated for 15 seconds in homogenization buffer, containing 25 mM Tris, 250 mM sucrose, and 5 µM flavin adenine dinucleotide (FAD). Homogenates were then centrifuged at 10 000 x g for 10 minutes and supernatants were used to determine activity of NQO1 in each sample. NQO1 catalytic activity assay was assessed using the reduction of 2,6-dichlorophenol-indophenol (DCPIP; Sigma–Aldrich). For each sample, absorbance at 600 nm of DCPIP was read over 1 minute in the presence or absence of 2 mM dicumarol (inhibits the reduction of DCPIP by NQO1). The rate obtained in the presence of dicumarol was subtracted.
from that obtained in the absence of dicumarol, and activity was expressed per mg of protein. Protein levels in each sample were determined by a Bradford assay.

2.3.5 qRT-PCR for NRF2-regulated Genes

CD-1 mouse GD14 fetal liver cells were plated at a density of 0.5 million cells/ml in a 6 well plate, and exposed to 0, 0.5, 1, 2.5, or 5 µM SFN for 24 hours. Cells were collected, centrifuged at 1200 rpm for 3 minutes, washed once in PBS, flash frozen in liquid nitrogen, and stored at -80°C until used. Total RNA was extracted using the RNeasy RNA extraction kit (Qiagen, Mississauga, ON) according to the manufacturer’s instructions. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Burlington, ON) and used in subsequent quantitative real-time PCR (qRT-PCR) reactions. qRT-PCR reactions were carried out using specific TaqMan® gene expression probes (Life Technologies) for mouse glutathione-s-transferase mu 1 (Gstm1; Mm00833915_g1), NAD(P)H quinone oxidoreductase 1 (Nqo1; Mm01253561_m1), glutathione cysteine ligase c (Gclc; Mm00802655), and heme oxygenase 1 (Ho-1; Mm00516005_m1). The geometric mean of the Ct values of TATA binding protein (Tbp; Mm00446973) and RNA polymerase IIa (Mm00839502_m1) were used as normalization controls as outlined in [309], and graphed using the delta-delta Ct method.

2.3.6 DCFDA ROS Detection Assay with SFN or PEG-catalase Pretreatment

To assess whether SFN and/or PEG-catalase could protect against observed increases in ROS levels induced by benzoquinone, the same flow cytometry protocol as discussed above was utilized [307], however, cells were exposed to either 1 or 2.5 µM SFN or 400 units/ml PEG-catalase for 24 hours, followed by a 0.5 hour exposure to 0, 15 or 25 µM benzoquinone (the time at which maximum levels of DCF fluorescence were observed following benzoquinone treatment). These concentrations of SFN and benzoquinone were chosen based on data attained
from above experiments (Figures 1-3), and were used for the remainder of experiments. The concentration of PEG-catalase used was based on concentrations previously used in our laboratory [191].

2.3.7 Measurement of Oxidative DNA Damage (8-OHdG)

Cells were plated at a density of 0.5 million cells/ml in a 10 cm cell culture dish and exposed to 0, 1, or 2.5 µM SFN at the time of plating. Twenty-four hours following exposure, plated cells were exposed to 0 or 25 µM benzoquinone for 0.5 or 6 hours. Cells were collected, washed with PBS, and flash frozen in liquid nitrogen and stored at -80°C until used. DNA was extracted from cell pellets using the Qiagen DNeasy DNA Extraction Kit (Qiagen, Mississauga, ON). DNA concentrations were determined using the NanoView (GE Healthcare Lifesciences, Mississauga, ON). The amount of 8-OHdG formed was determined using the Epigentek EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (Colorimetric). DNA (300 ng) was loaded into each well and the manufacturers instructions were followed. The percent of 8-OHdG present was calculated according to the manufacturers instructions.

2.3.8 Measurement of DNA Double Strand Breaks (γH2AX)

Cells were plated at a density of 0.5 million cells/ml in a 6 well plate, and at the time of plating were exposed to 0, 1, or 2.5 µM of SFN. After 24 hours SFN exposure, cells were exposed to 0, 15, or 25 µM of benzoquinone for 0.5, 2, or 6 hours after which cells were collected and fixed in 20% methanol for 10 minutes at room temperature. Cells were washed twice with 0.5% BSA in PBS and incubated with the phosphorylated histone H2AX (Serine 139) antibody (1:50 dilution in 0.5% BSA in PBS; Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature. Cells were once again washed with 0.5% BSA in PBS and incubated with R-Phycoerythrin-conjugated F(ab’)2 secondary antibody (Jackson ImmunoResearch, WestGrove,
PA; 1:100 dilution) for 0.5 hours at room temperature in the dark. Subsequently, cells were washed with PBS and re-suspended in 400 µl PBS. Flow cytometry was used to determine the percentage of cells in each sample that were positive for γH2AX.

2.3.9 qRT-PCR for DNA Repair Genes

Cells were plated at a density of 0.5 million cells/ml in 10 cm plates and were exposed to 0, 1, or 2.5 µM SFN at the time of plating. After 24 hours of SFN exposure, cells were exposed to 0, 15, or 25 µM benzoquinone for 0.5 or 6 hours. Cells were collected by centrifugation and RNA was extracted using the RNeasy RNA extraction kit (Qiagen) and cDNA was, once again, synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Qiagen’s RT² qPCR Primer Assays were purchased and used to measure RNA transcript levels of Breast cancer resistance 1 (Brcal; PPM03442A), Breast cancer resistance 2 (Brcal2; PPM03704E), 8-oxoguanine glycosylase (OggI; PPM05282A), poly (ADP-ribose) polymerase 1 (Parp-1; PPM05150C), and X-ray repair complementing defective repair in Chinese hamster cells 4 (Xrcc4; PPM04389A). These primers were tested for efficiency in CD-1 mouse fetal liver cells, and efficiencies were confirmed to be between 90 and 100%. Hypoxanthine-guanine phosphoribosyltransferase (Hprt; PPM03559F) was used as a control gene in these samples and analyzed using the delta delta Ct method.

2.3.10 Statistical Analysis

Two-way ANOVAs were conducted for all analyses with variables being either benzoquinone exposure concentration and time, or benzoquinone exposure concentration and SFN pretreatment. All statistical analyses were conducted and graphs were made using GraphPad Prism 6. A sample size of 3 was used for each experiment. Statistical significance was stated for any test in which the p ≤ 0.05.
2.4 Results

2.4.1 Increased ROS Levels and Cell Death following Exposure to Benzoquinone

Exposure of cultured GD14 fetal liver cells to 15 or 25 µM benzoquinone for 0.5, 1, 2, and 6 hours led to increased DCF fluorescence, representative of increased ROS, however this increase was only statistically significant following 0.5 hours of exposure to 25 µM benzoquinone (Figure 2.1). Benzoquinone exposure also led to a statistically significant, but marginal, increase in cell death at the highest concentration of benzoquinone (Figure 2.1 inset).

2.4.2 Increased mRNA Levels of NRF2-regulated Genes and NQO1 Activity following SFN Exposure

SFN exposure led to a statistically significant increase in both the expression of NRF2-regulated genes, as well as NQO1 catalytic activity in cultured GD14 fetal liver cells. Exposure of GD14 fetal liver cells to 2.5 or 5 µM SFN led to a concentration-dependent increase in NQO1 catalytic activity 24 hours post-exposure (Figure 2.2). Similarly, treatment with 2.5 or 5 µM SFN led to an increase in gene expression of Nqo1 (Figure 2.3A), Gclec (Figure 2.3B), Ho-1 (Figure 2.3C), and Gstm1 (Figure 2.3D). A statistically significant increase in cell death was observed at 24 h following exposure to 5 µM SFN (inset Figure 2.2), therefore for subsequent experiments, concentrations of 1 and 2.5 µM SFN were used to determine whether SFN was able to confer protection.

2.4.3 Increased ROS Levels following Benzoquinone Exposure not Prevented by SFN or PEG-catalase Pretreatment

Once again, increased ROS levels were observed following 15 and 25 µM exposure to benzoquinone. However, pretreatment with 1 or 2.5 µM SFN (Figure 2.4) or 400 U/ml PEG-catalase (Figure 2.5) for 24 hours did not reduce benzoquinone-induced increases in ROS levels.
Figure 2.1: Benzoquinone increases ROS levels in cultured CD-1 mouse fetal liver cells.

Levels of 2,7-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, a measure of reactive oxygen species, detected by flow cytometry, following exposure of cultured CD-1 mouse gestational day 14 fetal liver cells to 0 to 25 µM benzoquinone (BQ) for 0.5 to 6 hours. Inset: percentage of cells, following the same exposure protocol, negative for propidium iodide (PI); a measure of cell death. * denotes statistical significance (p<0.05).

Figure 2.2: SFN increases NQO1 activity in cultured CD-1 mouse fetal liver cells.

Activity of NAD(P)H quinone oxidoreductase (NQO1) following exposure to 0 to 5 µM sulforaphane (SFN) for 2 to 24 hours normalized to the vehicle-exposed control. Inset: Cell death as measured by the trypan blue exclusion assay following exposure to 0 to 5 µM SFN for 24 hours. * denotes statistical significance (p<0.05).
Figure 2.3: SFN increases expression of NRF2-regulated gene mRNA levels in cultured CD-1 mouse fetal liver cells.

mRNA levels of Nrf2-mediated genes *Nqo1* (A), *Gclc* (B), *Ho-1* (C), and *Gstm1* (D) extracted from cultured CD-1 mouse gestational day 14 fetal liver cells following exposure to 0 to 5 µM SFN for 24 hours. Values of treated samples were normalized to values of the vehicle control. * denotes statistical significance (p<0.05).
Figure 2.4: Benzoquinone-mediated increased ROS levels in cultured CD-1 mouse fetal liver cells not affected by SFN pretreatment.

Levels of 2,7-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, a measure of reactive oxygen species, detected by flow cytometry in living cells (propidium iodide negative; PI) following exposure of cultured CD-1 mouse gestational day 14 fetal liver cells to 24 hour pretreatment with sulforaphane (SFN), followed by 0, 15, or 25 µM benzoquinone (BQ) for an additional 0.5 hours. * denotes statistical significance (p<0.05).

Figure 2.5: Benzoquinone-mediated increased ROS levels in cultured CD-1 mouse fetal liver cells not affected by PEG-catalase pretreatment.

Levels of 2,7-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, a measure of reactive oxygen species, detected by flow cytometry in living cells (propidium iodide negative; PI) following exposure of cultured CD-1 mouse gestational day 14 fetal liver cells to 24 hour pretreatment with PEG-catalase, followed by 0, 15, or 25 µM benzoquinone (BQ) for an additional 0.5 hours. * denotes statistical significance (p<0.05).
2.4.4 SFN does not Protect against Benzoquinone-induced Cytotoxicity

Pretreatment with 2.5 μM SFN for 24 hours did not protect against cytotoxicity caused by 25 μM benzoquinone exposure. In contrast SFN pretreatment exacerbated cell death, resulting in a statistically significant increase in cell death following 6 hours of exposure (Figure 2.6).

2.4.5 Benzoquinone Exposure Increases Levels of 8-OHdG, which is not Prevented by SFN Pretreatment

Exposure of cultured fetal liver cells to 25 μM benzoquinone had no impact on levels of oxidized DNA, as measured by increased percentage of 8-OHdG, after 0.5 hours of exposure (Figure 2.7A). A statistically significant increase in 8-OHdG levels was observed following 6 hours of benzoquinone exposure (Figure 2.7B). SFN pretreatment for 24 hours did not prevent increased 8-OHdG levels found after 6 hours of benzoquinone exposure.

2.4.6 Benzoquinone Exposure Increases Levels of γH2AX, which is not Prevented by SFN Pretreatment

Exposure of GD14 fetal liver cells to 25 μM benzoquinone for 0.5 (Figure 2.8A) or 2 hours (Figure 2.8B) resulted in a statistically significant increases in γH2AX. However, no difference in γH2AX levels was observed following 6 hours of benzoquinone exposure (Figure 2.8C). Pretreatment with SFN conferred no protection against increased levels of γH2AX observed following benzoquinone exposure (Figure 2.8A,B).

