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# Benzene-initiated oxidative stress: Effects on embryonic signaling pathways

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ABSTRACT

Approximately 90% of childhood cancers are of unknown etiology; however, it is hypothesized that *in utero* carcinogen exposure may contribute. Epidemiological studies have correlated parental exposure to benzene with an increased incidence of childhood leukemias. However, mechanisms of benzene-induced carcinogenesis following *in utero* exposure remain unknown. We hypothesize that *in utero* exposure to benzene causes alterations in the redox-sensitive signaling pathways involving c-Myb, Pim-1, AKT, ERK-MAPK, p38-MAPK, and NF-κB via the production of reactive oxygen species (ROS) as a possible mechanism of *in utero*-initiated carcinogenesis. Using a CD-1 mouse model we have shown increased oxidative stress in fetal tissue from embryos exposed *in utero* to benzene by measuring reduced to oxidized glutathione ratios, and increased levels of ROS in male fetuses using flow cytometry and the ROS sensitive fluorescent probe dichlorofluoroscein diacetate (DCFDA). In addition, using Western blotting techniques we observed increased expression of fetal Pim-1, Pim-1 phosphorylation, c-Myb, and phosphorylated p38-MAPK (activated form) and lower protein levels of IκBα, while phosphorylated ERK-MAPK and AKT protein levels did not change. Interestingly, we found male fetuses more susceptible to benzene-induced oxidative stress, which is in agreement with the literature suggesting that males are more susceptible to benzene toxicity. Further studies evaluating the reason for this gender difference are ongoing.



## 1. Introduction

Concerns associated with exposure to environmental chemicals during pregnancy are typically related to worries about structural defects such as cleft lip, missing limbs or neurological anomalies, however another serious adverse outcome associated with *in utero* exposure to xenobiotics is the development of cancer after birth [1]. Benzene is a known adult carcinogen and in the case of *in utero* benzene exposure, several epidemiological studies have linked parental exposure to benzene with an increased risk of developing childhood leukemia [2–6]. In mechanistic animals studies, we and others have demonstrated that *in utero* exposure to benzene increases micronuclei formation in mouse fetal liver and bone marrow tissues [7–9], and alters fetal progenitor cell numbers [10,11], consistent with other studies demonstrating the development of cancer in adult mice after benzene exposure (reviewed in Ref. [12]). While the mechanism of how benzene exerts this toxicity remains unknown, using a mouse model we have been focusing on the effects of benzene-initiated reactive oxygen species (ROS) production on embryonic signaling pathways during the period of fetal hematopoiesis as a potential mechanism of *in utero*-initiated cancer (Fig. 1).

## 2. Benzene-induced oxidative stress

In several different laboratories, using a variety of experimental systems, benzene-initiated toxicity has been linked to the production of ROS including superoxide radical anions, hydroperoxyl radicals, hydrogen peroxide ($H_2O_2$), and the highly reactive hydroxyl radicals [13–16]. These species can act as signaling molecules affecting the regulation of gene expression, cell growth and cell death. Under normal physiological conditions, specific metabolizing and scavenging systems tightly control the concentrations of ROS formed by cells and tissues. However, in the case of excessive ROS production leading to oxidative stress, aberrant redox-sensitive signaling can occur, which would be particularly deleterious during embryonic development given the rapid growth, developmental changes and reliance on unique cellular signaling processes that occur during this period of life. Supporting a role for benzene-induced ROS in mediating *in utero*-initiated toxicity, our laboratory has shown in mice that *in utero* exposure to benzene leads to a decrease in the ratio of reduced to oxidized glutathione which is prevented by maternal pretreatment

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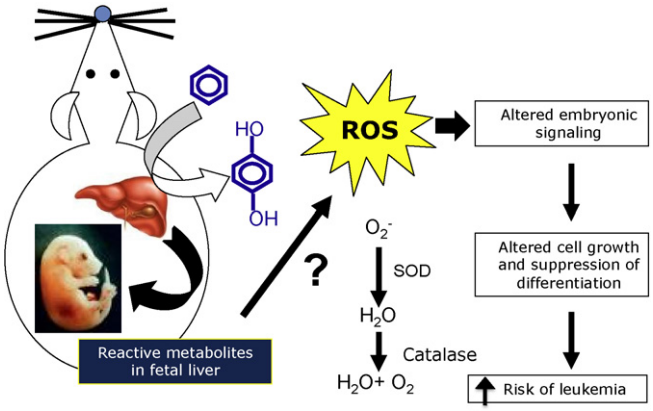


**Fig. 1.** Hypothesis. We hypothesize that *in utero* exposure to benzene leads to the formation of reactive oxygen species (ROS), that if not detoxified can alter embryonic signaling pathways leading to abnormal hematopoiesis, which can potentially lead to leukemogenesis.

with the anti-oxidative enzyme catalase (Fig. 2) [17]. In addition, we have shown a significant increase in ROS production as measured by 5- (and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) in male, but not female fetal liver tissue (site of hematopoiesis) 2 h after *in utero* exposure to benzene (Fig. 3). Interestingly, both male and female fetuses showed a significant decrease in ROS presence 4 h after *in utero* exposure to benzene (Fig. 3). This result is most likely due to activation of fetal oxidative defense systems, however further investigation is warranted. Together these data support the hypothesis that *in utero* exposure to benzene causes fetal oxidative stress. However, how fetal oxidative stress contributes to hematotoxicity is uncertain.

