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Investigating the effects of *in utero* benzene exposure on epigenetic modifications in maternal and fetal CD-1 mice☆

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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 tumor 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. These results suggest that the methodology used in the present study did not reveal alterations in DNA methylation and histone modifications following *in utero* exposure to benzene; however further experimentation investigating these modifications at the whole genome/epigenome level, as well as at later stages of benzene-induced carcinogenesis, are warranted.

1. 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 (van Steensel-Moll et al., 1985, Steffen et al., 2004). 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 tumor incidence in the offspring one year after birth (Badham et al., 2010). The mechanisms of how benzene exposure leads to leukemia in adults and children are not well understood, although evidence suggests that increased oxidative stress (Badham and Winn, 2010, Subrahmanyam et al., 1991) and DNA/chromosomal damage are involved (Tice et al., 1980, Tung et al., 2012, Zhang et al., 1993). 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 (Feinberg and Vogelstein, 1983). Global DNA hypomethylation, as well as gene-specific hypo- and hypermethylation, are associated with numerous types of cancer, including some forms of leukemia (Deneberg et al., 2010, Galm et al., 2006, Roman-Gomez et al., 2006). DNA hypomethylation is generally associated with increased gene expression *via* increased transcription, whereas hypermethylation is generally associated with transcriptional repression (Jones and Takai, 2001).

Similarly, specific histone modifications can also be associated with transcriptional repression and activation, and these mechanisms may

Abbreviations: γH2AX, phosphorylated histone H2A variant X; TSA, trichostatin A; Parp-1, poly[ADP-ribose] synthase 1; HAT, histone acetyltransferase; Pten, phosphatase and tensin homolog; MAGE1, melanoma antigen 1; HDAC, histone deacetylase; DNMT, DNA methyl transferase; ROS, reactive oxygen species; GD, gestational day; CDKN2B, cyclin-dependent kinase 4 inhibitor 2B; MTS2, tumor suppressor gene 2; CDK, cyclin dependent kinase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5b]pyridine; ABP, 4-aminobiphenyl.

☆ Preliminary results from these studies have been previously presented at the 45th Annual Symposium of the Society of Toxicology of Canada, December 2013 and the 54th Annual Meeting of the Teratology Society, June 2014.

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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 (Jones et al., 1998, Nan et al., 1998). 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 lysines 9 and 27 (H3K9 or H3K27) is associated with transcriptional repression, whereas methylation of H3K4 is associated with transcriptional activation (Berger, 2007). 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 (Downs et al., 2007, Escargueil et al., 2008). For example, phosphorylation of histone H2AX (γH2AX) is a well-known early marker of DNA double strand breaks (Rogakou et al., 1998). In addition, researchers have recently identified that acetylated histone H3K56 is another histone residue that becomes rapidly and reversibly modified in response to genotoxic cellular stress (Das et al., 2009, Tjeertes et al., 2009). 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 data (Das et al., 2009, Tjeertes et al., 2009).

Researchers are becoming increasingly aware of epigenetic modifications and their influence on normal and malignant biological processes. For example, the process of hematopoiesis is regulated by various histone acetyltransferases (HATs), enzymes responsible for acetylation of histones and non-histone proteins, both of which contribute to hematopoietic regulation (Mishima et al., 2011, Bruserud et al., 2006). Specifically, chromosomal translocations that are commonly found in hematopoietic malignancies often involve genes that encode HATs (Sun et al., 2015), suggesting that disruption of these genes can contribute to these malignancies. Similarly, fusion proteins that are specific to leukemias appear to physically interact with some HATs, likely contributing to leukemogenesis by disturbing acetylation regulation (Perez-Campo et al., 2013). While this evidence does not specifically implicate aberrant histone modifications as being causative to leukemia, the correlation between aberrant HAT expression and hematopoietic malignancies suggests that histone modifications are worth investigating.

Additionally, while numerous differences in epigenetic modifications have been noted in various types of cancer compared to normal tissue, this could be due to the increasing number of carcinogens that 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 (Liu et al., 2010). 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 Koturbash et al. (2011)).

Benzene exposure has been associated with changes in epigenetics, specifically, 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 tumor suppressor gene, *P15*, and hypomethylation of *MAGE1* (Bollati et al., 2007). *MAGE1* is a gene of unknown function; however it encodes a cell antigen that appears to be specifically expressed only on tumor cells and male germline cells (Reif, 1992). 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 (De Smet et al., 1996, Melki et al., 1999). Similarly, benzene metabolite exposure in cultured cells has been associated with hypermethylation of the DNA repair gene, *Parp-1* (Gao et al., 2010), as well as the candidate tumor suppressor gene, phosphatase and tensin homolog (*Pten*) (Yang et al., 2014). Exposure of cultured cells to the benzene metabolite hydroquinone or benzoquinone also led to global DNA hypomethylation (Hu et al., 2011, Ji et al., 2010). 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 tumor suppressors *p15* and *p16*, this decreased expression appeared to be independent of promoter methylation (Tian et al., 2012). 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′-eoxycytidine, a methyltransferase inhibitor, or trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor (Gao et al., 2011). The results of this study indirectly suggest that modifications in both methylation and histone acetylation patterns following exposure to benzene could be involved in the cytotoxicity observed following exposure to benzene or its metabolites.

Given the involvement of epigenetic mechanisms in carcinogenesis, including hematopoietic malignancies, as well as the emerging number of studies implicating epigenetic alterations following benzene exposure, we decided that it was important to assess the involvement of this mechanisms in the transplacental carcinogenicity associated with benzene. Therefore, 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.

2. Material and methods

2.1. 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

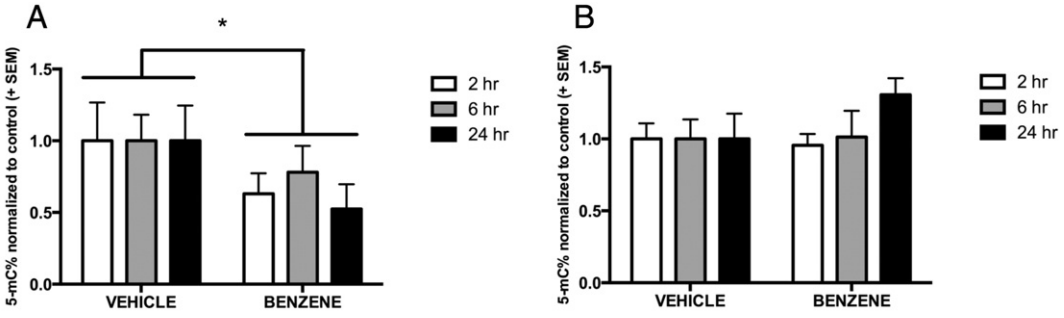

Fig. 1. The effects of *in utero* exposure to benzene (200 mg/kg) on gestational days (GDs) 8, 10, 12, and 14 *via* IP injection on global DNA methylation in (A) maternal bone marrow and (B) fetal livers 2, 6, and 24 h following the final dose of benzene. A significant decrease in global DNA methylation was observed in maternal bone marrow, but not fetal livers, following exposure to benzene. *$p < 0.05$.