2.4.7 Benzoquinone Exposure Significantly Decreases Ogg1 mRNA levels

Exposure of cultured CD-1 mouse GD14 fetal liver cells to benzoquinone for 0.5 hours had no effect on the expression of any of the 5 genes that were analyzed (data not shown). Benzoquinone exposure for 6 hours had no significant effect on mRNA levels of Brca1 (Figure 2.9A), Brca2 (Figure 2.9B) or Xrcc4 (Figure 2.9D). Parp-1 (Figure 2.9C) mRNA levels
Figure 2.6: SFN exacerbates benzoquinone-mediated cell death in cultured CD-1 mouse fetal liver cells.

Percentage of cultured fetal liver cells negative for propidium iodide (PI) staining, a marker of cell death, following exposure to 0 or 2.5 µM sulforaphane (SFN) for 24 hours followed by exposure to 0 or 25 µM benzoquinone (BQ) for an additional 6 hours. * denotes statistical difference between 0 and 25 µM BQ exposure. ‡ denotes statistical difference between 0 µM BQ and 2.5 µM SFN + 25 µM BQ exposure.

Figure 2.7: Benzoquinone exposure increases 8-OHdG levels in cultured CD-1 mouse fetal liver cells.

Percentage of oxidized guanine, 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, in DNA extracted from cultured gestational day 14 fetal liver cells of CD-1 mice exposure to 0, 1, or 2.5 µM sulforaphane (SFN) for 24 hours followed by exposure to 0 or 25 µM benzoquinone (BQ) for an additional 0.5 (A) or 6 (B) hours. * denotes statistical significance (p < 0.05)
Figure 2.7: Benzoquinone exposure increases γH2AX formation in cultured CD-1 mouse fetal liver cells.

Levels of phosphorylated histone H2AX (γH2AX), a marker of DNA double stranded breaks, in cultured CD-1 mouse gestational day 14 fetal liver cells exposed to 0, 1 or 2.5 µM sulforaphane (SFN) for 24 hours, followed by 0, 15, or 25 µM benzoquinone (BQ) for an additional 0.5 (A), 2 (B), or 6 (C) hours. Levels of γH2AX in treated cells were normalized to control values. * denotes statistical significance (p<0.05).
Figure 2.8: Benzoquinone exposure decreases *Ogg1* mRNA levels in cultured CD-1 mouse fetal liver cells.

mRNA levels of DNA repair genes *Brca1* (A), *Brca2* (B), *Parp-1* (C), *Xrcc4* (D), and *Ogg1* (E) following exposure of cultured CD-1 mouse gestational day 14 fetal liver cells to 0, 1, or 2.5 μM sulforaphane (SFN) for 24 hours followed by 0, 15, or 25 μM benzoquinone for an additional 6 hours. Values of treated samples were normalized to values of the vehicle control. * denotes statistical significance (p<0.05).
were non-significantly decreased (p=0.058) and Ogg1 (Figure 9E) mRNA levels were significantly decreased following exposure to 25 μM benzoquinone for 6 hours. Pretreatment with SFN for 24 hours had no significant effect on gene expression levels of any of the measured genes.

2.5 Discussion

We investigated whether benzoquinone exposure induced oxidative DNA damage and DSBs in primary cultures of mouse fetal liver cells, and evaluated the effects of benzoquinone exposure on the mRNA levels of DNA repair genes. We also assessed the potential protective ability of the proposed anti-carcinogenic agent, SFN, against benzoquinone-induced toxicity in these cells. Overall, we found that benzoquinone exposure resulted in an increase in ROS levels, oxidative DNA damage, and DSBs, as well as a decrease in Ogg1 gene expression, and that SFN did not confer any protection.

Increased ROS production following exposure to benzene and its metabolites has previously been demonstrated in several studies using cultured cells [121, 190, 191], and benzene cause increased ROS levels in livers of C57Bl/6 and CD-1 mouse fetuses following in utero exposure [194, 195]. Therefore, we expected to see an increase in ROS levels following exposure of cultured CD-1 mouse fetal liver cells to benzoquinone, which we confirmed. Likewise, our study confirmed the finding that benzene or benzene metabolite exposure results in an increased DSBs (γH2AX) in vitro [211, 214]. However, this was shown in fetal as opposed to adult-derived cells in the present study. Another study from our laboratory measured levels of γH2AX following in utero exposure to benzene in both fetal livers as well as post-natal day (PND) 2 bone marrow and, interestingly, found no difference between vehicle and benzene exposed animals [215]. However, this study measured γH2AX by Western blotting, a potentially less-sensitive
measure of γH2AX compared to flow cytometry and was conducted in C57Bl/6 mice, which have been shown to be resistant to benzene-induced transplacental carcinogenicity [122]. Whether in utero benzene exposure results in increased γH2AX in CD-1 mouse fetal livers remains to be determined.

Our study is the first to demonstrate increased 8-OHdG levels following benzoquinone exposure in primary fetal liver cells, which is consistent with previous studies demonstrating oxidative DNA damage following exposure to the benzene metabolite, 1,2,4-benzenetriol, in vitro [204], as well as in the bone marrow of mice following exposure to benzene [205]. We previously found no increase in 8-OHdG levels in C57Bl/6 primary fetal liver cells following benzoquinone exposure [193]. Given the susceptibility of CD-1 and not C57Bl/6 offspring to transplacental carcinogenesis, oxidative DNA damage may be critical to this process, although this hypothesis needs to be confirmed in vivo in CD-1 mice.

Our study demonstrates decreased Ogg1 gene expression levels following exposure to benzoquinone for 6 hours. This is a significant finding given that following DNA oxidation, OGG1 is responsible for the removal of the oxidized base [310], and its activity defines the rate-limiting step in the BER of 8-OHdG [311, 312]. Our results are consistent with those found in a study investigating the effects of cadmium exposure on OGG1 transcription in human fibroblasts and HeLa cells [313], wherein cadmium exposure resulted in decreased OGG1 transcription, which was also associated with increased overall mutation frequency [313].

While not significant, our study found that benzoquinone exposure also led to a decrease in Parp-1 mRNA levels. Parp-1 mRNA levels are decreased following exposure to benzoquinone in bone marrow cells extracted from mice [239]. Parp-1 is involved in a variety of cellular pathways, including DNA repair. It is described as a nick-sensor, binding to DNA
damage sites and facilitating DNA repair pathways by interacting with numerous mediators involved in BER, NHEJ, and HR [314, 315]. Mice lacking Parp-1 are highly sensitive to genotoxic agents, having increased sister chromatid exchanges and chromosome breaks following exposure to genotoxic agents [316]. Together, if decreased activities of OGG1 and PARP-1 are due to decreased transcription of Ogg1 and Parp-1 as observed in the present study, increased oxidative DNA damage and DSBs induced by benzoquinone exposure may disrupt multiple DNA repair pathways, which could increase the likelihood of a mutational event.

While benzoquinone exposure led to increased DNA damage and altered repair gene expression observed in the present study, we found no protective effects of SFN. SFN has been demonstrated to be a potent activator of the transcription factor NRF2 in numerous studies (reviewed in [290]), and we confirmed SFN-mediated induction of NRF2 target gene mRNA levels and NQO1 activity in fetal liver cells of CD-1 mice in our study. NRF2-regulated genes encode for a number of antioxidants and xenobiotic detoxification enzymes typically activated by cellular stress, including oxidative stress [317]. These genes include those measured in our study, Nqo1, Gclc, Ho-1, and Gstm1. Particularly relevant for the metabolism of benzene are both Nqo1 and Gstm1, which play important roles in the detoxification of benzene metabolites, including benzoquinone [133, 136]. Despite this, we found that SFN did not confer any protection against benzoquinone-induced toxicity in cultured fetal liver cells, which is contradictory to other studies that have found that SFN protects against increased ROS production and oxidative stress resulting from xenobiotic exposure in other cell types [318, 319]. A potential explanation for these observed differences is that fetal tissue has inherently lower expression of numerous detoxifying enzymes compared to adult expression and activity levels. For instance, expression levels of GST enzymes are significantly higher in neonatal and adult livers than in fetal livers in humans [162].
Specifically, GSTM protein levels are four times lower in fetal liver than in adult liver [162]. Therefore, it is possible that the doubling of gene expression and activity of NRF2-regulated genes we observed were not biologically sufficient to counteract the toxicity of benzoquinone. Interestingly, while SFN is protective against xenobiotic-induced cancers in adult mice [320], SFN conferred no protection, and in contrast, exacerbated carcinogenesis in offspring following in utero exposure to dibenzo[def,p]chrysene [266]. While that study offered no explanation for the observed detrimental effect of SFN, it may be due to fundamental differences between fetuses and adults. Further understanding of the disparity between SFN’s mechanisms of action at different life stages is clearly warranted.

Alternatively, emerging data suggest that SFN itself may be genotoxic. While evidence is conflicting, studies have found oxidative stress and increased DNA breaks following exposure to SFN. For example, one study demonstrated an increase in intracellular ROS production and an increased incidence of single stranded, but not double stranded, DNA breaks following 3 hours of exposure to 10 – 30 µM SFN in T lymphoblastoid Jurkat cells and human umbilical vein endothelial cells [321]. Similarly, another study showed a significant increase in ROS production and increased cell death following exposure to 10 - 20 µM SFN for 24 – 48 hours in osteocarcoma cells [322]. While the goal of cancer treatments is the induction of cell death, the prospect of a potential prophylactic exacerbating DNA damage and inducing cell death is concerning. In the present study, we did not observe an exacerbation of ROS production or DNA damage by pretreatment with SFN, however the concentrations used in our study were lower than those that induced negative effects. We did, however, see an increase in cell death following 5 µM exposure to SFN for 24 hours in cultured CD-1 mouse GD14 fetal liver cells; whereas, this same exposure did not induce cell death in cultured smooth muscle cells [318], suggesting
increased sensitivity of fetal cells to SFN. While non-significant, 2.5 µM SFN exposure in cultured fetal liver cells led to a reduction in mRNA levels of Parp-1. While limited data exist on the effects of SFN on DNA repair pathways, one study evaluated the effects of SFN on formation and repair of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) adducts in human liver cells, and found that while SFN decreased adduct formation, it has no effect on the mRNA expression of the BER genes, apurinic endonuclease and DNA polymerase β [323]. However, in a study investigating the effect of SFN on NER activity, the authors found a concentration-dependent inhibition of repair of adducts induced by (+)-anti-benzo[a]pyrene 7,8-diol-9,10-epoxide in vitro, implicating XPA as a target of SFN [324]. No data exist on the effects of SFN on Parp-1 expression; therefore, further investigation into this potential inhibition is warranted.

In conclusion, we demonstrated that benzoquinone leads to increased ROS, oxidative DNA damage, DSBs, and decreased expression of the BER gene, Ogg1, in cultured CD-1 mouse fetal liver cells. Furthermore, pretreatment with SFN conferred no protection against any of the effects observed benzoquinone-mediated effects.
Chapter 3
Sub-chronic Sulforaphane Exposure in CD-1 Pregnant Mice Enhances Maternal NAD(P)H Quinone Oxidoreductase 1 (NQO1) Activity and mRNA Expression of Nqo1, Glutathione S-transferase, and Glutamate-cysteine Ligase: Potential Implications for Fetal Protection Against Toxicant Exposure

Philbrook, N.A. and Winn, L.M. (2014) Reproductive Toxicology, 43, 30-37

3.1 Abstract

Sulforaphane (SFN) is a phytochemical that has been shown to protect against certain cancers in animal models. SFN induces nuclear factor (erythroid-derived 2) like-2 (NRF2)-controlled genes, which are involved in detoxification of many carcinogens. Whether SFN induces NRF2-mediated enzymes, including glutathione S-transferase (GST), NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), and glutamate-cysteine ligase catalytic subunit (GCLC) in pregnant CD-1 mice remains to be determined. In this study, two dosing protocols were implemented to investigate this question. In an acute study, non-pregnant and pregnant CD-1 mice were exposed to a single, acute dose of SFN (0, 50, or 100 mg/kg) on gestational day (GD) 14 via oral gavage. Animals were euthanized at 2, 6, or 24 hours following exposure and maternal liver, bone marrow, as well as fetal livers were removed. In a sub-chronic study, non-pregnant or pregnant female CD-1 mice were exposed to SFN (0 or 65 mg/kg) daily via oral gavage for 30 days. On GD14, mice were euthanized and liver, spleen, thymus, bone marrow, and fetal livers were removed. Nrf2, Nqo1, Gstm1, Ho-1, and Gclc mRNA levels were measured using qRT-PCR, as well as NQO1 activity was assessed using a colorimetric enzyme activity assay. Results demonstrated that acute exposure to SFN resulted in a statistically
significant increase in *Gstm1*, *Nqo1*, *Ho-1*, and *Gclc* mRNA transcript levels in adult liver from both non-pregnant and pregnant animals, however SFN treatment did not change NQO1 activity. Sub-chronic exposure to SFN, however, resulted in a statistically significant two to three-fold increase (p < 0.05) in *Gstm1*, *Gclc* and *Nqo1* transcript levels, as well as a two-fold increase in NQO1 activity in adult livers. No effects of maternal SFN treatment on fetal liver *Nrf2*, *Gstm1*, *Nqo1*, *Ho-1*, or *Gclc* transcript levels or NQO1 enzyme activity were observed. These results suggest that maternal treatment and enzyme induction by SFN could protect the fetus against exposure to various toxicants.

3.2 Introduction

Cancer chemoprevention is a strategy being developed to handle rising incidences of cancer. In particular, childhood cancers are a group of cancers for which there would be a great benefit from preventative measures that could inhibit cancer initiation. For example, leukemia is the most prevalent cancer in children, and its etiology remains largely unknown [325]. It has been hypothesized that childhood leukemia could be a result of prenatal exposure to certain environmental chemicals. While this association has not been conclusive in the literature (addressed in [326, 327]), numerous studies do support this hypothesis for some chemicals including insecticides [34, 328], and aromatic hydrocarbons, including benzene [329].

In recent years, many compounds found in fruits and vegetables have been determined to be bioactive, with now over 1000 different phytochemicals having been identified as having anti-cancer activity [258]. Dietary phytochemicals are thought to protect against the initiation, and progression of cancer via numerous mechanisms, including inhibition of the cell cycle, initiating apoptosis, inhibition of angiogenesis and metastasis, diminishing expression of various oncogenes, as well as behaving as antioxidants [330]. One such phytochemical that has been
demonstrated to be effective in preventing carcinogenesis in many animal models is sulforaphane (SFN; reviewed in [280, 300]).

There are numerous suggested mechanisms as to how SFN exerts its protective effects, including but not limited to inhibition of Phase I bioactivating enzymes [283, 331], initiation of apoptosis [302, 332, 333], changes in epigenetic modifications (reviewed in [303]), inhibition of metastasis and angiogenesis [334, 335], as well as induction of a number of detoxifying enzymes [285, 336]. SFN has been demonstrated to activate the redox sensitive transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2), which modulates a number of enzymes involved in the detoxification of many carcinogens and their metabolites. Increased tumor incidence is associated with reduced function and activity of NRF2, as seen in Nrf2 knockout mouse models [337]. Accordingly, activation of NRF2 by compounds such as SFN and oltipraz, a known NRF2 inducer, is associated with reduction in DNA adducts and tumor formation in animal models [338, 339].

Glutathione S-transferase (GST), heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), and NAD(P)H quinone oxidoreductase 1 (NQO1) are all inducible enzymes that, when activated, help the cell respond to oxidative stress. Activation by a few different transcription factors has been demonstrated to increase transcription levels and activity of some of these enzymes, including Activator Protein-1 (AP-1), nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) [340, 341], as well as NRF2, which increases expression of all four of these proteins. GST is an enzyme that catalyzes the conjugation of reactive compounds with GSH, stabilizing the previously reactive molecule. There are a number of isoforms of GST, including mu (GSTM), which is thought to be important in the repair of carcinogenic products formed from oxidative stress [342]. GSTM1 null polymorphisms in
humans are associated with increased risk of various types of cancer, indicating this enzyme’s importance in chemoprevention [342-344]. Both HO-1 and GCLC are induced in response to cellular stress, increasing the antioxidant capabilities of the cell; HO-1 by producing the endogenous antioxidant bilirubin [345], and GCLC by producing GSH [345, 346]. Lastly, NQO1 is an enzyme responsible for two-electron reduction of quinones, which converts these highly reactive molecules into hydroquinones, that upon further conjugation produces a stable, water-soluble conjugate that can be excreted in the urine [336]. Polymorphisms leading to reduced function of NQO1 are also associated with increased susceptibility to some cancers, including childhood leukemia [347].

In utero initiated cancer is studied in a number of different animal models using a variety of carcinogens. A previous study in our laboratory demonstrated that in utero exposure of CD-1 pregnant dams to 200 mg/kg benzene leads to a statistically significant increase in hematopoietic and hepatic tumors in offspring one year after birth [122]. With this under consideration, the purpose of this study was to determine whether SFN treatment in CD-1 mice could alter expression of NRF2 and the expression and/or activity of four of the genes it regulates, in particular, Gstm1, Ho-1, Gclc, and Nqo1. SFN was given to pregnant dams to determine its potential use as a preventative treatment for our previously established benzene model of transplacental carcinogenicity [122] and other models of developmental toxicity. Additionally, the effect of SFN in pregnant dams was compared to that in non-pregnant females of the same age to determine whether pregnancy as a physiological state influenced SFN’s effect on NRF2-mediated enzyme activity.
3.3 Materials and Methods

3.3.1 Animals and Sulforaphane

Four to six week old CD-1 mice were purchased from Charles River Canada (Montreal, Quebec). All practices were in accordance with the guidelines of the Canadian Council on Animal Care and experimental procedures were approved by the Animal Care Committee (Queen’s University). Mice were fed standard rodent chow and provided continual access to water. Half of the female mice was housed overnight with male mice in a 2:1 ratio, and the presence of a vaginal plug the next morning was considered gestational day (GD) 1. The other half was not bred and was used as the non-pregnant counterparts. R-sulforaphane was purchased from LKT Laboratories (St. Paul, Minnesota) and diluted in phosphate buffered saline (PBS; pH 7.4) for oral gavage, aliquoted, and stored at -20°C until used.

3.3.2 Acute dosing: Non-pregnant and Pregnant CD-1 Mice

Non-pregnant and pregnant (GD14) CD-1 female mice were exposed to a single dose of 0, 50, or 100 mg/kg SFN (dissolved in PBS) via oral gavage for 2, 6, or 24 hours (n ≥ 3 per treatment group; per time point for each of non-pregnant and pregnant mice). Mice were euthanized via cervical dislocation, and adult liver and bone marrow were extracted, as well as fetal livers from pregnant dams. Tissue was either stored in RNAlater® (Sigma-Aldrich, St. Louis, MI) at -20°C for RNA extraction, or flash frozen in liquid nitrogen and stored at -80°C until used for the NQO1 activity assay.

3.3.3 Sub-chronic dosing: Non-pregnant and Pregnant CD-1 Mice

A total of 24 CD-1 females (4-6 weeks) were dosed daily with 65 mg/kg/day SFN or the vehicle control (PBS) via oral gavage for a total of 4 weeks (30 days). After the first two weeks, 12 of the mice (6 treated and 6 control) were bred in a 1:1 ratio with males to attain pregnancy...
(presence of a vaginal plug indicated GD1). All mice, pregnant and non-pregnant, were dosed daily for the 2 remaining weeks in the study, at which point all were euthanized via cervical dislocation when pregnant dams reached GD14. Weights were recorded once a week throughout dosing. Following euthanasia adult liver, bone marrow, spleen, thymus, as well as fetal liver (from pregnant dams), were excised. In addition, litter size and numbers of resorptions in each litter attained from pregnant females were recorded. Portions of maternal liver and fetal liver were stored in RNAlater® for RNA extraction, and all other tissues were flash frozen and stored at -80°C until used in the NQO1 catalytic activity assay.

### 3.3.4 Quantitative Real-Time PCR

RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Burlington, ON). Quantitative real-time PCR (qRT-PCR) reactions were carried out using specific TaqMan® gene expression probes for mouse Nrf2 (Mm00477784_m1), Gstm1 (Mm00833915_g1), Ho-1 (Mm00516005_m1), Gclc (Mm00802655_m1), and Nqo1 (Mm01253561_m1). The geometric mean of Mouse Tata Binding Protein (Tbp; Mm00446973) and RNA Polymerase IIa (Mm00839502_m1) Ct values were used as normalization controls as outlined in [309]. All TaqMan® probes were purchased from Life Technologies. Gene expression (mRNA transcript levels) was expressed relative to the geometric mean of the two housekeeping genes (Tbp, and RNA polymerase II) using the delta-delta Ct method.

### 3.3.5 NAD(P)H Quinone Oxidoreductase 1 (NQO1) Catalytic Activity Assay

Sample preparation was completed using a protocol adapted from Current Protocols in Toxicology [308]. Approximately 20 mg of tissue (maternal or fetal) was mechanically homogenized in 150 µl homogenization buffer (25 mM Tris, 250 mM sucrose, 5 µM FAD). Bone
marrow samples were suspended in 100 µl homogenization buffer. Maternal or fetal samples were sonicated for 30 sec and centrifuged for 15 min at 10000 x g, whereas bone marrow samples were sonicated for 15 seconds and centrifuged for 10 min at 10000 x g. Supernatants of maternal or fetal samples were then transferred to a 10-ml ultracentrifuge tube and centrifuged for 60 min at 30000 x g. The supernatants were then collected and used to determine NQO1 enzyme activity in tissues of interest.

NQO1 catalytic activity assay was assessed using the reduction of 2,6-dichlorophenol-indophenol (DCPIP; Sigma-Aldrich, St. Louis, MI) by NQO1 present in each sample. For each sample, absorbance at 600 nm of DCPIP was read over 1 minute in the presence or absence of 2 mM dicumarol (inhibits the reduction of DCPIP by NQO1). Activity of NQO1 was determined by subtracting the rate obtained in the presence of dicumarol from the rate obtained in the absence of dicumarol [308].

3.3.6 Statistical Analysis

For the acute animal study, three-way ANOVAs were used to analyze qRT-PCR and NQO1 activity results for adult liver and bone marrow; the three independent variables were SFN dose (0, 50, 100 mg/kg), time (2, 6, 24 hours), and pregnancy status. For fetal tissue from the acute dosing study, a two-way ANOVA was used to analyze qRT-PCR and NQO1 activity data; the two independent variables were SFN dose (0, 50, 100 mg/kg) and time (2, 6, 24 hours). For the sub-chronic dosing study, weight gain and average litter size were analyzed using a student’s t-test. Incidences of resorptions were analyzed using Fisher’s Exact Test. qRT-PCR and NQO1 activity results were analyzed using a two-way ANOVA, with the independent variables being treatment (PBS or SFN) and pregnancy status in adult tissue, whereas a student’s t-test was used to evaluate any differences between treatment groups (PBS or SFN) in fetal tissue. GraphPad
Prism 5 (t-tests, Fisher’s Exact test, and two-way ANOVAs) and SPSS (three-way ANOVAs) were used to run statistical tests, and GraphPad Prism 5 was used to graph all results.

3.4 Results

3.4.1 Acute study: Non-pregnant and Pregnant Mice

In the livers of both non-pregnant and pregnant CD-1 mice, there were no differences detected in Nrf2 mRNA levels between SFN-treated female mice compared to vehicle controls, at any of the time points analyzed (Figure 3.1A). However, a statistically significant increase (p < 0.05) in relative Gstm1 mRNA levels was observed at 6 hours in both non-pregnant and pregnant animals, and 24 hours in non-pregnant animals following a single oral dose of SFN (Figure 3.1B). Gstm1 mRNA levels were increased 2.7 and a 2.5 fold after 6 hours exposure to a single dose of 50 or 100 mg/kg SFN, respectively. A 2-fold increase was also maintained at 24 hours following exposure to 100 mg/kg SFN in liver. Livers of pregnant dams exhibited a significant 2.5 fold increase (p < 0.05) in Gstm1 mRNA levels only after 6 hours exposure to 50 mg/kg SFN. A statistically significant (p < 0.05) increase in Ho-1 mRNA levels was observed in the livers of pregnant adult females 2 hours post-exposure to both 50 (2.1 fold increase) and 100 mg/kg SFN (2.1 fold increase), and a trend towards an increase in Ho-1 mRNA levels was observed in the livers of non-pregnant adult females as well (Figure 3.1C). Gclc mRNA levels were also statistically increased (p < 0.05) in pregnant adult livers following a 6-hour exposure to 50 (2.2 fold increase) and 100 mg/kg SFN (3.1 fold increase), as well as non-pregnant livers exposed to 100 mg/kg SFN (3.3 fold increase; Figure 3.1D). Similarly, a significant increase (p < 0.05) in relative Nqo1 mRNA levels was also observed at 6 hours in both non-pregnant (3.4 fold increase) and pregnant animals (1.8 fold increase) following a single 100 mg/kg oral dose of SFN (Figure 3.1E). There was also a significant interaction (p < 0.05) between pregnancy state and treatment...
Figure 3.1: Acute SFN exposure increases mRNA levels of NRF2-mediated genes in livers of non-pregnant and pregnant CD-1 mice.

qRT-PCR analysis of livers obtained from adult non-pregnant or pregnant CD-1 mouse exposed to a single, acute dose of 0, 50, or 100 mg/kg SFN. All data were quantified using the delta delta Ct method utilizing Tata Binding Protein and RNA polymerase 2A as housekeeping gene controls. Bar graphs showing the relative Nrf2 (A), Gstm1 (B), Ho-1 (C), Gclc (D), and Nqo1 (E) mRNA levels determined in livers from non-pregnant or pregnant adult CD-1 mice exposed in vivo to SFN for 2, 6, or 24 hours. (* indicates p < 0.05).
suggesting that, in the mouse, pregnancy alters \textit{Nqo1} mRNA levels to some extent. Finally, a significant interaction (p < 0.05) between treatment and time was also observed (Figure 3.1B-E) for all genes analyzed, except for \textit{Nrf2}, indicating that the effects of SFN treatment were dependent on the time point analyzed.

Despite significant increases in mRNA levels, when NQO1 enzymatic activity was assessed using a colorimetric enzyme activity assay, there were no statistical differences observed between any of the treatment groups at any of the time points assessed in either non-pregnant or pregnant mice (Figure 3.2). In fetal livers, relative gene expression of \textit{Nrf2}, \textit{Gstm1}, \textit{Ho-1}, \textit{Gclc}, and \textit{Nqo1}, (Figure 3.3) as well as NQO1 catalytic activity (Figure 3.4) was not different than control levels. Similarly, no significant effect of SFN was observed for NQO1 activity in adult bone marrow samples (data not shown).

3.4.2 Sub-chronic study: Pregnant and Non-pregnant CD-1 Mice

In our sub-chronic studies, results demonstrated that daily exposure to SFN for 4 weeks had no significant effect on weight of female non-pregnant adult CD-1 mice, and had no significant effect on weight gain in pregnant CD-1 mice from GD1 to GD14 (data not shown). Similarly, daily SFN treatment did not significantly affect the litter size of CD-1 dams, however did result in a significant decrease in the number of resorptions observed compared to PBS-treated dams (Total of 9 resorptions out of 68 pups in the PBS group and 1 resorption out of 59 pups in SFN group; p < 0.05).

Our results also showed that sub-chronic exposure to SFN resulted in no significant differences in \textit{Nrf2} mRNA levels in adult livers taken from either non-pregnant or pregnant mice (Figure 3.5A). Sub-chronic SFN treatment did, however, result in a statistically significant increase (p < 0.05) in \textit{Gstm1}, \textit{Gclc}, and \textit{Nqo1} relative mRNA levels as compared to PBS-treated
Figure 3.2: NQO1 activity in livers of non-pregnant and pregnant CD-1 mice not affected by acute SFN exposure.

A bar graph showing normalized adult NQO1 catalytic activity in liver from either non-pregnant or pregnant CD-1 mice exposed \textit{in vivo} to 0, 50, or 100 mg/kg SFN for 2, 6, or 24 hours.
Figure 3.3: NRF2-regulated genes in CD-1 mouse fetal livers not affected by an acute exposure to SFN in vivo.

qRT-PCR analysis of fetal livers obtained from pregnant CD-1 mice exposed to a single dose of 0, 50, or 100 mg/kg SFN for 2, 6, or 24 hours. Bar graphs showing relative Nrf2 (A), Gstm1 (B), Ho-1 (C), Gclc (D), and Nqo1 (E) mRNA levels in fetal livers from pregnant animals.
Figure 3.4: NQO1 activity in CD-1 mouse fetal livers not affected by an acute exposure to SFN in vivo.

A bar graph showing normalized NQO1 catalytic activity in fetal liver of CD-1 mice exposed in utero to 0, 50, or 100 mg/kg SFN for 2, 6, or 24 hours.
controls in both non-pregnant and pregnant adult liver tissue (Figure 3.5B,C, and E), as well as there was a trend towards an increase in Ho-1 expression in SFN-exposed livers, although this difference was not significant (Figure 3.5D). In non-pregnant mice, both Gstm1 and Nqo1 mRNA levels were increased 1.9 fold, and Gclc mRNA levels were increased 2-fold. In pregnant animals, Gstm1 mRNA levels were increased 2.4 fold, Gclc mRNA levels were increased 1.7 fold, and Nqo1 mRNA levels were increased 1.9 fold. Unlike our acute studies, with sub-chronic SFN treatment there was also a statistically significant 2-fold increase (p < 0.05) in liver NQO1 enzyme activity in both pregnant and non-pregnant mice following daily oral exposure to 65 mg/kg SFN for 30 days (Figure 3.6). In fetal livers, exposure to SFN throughout gestation, however, had no significant effect on Nrf2 (Figure 3.7A), Gstm1 (Figure 3.7B), Ho-1 (Figure 3.7C), Gclc (Figure 3.7D), or Nqo1 (Figure 3.7E) mRNA levels, or NQO1 activity (Figure 3.8) compared to PBS-treated controls. Similarly, daily SFN treatment for 30 days had no effect on NQO1 activity in the bone marrow, spleen, or thymus of adult mice (data not shown).

3.5 Discussion

The present study aimed to determine whether acute or sub-chronic oral SFN treatment affected NRF2-mediated enzyme gene expression and activity levels in maternal and fetal CD-1 mice, and how any effect compared to that measured in non-pregnant female CD-1 mice. As a whole, an increase in Gstm1, Ho-1, Gclc, and Nqo1 mRNA expression was observed in non-pregnant and pregnant adult liver from animals exposed to a single or repeated daily doses of SFN. However, a functional change in NQO1 activity was only observed in animals exposed to SFN daily for 30 days. No effect of SFN treatment was seen in any other tissue analyzed, including fetal liver, and pregnancy status did not significantly influence SFN’s effect on the enzyme expression or activity or NRF2-regulated enzymes.
Figure 3.5: Sub-chronic SFN exposure in vivo increases mRNA levels of NRF2-regulated genes in livers of non-pregnant and pregnant CD-1 mice.

qRT-PCR analysis of livers obtained from non-pregnant or pregnant CD-1 mice exposed daily for 30 days to 0 or 65 mg/kg SFN via oral gavage. Bar graphs showing relative Nrf2 (A), Gstm1 (B), Ho-1 (C), Gclc (D), and Nqo1 (E) mRNA levels in adult livers from either non-pregnant or pregnant animals. (* indicates p < 0.05).
Figure 3.6: Sub-chronic SFN exposure *in vivo* increases NQO1 activity in livers of pregnant and non-pregnant CD-1 mice.

A bar graph showing normalized adult NQO1 catalytic activity in liver from either non-pregnant or pregnant CD-1 mice exposed *in vivo* to 0 or 65 mg/kg SFN for 30 days.

(* indicates p < 0.05).
Figure 3.7: NRF2-regulated genes in CD-1 mouse fetal livers not affected by an acute exposure to SFN in vivo.

qRT-PCR analysis of fetal livers obtained from pregnant CD-1 mice exposed daily for 30 days to 0 or 65 mg/kg SFN via oral gavage. Bar graphs showing relative Nrf2 (A), Gstm1 (B), Ho-1 (C), Gclc (D), and Nqo1 (E) mRNA levels in fetal livers.
Figure 3.8: NQO1 activity in CD-1 mouse fetal livers not affected by an acute exposure to SFN in vivo.

A bar graph showing normalized NQO1 catalytic activity in fetal CD-1 mouse liver following daily exposure in utero via maternal oral gavage to 0 or 65 mg/kg SFN for 30 days.
Our findings showing that SFN induces the expression of mRNA of genes regulated by NRF2 are well supported in the literature. Zhang et al. (1992) also reported an increase in GST and NQO1 activities in CD-1 mice following oral exposure to a higher dose of SFN, although in this study, only non-pregnant CD-1 females were used. Similarly, exposure of rats to as little as 40 µmol/kg/day (approximately 7 mg/kg/day) in one study [348] and at doses as high as 1000 µmol/kg/day (approximately 177 mg/kg/day) in another study [336], led to significantly higher GST and NQO1 activities in numerous tissues examined following SFN treatment. In each of these studies, SFN treatment was given repeatedly, consistent with our study.

The doses of SFN chosen for this study were based on results obtained during pilot studies conducted in our laboratory. In these initial experiments we evaluated the effects of 1, 10, and 50 mg/kg SFN given via IP injection or oral gavage, on alternating days throughout gestation or everyday throughout gestation (up to two weeks), looking primarily at NQO1 activity as an endpoint. No changes were observed at any of these doses or dosing protocols although a dose of 50 mg/kg SFN given by oral gavage led to an increasing trend in NQO1 activity (Appendix C). Therefore, this led us to evaluate 50 mg/kg and 100 mg/kg at the varying time points (2, 6, 24 hours) in the present study. While others have observed effects at lower SFN doses, it is not clear to us why our pilot studies did not demonstrate positive results. As for the sub-chronic dosing, an intermediate dose was chosen for the current study based on the study by Priya and Sakthisekaran (2011), which demonstrated that chronic treatment in mice with 9 µmol/mouse/day SFN over many weeks had no negative consequences and produced a favourable response in preventing cancer initiation [349]. Given the fact that in our study half of the animals were pregnant, thus having weight changes over the course of dosing, we converted 9 µmol/mouse/day to the equivalent mg/kg/day.
Increases in \textit{Gstm1, Ho-1, Geclc, Nqo1} expression, as well as NQO1 activity were modest, averaging around a 2 to 3 fold increase for each gene. However, this doubling of enzyme activity, particularly enzymes involved in detoxification of carcinogens, could have a substantial impact \textit{in vivo}. For example, in a study of benzo[a]pyrene and nicotine-derived nitrosamine ketone (NNK)-induced lung carcinogenesis, treatment with an organoselenium compound resulted in a reduction in lung tumour multiplicity seen in mice [350]. This anti-cancer effect was associated with a 2 to 2.5 fold increase in activity of total GST as well as glutathione peroxidase, which was associated with a reduction in lung tumour multiplicity [350]. While this does not necessarily indicate a causative relationship between the two, it does suggest that a modest increase in detoxification enzymes, including those regulated by NRF2, can lead to a biologically significant change.

In the present study, mRNA levels of genes analyzed were increased at distinct time points following acute exposure to SFN, for example, \textit{Gstm1} expression was increased at both 6 and 24 hours, whereas \textit{Nqo1} expression was increased only at 6 hours post-exposure. Transient changes in expression levels that do not persist could be biologically significant, particularly with regard to developmental landmarks. Given that development is a very strict and time-regimented process, interfering with levels of key proteins and enzymes at distinct times may have significant downstream effects. In this study, an increase in mRNA levels corresponded with an increase in NQO1 enzyme activity in the adult livers from chronically dosed mice, in mice given a single dose of SFN, increases in mRNA levels at 6 and 24 hours did not result in an increase in NQO1 activity. It is important to note that mRNA levels and protein expression/enzyme activity levels do not always parallel each other, as there are several processing steps involved in translating mRNA to protein [351, 352]. It is possible that a single dose of SFN is insufficient to produce a
persistent enough modification in mRNA levels to lead to an increase in NQO1 enzyme activity. Further studies to confirm this are warranted. Similarly, while the observed increase in Gstm1, Gclc, and Nqo1 gene expression and activity suggest that NRF2 activity is increased, we did not see a corresponding increase in Nrf2 mRNA levels, providing another example of how mRNA levels and protein/activity levels do not always correspond. Previous studies have shown that SFN interacts with KEAP1, the protein that is responsible for targeting and degrading NRF2 in the cytoplasm [353]. It is known that SFN interacts with KEAP1, preventing NRF2 degradation, and thus increasing NRF2 stability and translocation into the nucleus [353]. Thus, since SFN affects the NRF2 protein post-translationally, it was not unexpected that an increase in Nrf2 mRNA levels was not observed in the present study. Additionally, since GSTs and NQO1 have been shown to be modulated by other transcription factors, including AP-1 and NFKB, it is possible that SFN induces these genes through one of these pathways, which were not investigated in the present paper.

Significant increases in Gstm1, Gclc, and Nqo1 mRNA expression, as well as NQO1 activity was observed in maternal liver from mice exposed to SFN for 30 days, however no change was observed in fetal liver. While this could be interpreted to be due to the failure of SFN to reach the fetus, one study demonstrated that dietary SFN administered to pregnant B6129SF1 mice led to measurable SFN metabolite concentrations in post-natal day 0 pups, indicating that at least SFN metabolites do indeed pass through the placenta and reach the fetus [266]. Given that we were able to detect measurable levels of these transcripts in fetal livers, as well as measurable NQO1 activity, the lack of fetal induction does not appear to be due to too little enzyme levels in fetal tissue.
Despite seeing no effect of SFN in fetal tissue, it is still possible that maternal SFN treatment could be protective against transplacental carcinogenesis. Administration of other dietary compounds, such as green tea, garam masala, and indole-3-carbinol, another phytochemical derived from cruciferous vegetables, have demonstrated transplacental chemoprevention in different animal models [266, 354, 355]. In B6129SF1 females, treatment with caffeinated green tea, and caffeine alone decreased mortality caused by dibenzo[a,l]pyrene-induced lymphoma in offspring exposed in utero. The authors indicate that the induction of maternal liver enzymes, not fetal enzymes, may reduce bioavailability of this carcinogen to the fetus, suggesting this as a mechanism for chemoprevention [354]. Interestingly, in a study of transplacental carcinogenesis induced by dibenzo[def,p]chrysene, dietary SFN exacerbated tumourigenesis and morbidity caused by this carcinogen in offspring [266]. Whether or not SFN will have a similar effect in our benzene model of transplacental carcinogenesis in CD-1 mice will require further experimentation.

The use of dietary phytochemicals for chemoprevention is growing in popularity at both the basic science level and in clinical trials, however the utility of these compounds in preventing transplacental carcinogenesis is still relatively new. Our study demonstrating that oral SFN treatment to pregnant CD-1 mice induces maternal Gstm1, Ho-1, Gclc, and Nqo1 gene expression and NQO1 activity gives reason to investigate SFN as a potential preventative agent against known and suspected transplacental carcinogens, including our laboratory’s model of benzene-induced transplacental carcinogenesis.
Chapter 4

Investigating the Effects of *In Utero* Benzene Exposure on Epigenetic Modifications in Maternal and Fetal CD-1 Mice


4.1 Abstract

Exposure to the ubiquitous environmental pollutant benzene is positively correlated with leukemia in adults and may be associated with childhood leukemia following *in utero* exposure. While numerous studies implicate oxidative stress and DNA damage as playing a role in benzene-mediated carcinogenicity, emerging evidence suggests that alterations in epigenetic regulations may be involved. The present study aimed to determine whether DNA methylation and/or various histone modifications were altered following *in utero* benzene exposure in CD-1 mice. Global DNA methylation and promoter-specific methylation of the tumour suppressor gene, *p15*, were assessed. Additionally, levels of acetylated histones H3, H4, and H3K56, as well as methylated histones H3K9 and H3K27 were assessed by Western blotting. A significant decrease in global DNA methylation of maternal bone marrow was observed following benzene exposure, however no effect on global DNA methylation was detected in fetal livers. Additionally, no effect of benzene exposure was observed on *p15* promoter methylation or any measured histone modifications in both maternal bone marrow and fetal livers. The results from this study suggest that DNA methylation and histone modifications do not play a significant role in the early stages of transplacental carcinogenesis observed in CD-1 mice following *in utero* exposure to benzene, however further experimentation is warranted to confirm whether these modifications play a role at later stages of benzene-induced carcinogenesis.
4.2 Introduction

Benzene is a pollutant that has a pervasive presence in the environment. It has long been known that benzene causes various blood disorders, including leukemia. Additionally, epidemiological evidence suggests that in utero exposure to benzene is also associated with increased incidence of childhood leukemia [89, 105]. In support of this, a study from our laboratory found that in utero exposure of CD-1 mice to 200 mg/kg benzene on gestational days (GDs) 8, 10, 12, and 14 resulted in increased tumour incidence in the offspring one year after birth [122]. The mechanisms of how benzene exposure leads to leukemia in adults and children are not well understood, although evidence suggests that increased oxidative stress [194, 356] and DNA/chromosomal damage are involved [193, 204, 357]. There is also increasing evidence supporting the role of epigenetic modifications in the process of carcinogenesis, however, this has yet to be extensively explored with respect to benzene-induced carcinogenicity, and in particular benzene-induced transplacental carcinogenesis.

Epigenetic modifications refer to heritable alterations in DNA or associated proteins that do not arise from changes to the gene sequence. Two of the most widely studied epigenetic changes are DNA methylation and histone modifications, including methylation and acetylation. DNA methylation plays an important role in gene expression in both normal and transformed cells, and it is known that DNA methylation patterns may become aberrant in cancer cells [243]. Global DNA hypomethylation, as well as gene-specific hypo- and hypermethylation, are associated with numerous types of cancer, including some forms of leukemia [244-246]. DNA hypomethylation is generally associated with increased gene expression via increased transcription, whereas hypermethylation is generally associated with transcriptional repression [247].
Similarly, specific histone modifications can also be associated with transcriptional repression and activation, and these mechanisms may be dependent on DNA methylation. For example, DNA methylation at CpG islands may interact with histone modifying enzymes, such as histone deacetylases (HDACs), that are involved in chromatin remodeling [249, 250]. Consequently, increased histone acetylation is associated with increased transcription, and the reverse is also generally true. In addition to acetylation, methylation of histones at specific residues has also been found to be associated with either gene silencing or increased gene expression. Methylation of histone H3 at lysine 9 and 27 (H3K9 or H3K27) is associated with transcriptional repression, whereas methylation of H3K4 is associated with transcriptional activation [251]. Other histone modifications have more specific cellular roles, and it is becoming increasingly apparent that certain histone modifications are involved in the cellular response to DNA damage [358, 359]. For example, phosphorylation of histone H2AX (γH2AX) is a well-known early marker of DNA double strand breaks [360]. In addition, researchers have recently identified acetylated histone H3K56 as another histone residue that becomes rapidly and reversibly modified in response to genotoxic cellular stress [361, 362]. At this point, it is unclear whether there is an increase or decrease in acetylation at this residue following DNA damage, as different studies report opposing results [361, 362].

While numerous differences in epigenetic modifications have been noted in various types of cancer compared to normal tissue, it remains to be answered whether these modifications are involved in initiating carcinogenesis or whether they are a consequence of carcinogenesis. However, an increasing number of carcinogens have been shown to result in epigenetic alterations, suggesting that the former may be true. Tobacco smoke, for example, leads to DNA hypomethylation, gene-specific promoter DNA hypermethylation, and alterations in acetylation
and methylation of various histone residues [253]. Similarly, exposures to the known carcinogenic metals, arsenic, nickel, and chromium are associated with changes in DNA methylation patterns, including DNA hypermethylation at a number of specific gene promoters, as well as altered levels of methylation and acetylation of a number of histone residues (reviewed in [363]).

Benzene exposure is also associated with changes in DNA methylation profiles. One study demonstrated that benzene exposure in humans was associated with genome-wide hypomethylation, as well as gene specific hypermethylation of the tumour suppressor gene, P15, and hypomethylation of MAGE1 [254]. MAGE1 is a gene of unknown function, however it encodes a cellular antigen that appears to be specifically expressed only on tumour cells and male germline cells [200]. It is important to note that both of these anomalies have also been observed in acute myelogenous leukemia, which is the type of leukemia most commonly seen in adults following exposure to benzene [257, 364]. Similarly, benzene metabolite exposure in cultured cells has been associated with hypermethylation of DNA repair gene, PARP-1 [239], as well as the candidate tumour suppressor gene, phosphatase and tensin homolog (PTEN) [365]. Exposure of cultured cells to the benzene metabolites hydroquinone or benzoquinone also led to global DNA hypomethylation [255, 256]. In contrast to these data, while exposure of cultured primary murine bone marrow cells to benzoquinone led to a decrease in gene expression of the tumour suppressors p15 and p16, this decreased expression appeared to be independent of promoter methylation [366]. Finally, a study conducted in primary rat bone marrow cells demonstrated that the cytotoxicity of benzene could be prevented by treatment with either 5-aza-2′-deoxycytidine, a methyltransferase inhibitor, or trichostatin A (TSA), a HDAC inhibitor [367]. The results of this
study indirectly suggest that modifications in both methylation and histone acetylation patterns could be involved in the cytotoxicity observed following exposure to benzene or its metabolites.

In the present study, we aimed to investigate whether exposure to a transplacentally carcinogenic dose of benzene led to modifications in DNA methylation or histone acetylation and methylation in fetal livers and maternal bone marrow in CD-1 mice.

4.3 Materials and Methods

4.3.1 Animal Breeding and Treatment

Four to six week old CD-1 mice were purchased from Charles River Canada (Montreal, Canada) and housed in the Queen’s University Animal Care Facilities for one week prior to breeding. CD-1 mice were maintained in a temperature-controlled room with a 12 hour light/dark cycle, were fed standard rodent chow (Purina Rodent Chow, Ralston Purina International, Strathroy, ON, Canada), and provided with tap water ad libitum. Females were bred in a 2:1 ratio with male CD-1 mice overnight, and presence of a vaginal plug the next morning was designated GD1. Beginning on the morning of GD8, mice were dosed with 200 mg/kg benzene dissolved in corn oil, or just the vehicle, via IP injection. Dams were dosed on GDs 8, 10, 12, and 14, and euthanized via cervical dislocation 2, 6, or 24 hours following the final dose. Maternal bone marrow was flushed from humeri with ice cold PBS (pH 7.4) and centrifuged at 1200 rpm for 3 minutes. Supernatant was removed and discarded and the bone marrow pellet was flash frozen before storage at -80°C. Fetal livers were also excised, flash frozen, and stored at -80°C until use. All animal handling and procedures were done in accordance with the guidelines of the Canadian Council on Animal Care and the Queen’s University Animal Care Committee.
4.3.2 DNA Extraction and Global DNA Methylation Quantification

DNA was extracted from maternal bone marrow and fetal livers using the Qiagen DNeasy Kit (Qiagen, Mississauga, ON) according to the manufacturers instructions. Global methylation was detected using MethylFlash™ Methylated DNA Quantification Kit (Colorimetric; Epigentek, NY). For our samples, 100 ng DNA was loaded into each well in duplicate, and the protocol was followed according to the manufacturers instructions. The percentage of 5-methylcytosine of total DNA was calculated as outlined by the manufacturer.

4.3.3 Methylation of the p15 Promoter

Methylation of the p15 gene promoter region was assessed using the Qiagen EpiTect Methyl II PCR assay for p15 according to the manufacturers instructions (Qiagen, Mississauga, ON). Briefly, 125 ng genomic DNA was incubated with methylation-sensitive and/or methylation-dependent restriction enzymes or neither. These restriction enzymes digest unmethylated and methylated DNA, respectively. Using p15 promoter region-specific primers (Qiagen; EPMM107784-1A), the DNA product of each enzyme reaction was amplified using real time PCR and the relative fractions of methylated and unmethylated DNA were calculated by comparing the amount of each digest with the amount of DNA in each sample after no enzyme digestion using a delta Ct method.

4.3.4 Histone Western Blotting and Quantification

Histones were acid extracted according to a protocol produced by Abcam (Cambridge, United States). Briefly, maternal bone marrow and fetal livers were homogenized in triton extraction buffer, centrifuged at 4°C, and extracted in 0.2 N HCl overnight. Samples were centrifuged the next morning and a Bradford assay was used to quantify protein concentrations. A total of 2 µg histones were separated on a 15% polyacrylamide gel, transferred to a PVDF
membrane, blocked for 1 hour in 5% BSA, and probed with antibodies detecting acetylated histone H3 (1:15000; Upstate Biotechnology, Billerica, United States), hyperacetylated histone H4 (1:15000; Upstate Biotechnology), anti-trimethyl histone H3K27 (1:40000; Abcam), anti-monomethyl histone H3K9 (1:10000; Abcam), anti-trimethyl histone H3K9 (1:20000; Abcam), or anti-trimethyl H3K27 (1:40000; Millipore, Billerica, MA) antibodies overnight at 4°C. For anti-acetyl H3K56, 5 µg histones were separated on a 15% polyacrylamide gel, transferred to a PVDF membrane, and blocked in 5% skim milk for 1 hour, followed by incubation with the primary antibody (1:400; Millipore) overnight. All membranes were incubated with anti-rabbit secondary antibodies for 1 hour (1:10000) in their respective blocking solutions and visualized using an enhanced chemiluminescence kit (Perkin Elmer, Boston, United States). Total histone H3 or histone H4 were probed as loading controls (dilution 1:5000; Upstate Biotechnology). Densitometry was conducted using ImageJ software (NIH, Bethesda, MD) to quantify the relative optical densities of the bands.

4.3.5 Statistical Analysis

Global methylation levels and levels of histone modifications were measured separately in three fetal livers and values were averaged to equal an n of 1. Five dams were used per treatment group per time point (2, 6, and 24 hours). Two-way ANOVAs were used to determine whether any statistical differences were present between treatment groups and time points for maternal bone marrow and fetal livers separately. GraphPad Prism 6 was used to conduct all statistical analyses.
4.4 Results

4.4.1 Global DNA Methylation

A significant decrease in global DNA methylation was observed in bone marrow DNA extracted from dams exposed to 200 mg/kg benzene throughout gestation (Figure 4.1A). *In utero* benzene exposure, however, had no effect on global methylation of DNA extracted from GD14 fetal livers (Figure 4.1B).

4.4.2 Methylation of the *p15* Promoter

No differences in the methylation of the *p15* promoter were found between extracted bone marrow from dams exposed to benzene and the vehicle control (Figure 4.2A). Similarly, no effect of benzene exposure was found on the level of *p15* methylation in fetal livers of exposed mice compared to those exposed to the vehicle control (Figure 4.2B).

4.4.3 Acetylated Histones H3, H4, and H3K56

Levels of acetylated histone H3 were not different between benzene-exposed maternal bone marrow (Figure 4.3A) or fetal livers (Figure 4.3B) compared to their vehicle-exposed counterparts. Similarly, no difference in levels of acetylated histone H4 was observed in benzene exposed maternal bone marrow (Figure 4.4A) or fetal livers (Figure 4.4B) at any of the measured time points. No difference in levels of acetylated H3K56 was observed in the bone marrow (Figure 4.5A) or fetal livers (Figure 4.5B) of mice exposed to benzene.

4.4.4 Methylated Histone H3K9 and H3K27

No differences in levels of mono- or tri-methylated histone H3K9 were observed in benzene exposed maternal bone marrow (Figure 4.6A and 4.7A) or fetal livers (Figure 4.6B and 7B) at any of the measured time points. Similarly, no differences in levels of tri-methylated
Figure 4.1: *In vivo* benzene exposure decreases global DNA methylation in CD-1 mouse maternal bone marrow.

The effects of *in utero* exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on global DNA methylation in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. *p* < 0.05.

Figure 4.2: *p15* promoter DNA methylation in CD-1 mouse maternal bone marrow or fetal livers not affected by *in vivo* benzene exposure.

The effects of *in utero* exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on *p15* promoter DNA methylation in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene.
Figure 4.3: Levels of acetylated histone H3 in CD-1 mouse maternal bone marrow or fetal livers not affected by \textit{in vivo} benzene exposure.

The effects of \textit{in utero} exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on levels of acetylated histone H3 in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. Western blots of acetylated histone 3 levels were normalized to total H3 levels and then expressed relative to control levels.

Figure 4.4: Levels of acetylated histone H4 in CD-1 mouse maternal bone marrow or fetal livers not affected by \textit{in vivo} benzene exposure.

The effects of \textit{in utero} exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on levels of acetylated histone H4 in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. Western blots of acetylated histone 4 levels were normalized to total H4 levels and then expressed relative to control levels.
Figure 4.5: Levels of acetylated histone H3K56 in CD-1 mouse maternal bone marrow or fetal livers not affected by \textit{in vivo} benzene exposure.

The effects of \textit{in utero} exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on levels of acetylated histone H3K56 in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. Western blots of acetylated histone H3K56 levels were normalized to total H3 levels and then expressed relative to control levels.
Figure 4.6: Levels of monomethylated H3K9 in CD-1 mouse maternal bone marrow or fetal livers not affected by in vivo benzene exposure.

The effects of in utero exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on levels of monomethylated histone H3K9 in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. Western blots of monomethylated H3K9 levels were normalized to total H3 levels and then expressed relative to control levels.

Figure 4.7: Levels of trimethylated histone H3K9 in CD-1 mouse maternal bone marrow or fetal livers not affected by in vivo benzene exposure.

The effects of in utero exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on levels of trimethylated histone H3K9 in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. Western blots of trimethylated H3K9 levels were normalized to total H3 levels and then expressed relative to control levels.
histone H3K27 were detected in benzene exposed maternal bone marrow (Figure 4.8A) or fetal livers (Figure 4.8B) at any of the measured time points compared to vehicle-exposed maternal bone marrow and fetal liver.

4.5 Discussion

In the present study, we investigated the effects of in utero benzene exposure in CD-1 mice on DNA methylation and histone modifications, specifically methylation and acetylation, in maternal bone marrow and fetal livers. While hypomethylation was observed in maternal bone marrow following exposure to benzene, no other differences were detected between benzene-exposed and vehicle control groups in maternal or fetal tissue.

Decreased global DNA methylation has been observed following benzene exposure in both in vitro studies [255, 256], as well as in the lymphocytes of individuals exposed to low doses of benzene [254, 368]. Our study, however, demonstrates for the first time that this finding can be repeated following in vivo administration of benzene in an animal model. Global DNA hypomethylation has been found in numerous types of cancer, including acute myelogenous leukemia (AML; reviewed in [369]), the subtype of leukemia most commonly found in humans following exposure to benzene. While the mechanism of how benzene exposure results in genome-wide hypomethylation is not yet elucidated, one study did demonstrate inhibition of the enzyme DNA methyltransferase (DNMT) following exposure to benzoquinone, a metabolite of benzene [255]. This, however, contradicts the finding that DNMT activity is typically increased in AML [370]. Another mechanism by which benzene exposure may lead to DNA hypomethylation is by increasing reactive oxygen species (ROS) levels. For example, ROS can cause oxidative DNA damage, which can physically interfere with the ability of DNMT, the enzyme responsible for transferring methyl groups to DNA, to interact with DNA [189]. ROS
Figure 4.8: Levels of trimethylated histone H3K27 in CD-1 mouse maternal bone marrow or fetal livers not affected by *in vivo* benzene exposure.

The effects of *in utero* exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 via IP injection on levels of trimethylated histone H3K27 in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. Western blots of trimethylated H3K27 levels were normalized to total H3 levels and then expressed relative to control levels.
have also been found to increase levels of DNMT activity during malignant transformation of melanocytes [188]. Despite demonstrating decreased global DNA methylation in maternal bone marrow following benzene exposure, no effect was seen in livers of fetuses exposed in utero to benzene in the present study. This was unexpected, given the observed decrease in maternal bone marrow global DNA methylation, as well as the finding that in utero exposure to cigarette smoke in humans was associated with global DNA hypomethylation in buccal cells of exposed children [371]. Cigarette smoke, however, contains numerous chemicals in addition to benzene, which may be contributing to this finding. The negative results in our study could be an issue of benzene dose, although the dose used in this study is high and is known to reach fetal liver in this animal model [122]. Alternatively, the lack of effect of benzene on DNA methylation and other measures of epigenetic modifications in the present study may be due to the timing of tissue analysis. GD14 mouse fetal livers were chosen for analysis in this study because hematopoietic stem cells are at their highest concentration on this GD in mice [305, 372]. Since benzene is both a hepatic and a hematopoietic carcinogen in CD-1 mice, both susceptible cell types are present in these samples at this time point. However, the process of carcinogenesis is lengthy, and it is possible that any epigenetic changes that may have occurred to a small number of critical cells during benzene exposure might not be detectable until later in the process of carcinogenesis. Measurements of DNA methylation and histone modification at later stages of carcinogenesis (ie: during post-natal life) may provide valuable insight as to the involvement of epigenetic modifications in the carcinogenesis caused by in utero exposure to benzene.

The tumour suppressor gene, \( p15 \), also known as cyclin-dependent kinase 4 inhibitor B (\( CDKN2B \)) or multiple tumour suppressor gene 2 (\( MTS2 \)), encodes a protein involved in cell-cycle regulation, specifically inhibiting cyclin-dependent kinase 4 (CDK4) and CDK6 involved in
cell cycle progression [373]. *P15* has been found to be deleted in numerous types of leukemias, primarily those derived from cells of the lymphoid lineage [374]. Additionally, some researchers have demonstrated a silencing of *P15* by hypermethylation of CpG islands in the promoter region of this gene in both adults [375, 376] and children [377] with hematological malignancies. Our data demonstrated no change in methylation levels of *p15* in either maternal bone marrow or fetal livers following exposure to benzene. There are a number of reasons that could contribute to this negative result. Firstly, while benzene has been demonstrated in some studies to cause hypermethylation of the *p15* promoter, this finding is not consistent. For example, one study did see decreased mRNA transcript levels of *p15* in mouse bone marrow cells following exposure to benzene metabolites, however this was not mediated by promoter methylation [366]. In addition, while low dose exposure to benzene resulted in hypermethylation of *P15* in exposed subjects in one study [254], another study found the opposite; a significant decrease in methylation of the *P15* promoter was correlated to benzene exposure in petrochemical workers [378]. Together, these studies indicate that *p15* is not consistently hypermethylated following exposure to benzene or its metabolites and that the precise timing at which promoter methylation is measured may be critical. Further study should investigate whether benzene exposure alters promoter methylation patterns of *p15* in later stages of carcinogenesis in the bone marrow of exposed offspring.

Secondly, *p15* promoter methylation has been found to be highly specific to hematopoietic malignancies in humans [375-377]. While *p15* promoter methylation has also been found in hematological malignancies in a murine model previously [379], benzene is not specifically a hematological carcinogen in mice. Instead, it is a multipotential carcinogen in adult mice, resulting in tumour formation in a number of organs [112, 113], and has been found to be both a hepatic and hematopoietic carcinogen to *in utero* exposed CD-1 mouse offspring [122]. Further
study should investigate whether benzene exposure alters promoter methylation of other epigenetically-regulated tumour suppressor genes. Lastly, the detection method used in this study looked at one specific CpG island in the promoter region of \textit{p15}. It is possible that there are other CpG islands in the promoter region of \textit{p15} that could be methylated and that were not detected with the method used in this study.

Aberrant global histone modifications are characteristic of various cancers, having been identified in lung, prostate, pancreatic, ovarian, and breast cancers (reviewed in [380]). Additionally, some carcinogens have been shown to alter histone modifications, including benzo[a]pyrene [381, 382], carcinogenic metals [363], PhIP, and 4-aminobiphenyl (ABP) [383] in \textit{in vitro} models. Our study was the first to directly determine whether benzene exposure altered levels of global histone methylation or acetylation in murine bone marrow or fetal livers \textit{in vivo}, and results demonstrated no effect of benzene exposure on any form of histone modification measured in either tissue type. This contradicts the results of one study that found that treatment with the HDAC inhibitor, TSA, attenuated cytotoxicity of benzene in cultured cells [367], however levels of histone modifications were not directly measured. Although no change in global histone modifications were found following benzene exposure in the present study, it is possible that modifications at specific histone residues in the vicinity of specific gene promoters may be altered. Further experimentation is required to confirm these suspicions.

In conclusion, this study found few alterations in the DNA methylation or histone modifications measured in this study in the livers of CD-1 mouse fetuses exposed to benzene \textit{in utero}. While hypomethylation was observed in maternal bone marrow following exposure to benzene, the lack of evidence of epigenetic alterations in fetal tissue suggests that these global epigenetic modifications may not play a prominent role in the initial phases of benzene-mediated
transplacental carcinogenesis. However, future studies investigating these epigenetic marks during adulthood of the exposed offspring are warranted to determine whether epigenetic dysregulation occurs later in the process of carcinogenesis.
Chapter 5

General Discussion

5.1 Summary

The overall hypothesis of this thesis was that benzoquinone and/or benzene exposure would result in increased ROS, DNA damage, and epigenetic alterations following exposure to GD14 fetal liver cells in vitro or in utero exposure compared to appropriate non-treated controls. Additionally, we hypothesized that SFN treatment would induce NRF2-regulated genes in cultured fetal liver cells or in fetal livers that would confer protection against benzene-mediated toxicity. The data presented in Chapter 2 demonstrate that benzoquinone exposure led to increased ROS levels, increased oxidative DNA damage, and increased DSBs in cultured CD-1 mouse GD14 fetal liver cells. Additionally, benzoquinone exposure significantly decreased gene expression of the BER gene, Ogg1. None of these observed benzoquinone-induced changes were prevented by pretreatment with SFN. Chapter 3 describes our evaluation of whether SFN would induce NRF2-mediated gene expression and NQO1 activity in pregnant CD-1 mice and fetal livers, and whether this differed from induction in non-pregnant CD-1 mice. SFN induced NRF2-mediated genes in the livers of both pregnant and non-pregnant CD-1 mice, with no statistically significant differences between the two. However, SFN had no effect on gene expression or activity of NRF2-regulated genes in fetal livers. Finally Chapter 4 describes our investigation of the effects of benzene exposure on DNA methylation and histone methylation or acetylation in maternal and fetal CD-1 mice. While benzene exposure led to a significant decrease in global DNA methylation in maternal bone marrow, no change in global DNA methylation was observed in fetal livers. Additionally, benzene had no effect on promoter methylation of the gene, p15, or
global histone methylation or acetylation in either CD-1 mouse maternal bone marrow or fetal livers.

5.2 Mice as a Model for Benzene-Mediated Carcinogenesis

In humans, benzene exposure is strictly associated with neoplasms of the hematopoietic system, primarily AML and more recently identified, NHL [74, 75]. While benzene has been found to cause leukemia and lymphoma in mice [113-117], it is also a multipotential carcinogen in mice, resulting in tumour formation in the Zymbal gland, oral and nasal cavities, forestomach, skin, mammary glands, liver, and lungs [112, 114]. This raises the question of whether mice are an appropriate surrogate for studying benzene-induced hematotoxicity and leukemogenesis in humans.

There are numerous similarities between mice and humans that contribute to the suitability of mice as a model organism for the study of transplacental benzene exposure. For example, of all the CYP enzymes, CYP2E1 expression and activity are the most conserved between mice and humans [384, 385]. Specifically, CYP2E1 is 80% conserved between the two species, in contrast to other CYP isoforms [385], for example CYP2C, that has distinct members and varying roles within each species [384]. Additionally, the ontogenies of numerous drug-metabolizing enzymes (both Phase I and II enzymes) show similar patterns of expression throughout development between both humans and mice (discussed in section 1.4.2), suggesting that metabolism of benzene would closely parallel each other in both species.

Studies using mice to investigate the mechanisms of benzene-mediated hematotoxicity and carcinogenicity have provided a large body of the mechanistic information that we currently have about the negative health effects associated with benzene exposure. For example, mouse studies confirmed the causative association between benzene exposure and carcinogenicity [112],
as well as identified the importance of CYP2E1 metabolism in benzene-induced carcinogenesis [168-170]. Additionally, exposure of mice to benzene identified oxidative DNA damage as a consequence of benzene exposure [205], which was subsequently confirmed in samples extracted from benzene-exposed humans [209]. Finally, exposure of benzene in animals has provided insight into new directions to investigate with respect to benzene carcinogenicity. For example, studies conducted in mice were the first to suggest the importance of properly functioning DNA repair pathways to counteract hematotoxicity associated with benzene exposure [224], which was later confirmed in humans, where an association between SNPs of certain repair genes and increased incidence of benzene-associated hematotoxicity was observed [241, 242].

However, while utilizing mice has been critical to our understanding of benzene hematotoxicity and carcinogenicity, some differences in the effects of benzene at the molecular level in mice and humans exist. For example, DNA adducts following benzene exposure appear to lead to different mutation types in mice and humans [218], although the impact of this difference has not yet been explored. Interestingly, the physiological or cellular differences that may be contributing to disparate effects of benzene in mice and humans have not been well studied. With regards to benzene’s developmental effects, there are a number of distinctions that could contribute to the varying effects observed between humans and mice. For example, morphogenesis of the placenta differs considerably between mice and humans [386], which may contribute to differential transfer of benzene or its metabolites to the fetus. Similarly, while the transition between tissues responsible for hematopoiesis throughout development is remarkably similar between mice and humans (section 1.2.1), the time frame differs considerably, with the bone marrow becoming the primary site of hematopoiesis around the midpoint in human gestation, whereas in mice it occurs primarily at the end of gestation and in post-natal life [29].
Thus it is important to consider that while the impact of these discrepancies on the fate of benzene and its effects on each species has yet to be defined, data attained from murine studies may need to be interpreted with some caution when considering the implications for the toxicity of benzene in humans.

5.3 Benzene-Induced Transplacental Carcinogenicity

The carcinogenicity associated with benzene has been studied for decades, however its association with \textit{in utero} initiated cancers has not been as well studied. In adults, it is becoming increasingly apparent that numerous mechanisms are likely contributing to benzene-mediated carcinogenesis, which is likely to be the case for \textit{in utero} initiated cancers by benzene as well. Many studies in both adult and \textit{in utero} animal models, as well as human samples, have found that benzene exposure causes increased ROS (discussed in section 1.5.1) and causes various types of DNA damage (discussed in section 1.5.2 and addressed in Chapter 2). Additionally some studies have suggested the involvement of altered epigenetics (discussed in section 1.5.4 and addressed in Chapter 4) or alterations in DNA repair (discussed in section 1.5.3 and addressed in Chapter 2) as being involved in benzene’s carcinogenesis. Other less studied mechanisms not discussed in this thesis may also play a role in benzene-mediated carcinogenesis, such as alterations in immune function mediators [387] or inhibiting gap-junction cellular communication [388]. Finally, expression levels of critical enzymes involved in benzene toxification (ie: CYP2E1 and MPO) as well as those involved in detoxification (ie: NQO1 and GST) appear to influence the susceptibility of an individual to benzene-mediated hematotoxicity or carcinogenicity.

Given the potential for several different variables associated with benzene-initiated toxicity, it is difficult to delineate the relative contribution of each to the processes of
carcinogenicity and hematotoxicity. It is likely, however, that a “multi-hit” model may be necessary to induce carcinogenicity by benzene [134]. If this is the case in adults, then it is also likely to be the situation for benzene-associated transplacental carcinogenicity. If this were the case, an accumulation of cellular assaults from benzene and its metabolites could lead to cellular transformation of fetal hematopoietic progenitor cells and consequently the development of leukemia. This hypothesis is supported by the finding that in humans, childhood leukemias (both ALL and AML) are often initiated in utero, with characteristic chromosomal translocations and other genetic abnormalities present in the blood of newborns collected at birth [389, 390]. As previously stated, since the etiology of only 10% of childhood leukemia cases are known [42], it is plausible that in utero exposures, including exposure to chemicals such as benzene, may play a significant role in the development of childhood leukemias. Thus, while there is an extensive body of literature related to benzene carcinogenesis, the very possible association of benzene exposure resulting in in utero initiated leukemia in humans is much less well-studied, which is the basis for the research presented in this thesis.

5.4 Sulforaphane and NRF2 Paradox

There is no denying the abundance of studies reporting that consumption of SFN-containing cruciferous vegetables is protective against the development of cancer in humans [271-273, 275, 279]. Additionally, numerous studies have demonstrated the protective effects of SFN against carcinogen-mediated tumourigenesis in laboratory animals [267-270]. As outlined throughout this thesis, one of the major mechanisms by which SFN is proposed to exert its anti-carcinogenic effects is via the potent activation of NRF2 and the consequential upregulation of downstream targets involved in managing cellular stress. However, NRF2 has also been demonstrated to be upregulated in numerous types of cancers [391, 392], which is hypothesized
to contribute to acquired chemoresistance of malignant cells [393, 394]. Thus, the timing of SFN exposure could have opposing effects with respect to its effects on tumourigenesis. Specifically, if SFN is given prior to the initiation of the cancer process, it may be beneficial and confer protection. However, if given once cancer has been initiated and a cell has been transformed, NRF2 upregulation mediated by SFN may contribute to the malignancy of the tumour.

Studies investigating the biological effects of SFN have also reported conflicting data. Related to its anti-carcinogenic activity, SFN exposure has been associated with protection against increased ROS production and DNA damage levels associated with xenobiotic exposure [318, 319, 395, 396]. However, as discussed in Chapter 2, SFN induces or exacerbates ROS production, induces DNA damage, and increases cytotoxicity [321, 322]. These findings are at odds given that SFN is being touted as an anti-carcinogenic agent, yet these molecular events have all been implicated in cancer. A possible explanation for this contradiction is that the biological effects of SFN effects appear to be partially dose-dependent [397, 398]. For example, one study found protective effects of SFN at 0.25 and 1 µM in human mesenchymal stem cells, reducing senescence and apoptosis, however exposure of these cells to 5 and 20 µM SFN had opposing effects [397]. In another study, exposure of rat aortic smooth muscle cells to 1, 2.5, or 5 µM dose-dependently induced Phase II enzyme expression and protected against oxidative stress-induced cytotoxicity caused by exposure to a number of exogenous agents, including H₂O₂, finding no evidence of deleterious effects of SFN [318]. In contrast, as described in Chapter 2 of this thesis, cultured CD-1 mouse fetal liver cells exposed to 2.5 to 5 µM SFN induced Phase II enzyme gene expression and NQO1 activity, yet also resulted in cytotoxicity at a concentration of 5 µM. This suggests a very narrow therapeutic window for SFN’s protective effects in this model, with higher concentrations leading to deleterious effects. Taken together, the results
presented in this thesis in combination with other reported studies suggest that the effects of SFN may be cell type specific in addition to being concentration/dose-dependent, which may lead to difficulty in determining the utility of SFN in protecting or treating cancer. Therefore, before SFN can be employed as a chemopreventative agent or as a potential cancer treatment, its molecular effects in both \textit{in vitro}, and perhaps more importantly \textit{in vivo}, systems need to be more thoroughly addressed and elucidated.

5.5 Future Directions

5.5.1 Further Investigating SFN or Other Phytochemicals as Potential Protective Agents

While my thesis research investigated the potential protective effects of SFN \textit{in vitro} and found no protective effects against benzoquinone-mediated toxicity, these data are only partially predictive of what could occur in an \textit{in vivo} model. Given that benzene is thought to be extensively metabolized in the maternal liver prior to transport to the fetus, if maternal \textit{Nrf2} activation leads to increased detoxification of benzene before reaching the fetus, this could confer protection. Using the SFN dosing protocol delineated in Chapter 3 in conjunction with the benzene dosing protocol found to cause tumours following \textit{in utero} exposure, SFN could be tested as a potential anti-carcinogenic agent in this model. Only one other study has investigated SFN as a protective agent against cancer initiated \textit{in utero}, and this study found that SFN unexpectedly exacerbated tumour multiplicity and mortality of mouse offspring exposed to dibenzo[\textit{def,p}]chrysene \textit{in utero} [266]. Interestingly, this study did find a protective effect of indole-3-carbinol, another phytochemical also derived from cruciferous vegetables [266]. This is particularly perplexing given that most phytochemicals have a number of similarities in their molecular mechanisms that are thought to contribute to their anti-carcinogenic properties [399]. Therefore, I suggest that future studies in our laboratory should investigate other phytochemicals
in addition to SFN as potential preventative agents against benzene-induced carcinogenicity in *in utero* exposure studies, since it appears that there may be critical differences between the mechanisms of action of different phytochemicals.

5.5.2 Benzene-Associated DNA Damage and Repair

The study described in Chapter 2 investigated whether benzoquinone led to DNA damage, and measured transcript levels of DNA repair genes known to be involved in the repair of either oxidative DNA damage or DSBs *in vitro*. Our results demonstrated that *Ogg1* expression levels were significantly decreased following exposure to benzoquinone. In future studies, this relationship could be explored in more detail *in vitro*, confirming that *Ogg1* transcript levels result in decreased protein expression and activity of this protein. Further studies could also evaluate *Parp-1* levels, which were also decreased following benzoquinone exposure, although not significantly. Additionally, while benzoquinone is a major reactive metabolite of benzene, due to the complexity of benzene metabolism, it is more likely that a cell would be exposed to numerous benzene metabolites concurrently following *in vivo* exposure. Therefore, to better mimic the *in vivo* scenario, further studies using cultured cells should investigate the effects of mixtures of benzene metabolites as opposed to one metabolite specifically. Benzene metabolites have been demonstrated to perpetuate each others’ toxicities [400, 401], therefore concentrations of metabolite combinations at more relevant exposure levels may result in negative changes, perhaps more so than what would be seen following exposure to a single benzene metabolite.

Investigation of the effects of benzene on DNA damage and aberrations in DNA repair should also be conducted *in vivo*. Given that the direct exposure of fetal liver cells to benzoquinone in culture resulted in an increase in the mutagenic DNA lesions, 8-OHdG and
DSBs, it is important to confirm whether these same effects occur in our *in vivo* model following benzene exposure. Importantly, protein levels and/or activity levels of both OGG1 and PARP-1 should be measured in an *in vivo* system given that they appeared to be the most effected in the *in vitro* setting.

### 5.5.3 Characterization of GD14 Fetal Liver Cells

A couple of recent studies in our laboratory, including those described in Chapter 2, have been conducted in cultured murine GD14 fetal liver cells. These cells provide an interesting model for investigating the implications of benzene exposure on critical fetal tissues. As previously mentioned, mouse GD14 fetal livers contain the highest population of hematopoietic stem cells that the liver will possess during gestation prior to hematopoiesis migrating to the spleen, and then finally the bone marrow [305, 372]. Since benzene was found to be both a hematopoietic and hepatic carcinogen in offspring exposed to benzene *in utero*, culturing fetal livers from GD14 mice allows us to expose both susceptible cell types to benzene metabolites together. However, while much is known about the hematopoietic microenvironment of fetal liver, the cell types that exist and thrive in our culture system have not been characterized. While it is clear from our study that these cells are responsive to both benzoquinone and SFN exposure, the specifics of which cell types and the ratios that each cell type exist in in this culture is not defined, and should be focused on in future studies, particularly if our laboratory is going to continue culturing these cells to elucidate benzene’s mechanisms of action.

### 5.5.4 Next-Generation Sequencing of Benzene-Exposed Fetal Livers

Microarrays for gene expression have been conducted on both human samples from individuals exposed to benzene, as well as on murine bone marrow following exposure to benzene [402-404]. These data have provided valuable information pertaining to the effects of
benzene exposure; however, a large-scale analysis of gene expression of benzene-exposed murine fetal livers has not been conducted. Due to distinct differences between adults and fetuses, such as differential expression of benzene-metabolizing enzymes and differing locations of hematopoiesis, benzene may have varying effects on targets in fetuses compared to adult mice. Thus, conducting a wide scale analysis of gene expression using next-generation sequencing of fetal livers following exposure to benzene versus those exposed to the vehicle-control in utero could provide new insight into pathways that are affected by benzene exposure.

5.6 Conclusions

This thesis describes results obtained following the evaluation of the effects of benzene in both in vitro and in vivo models of developmental carcinogenesis. The data presented in this thesis partially support the proposed hypotheses. To reiterate, the first hypothesis was that benzene or benzoquinone exposure would result in increased ROS, DNA damage, and altered epigenetics in CD-1 mouse fetal livers. The second hypothesis was that SFN pretreatment would induce NRF2-mediated gene expression and activity, and would confer protection against any incurred toxicity. In addressing the first hypothesis, while we observed increased ROS levels and DNA damage, as well as altered gene expression of DNA repair genes following exposure to benzoquinone in cultured fetal liver cells, in utero benzene exposure had little effect on the evaluated epigenetic modifications in fetal livers of CD-1 mice. With respect to the second hypothesis, SFN exposure induced enzymes involved in benzene detoxification in cultured CD-1 mouse fetal liver cells, however only induced these enzymes in maternal liver following in utero exposure. Additionally, SFN conferred no protection against benzoquinone-induced toxicity in cultured CD-1 mouse fetal liver cells. The findings from this thesis do provide valuable information for the basis of future studies, suggesting that DNA damage and altered DNA repair
may be more involved in benzene’s toxicity rather than epigenetic alterations in this model. Additionally, the data presented in this thesis indicate that SFN needs to be further studied before being applied as a preventative treatment for \textit{in utero}-initiated cancer caused by benzene exposure.
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Appendix A

Introduction

Sulforaphane (SFN) is a phytochemical that is being pursued as a chemopreventative agent. SFN has been demonstrated to have numerous cellular effects that are thought to contribute to its protective effects. This includes evidence that *in vitro*, SFN exposure is linked to down-regulation of DNA methyltransferases (DNMTs) [1,2]. DNMTs are proteins that transfer methyl groups to DNA, leading to increased DNA methylation, typically associated with decreased gene expression when CpG islands of promoter regions of genes are methylated. We were interested in investigating whether SFN exposure also led to decreased expression of DNMTs in cultured CD-1 mouse fetal liver cells.

Materials and Methods

Gestational Day 14 CD-1 Mouse Fetal Liver Cell Culture

As described in Chapter 2, gestational day (GD) 14 fetal liver cells were extracted from pregnant CD-1 dams and cultured in supplemented IMDM. Cells were plated at a density of 0.5 million cells/ml in 10 cm treated cell culture plates and exposed to 0, 0.5, 1, 2.5, or 5 µM SFN. Cells were collected after 24 hours SFN exposure, and RNA was extracted using the RNeasy RNA extraction kit (Qiagen, Mississauga, ON). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Burlington, ON). qRT-PCR reactions were carried out using specific TaqMan gene expression probes (Life Technologies, Burlington, ON) for mouse DNA methyltransferase 1 (*Dnmt1*), DNA methyltransferase 3A (*Dnmt3a*) and DNA methyltransferase 3B (*Dnmt3b*). Data were normalized using the same methods as described in Chapter 2.
qRT-PCR

qRT-PCR reactions were carried out using specific TaqMan gene expression probes (Life Technologies, Burlington, ON) for mouse DNA methyltransferase 1 (Dnmt1), DNA methyltransferase 3A (Dnmt3a) and DNA methyltransferase 3B (Dnmt3b). Data was normalized using the same methods as described in Chapter 3.

Results

Exposure of cultured GD14 CD-1 mouse fetal liver cells to up to 5 µM SFN had no effect on gene expression of Dnmt1 (Figure A.1A) or Dnmt3a (Figure A.1B), however the 5 µM SFN concentration resulted in a significant decrease in gene expression of Dnmt3b (Figure A.1C).

![Graphs showing gene expression levels](image.png)

Figure A. 1: Relative Dnmt3b (A), Dnmt1 (B), and Dnmt3a (C) transcript levels following exposure of cultured GD14 fetal liver cells to 0 – 5 µM SFN in vitro for 24 hours.

Conclusions

High exposure concentrations (5 µM) of SFN resulted in a significant decrease in gene expression of Dnmt3b in cultured CD-1 mouse fetal liver cells, which is consistent with the
literature. Investigations into whether this decreased gene expression occurs in vivo are warranted.

References


Appendix B

Introduction

Stemming from my findings described in Appendix A, I was interested in investigating whether SFN had an effect on Dnmt gene expression of CD-1 mouse maternal or fetal liver exposed to SFN in utero.

Materials and Methods

In vivo CD-1 Mouse Chronic Exposure to SFN

As described in detail in Chapter 3, pregnant CD-1 mice were exposed to 65 mg/kg SFN daily via oral gavage for two weeks prior to breeding and two weeks post-breeding for a total of 4 weeks of exposure. Pregnant dams were euthanized on GD14 of pregnancy. RNA was extracted and cDNA was synthesized from maternal livers as well as fetal livers as described in Chapter 3.

qRT-PCR

qRT-PCR reactions were carried out using specific TaqMan gene expression probes (Life Technologies, Burlington, ON) for mouse DNA methyltransferase 1 (Dnmt1), DNA methyltransferase 3A (Dnmt3a) and DNA methyltransferase 3B (Dnmt3b). Data were normalized using the same methods as described in Chapter 3.

Results

Exposure of pregnant CD-1 mice to 65 mg/kg SFN for 4 weeks resulted in a statistically significant increase in Dnmt1 gene expression (Figure B.1A), no effect on Dnmt3a gene expression (Figure B.1B), and a non-significant increase in Dnmt3b (Figure B.1C) gene.
expression in maternal livers. *In utero* SFN exposure had no effect on gene expression of *Dnmt1* (Figure B.2A), *Dnmt3a* (Figure B.2B), or *Dnmt3b* (Figure B.2C) in fetal livers.

![Figure B. 1: Relative *Dnmt1* (A), *Dnmt3a* (B), and *Dnmt3b* (C) transcript levels in maternal liver following exposure to 65 mg/kg SFN or the vehicle, PBS, daily for 4 weeks.](image)

![Figure B. 2: Relative *Dnmt1* (A), *Dnmt3a* (B), and *Dnmt3b* (C) transcript levels from GD14 livers extracted from dams exposed to 65 mg/kg SFN or vehicle, PBS, daily for 4 weeks.](image)

**Conclusion**

Interestingly, while *in vitro* (Appendix A) exposure to SFN resulted in a decrease in *Dnmt* gene expression, *in vivo*, SFN exposure resulted in a significant increase in *Dnmt* gene expression, although this only occurred in maternal liver.
Appendix C

Prior to the study conducted in Chapter 3, a few different dosing regimens were conducted to determine whether sulforaphane (SFN) would induce Nrf2-mediated genes in CD-1 mice in vivo to investigate its utility as a protective agent against benzene-induced toxicity and/or carcinogenicity. The following outlines the dosing protocols conducted and the data attained from these pilot studies.

Intraperitoneal Injection of SFN: Alternating Days

Initially, we employed a dosing regimen that exposed pregnant CD-1 mice (n = 5) to 0, 1, 10, or 50 mg/kg SFN dissolved in PBS (pH7.4) on gestational days 7, 9, 11, and 13 and maternal liver, heart, as well as fetal livers were extracted. These gestational days were chosen based on the dosing protocol used in our lab for benzene exposure, which is IP injection of 200 mg/kg benzene on GDs 8, 10, 12, and 14. NQO1 activity assays were conducted as described in the Materials and Methods of Chapter 3. While maternal liver and fetal livers were the tissues of interest, maternal heart was also assayed as an internal positive control, as NQO1 activity is higher in heart than in liver. The NQO1 activity as a result of this dosing protocol can be seen in Figure C.1.
Intraperitoneal Injection of SFN: Daily

Following the results of the initial study shown above, we decided to next investigate whether 50 mg/kg SFN exposure via IP injection to pregnant CD-1 mice on GDs 7 to 13 inclusive would induce NQO1 activity in CD-1 mouse maternal or fetal liver. The NQO1 activity resulting from this dosing protocol can be seen in Figure C.2.

Figure C. 1: Nqo1 activity levels in maternal heart and liver, as well as livers extracted from male (M) GD14 fetuses and female (F) GD14 fetuses following in utero exposure to 0, 1, 10, or 50 mg/kg SFN on GDs 7, 9, 11, and 13 via intraperitoneal injection.

Figure C. 2: Nqo1 activity levels in maternal heart and liver, as well as livers extracted from male (M) GD14 fetuses and female (F) GD14 fetuses following in utero exposure to 0 or 50 mg/kg SFN on GDs 7 through to 13 daily via intraperitoneal injection.

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Oral Gavage of SFN: Daily

Following the negative results of the two studies shown above, we decided to next investigate whether 50 mg/kg SFN exposure via oral gavage to pregnant CD-1 mice on GDs 7 to 13 inclusive would induce NQO1 activity in CD-1 mouse maternal or fetal liver. The NQO1 activity resulting from this dosing protocol can be seen in Figure C.3.

Figure C. 3: Nqo1 activity levels in maternal heart and liver, as well as livers extracted from male (M) GD14 fetuses and female (F) GD14 fetuses following in utero exposure to 0 or 50 mg/kg SFN on GDs 7 through to 13 via oral gavage.

Conclusions

None of the dosing regimens described above resulted in induction of the activity of the Nrf2-mediated gene, Nqo1. Following these three pilot experiments, we conducted the study outlined in Chapter 3 of this thesis.