## 3. Effects on embryonic cell signaling pathways

While it is well known that excessive ROS production can cause developmental toxicity through oxidative damage to key cellular components such as DNA, proteins and lipids, interference with normal embryonic signaling also has deleterious consequences. Hematopoiesis during development is tightly controlled and involves orchestrated changes in gene expression. We have been focusing on a signaling pathway involving the c-Myb oncoprotein, which is over-expressed in many malignancies including

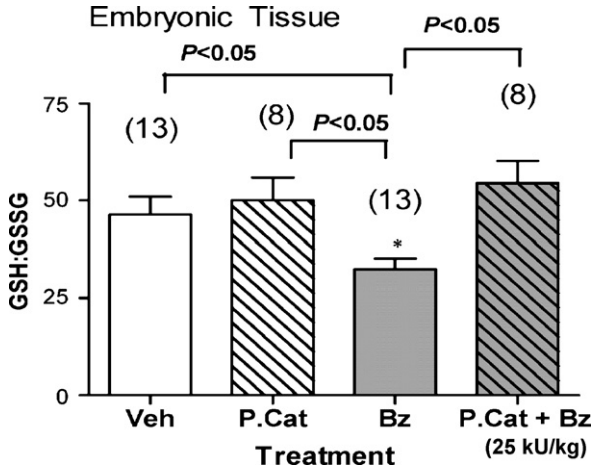


**Fig. 2.** Reduced to oxidized glutathione levels (GSH:GSSG) in headless mouse embryos 24 h after GD 10 and 11 exposure to 800 mg/kg benzene (Bz) and GD 9.5 and 10.5 pretreatment with 25 kU/kg PEG-catalase (P.Cat). The number of litters assessed is indicated above each bar. Statistically significant differences ($p < 0.05$) are indicated with brackets and as asterisk (*). Data from Wan and Winn [17].

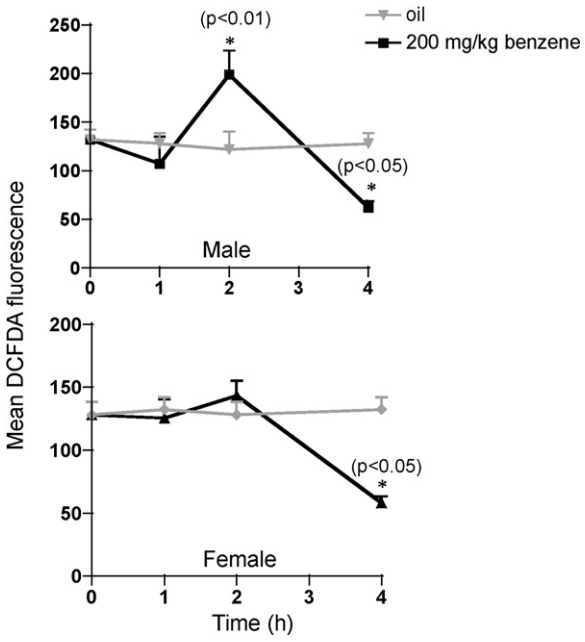


**Fig. 3.** The effect of *in utero* benzene exposure on reactive oxygen species (ROS) production in CD-1 mouse fetuses on gestational day (GD) 14. Pregnant CD-1 mice were injected with either corn oil (vehicle) or 200 mg/kg benzene i.p. on GD 8, 10, 12, and 14. Fetal liver cells were incubated with DCFDA at 0, 1, 2, and 4 h after the last injection on GD 14, and subsequently were analyzed by flow cytometry. Fetuses were sexed using Y chromosome specific PCR. *In utero* exposure to benzene caused a significant increase in ROS in male fetuses 2 h after exposure ($n = 4$, $p < 0.01$). In addition, both male and female fetuses had a significant reduction in DCFDA fluorescence 4 h after *in utero* exposure to benzene (male: $p < 0.05$; female $n = 4$, $p < 0.05$). Values significantly different from controls are denoted with an asterisk (*) ($p < 0.05$). DCFDA: 5- (and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate.

myelogenous leukemia [18]. This transcription factor is essential for hematopoiesis [19], and is important for a variety of biological processes including the regulation of cell proliferation, differentiation and apoptosis. c-Myb is highly expressed in all immature, proliferating cells, and its transcriptional activity is subject to stringent negative regulation [18]. The activity of c-Myb can be increased by Pim-1, a small (33 kDa) serine/threonine kinase, the altered activity of which has also been associated with leukemia [20]. Given the role of c-Myb and Pim-1 in leukemogenesis we hypothesize that *in utero* exposure to benzene may cause toxicity by altering this signaling pathway. We previously demonstrated in mice that *in utero* exposure to benzene leads to an induction in embryonic expression of c-Myb as well as increased levels of both phosphorylated and total Pim-1. These effects coincided with increased oxidative stress and were reduced by pretreatment with the anti-oxidative enzyme catalase, supporting our hypothesis [17].

Since several studies have demonstrated a positive relationship between Pim-1 and NF-κB [21,22], our most recent work has focused on the potential involvement of the NF-κB signaling pathway in ROS-initiated leukemogenesis. NF-κB is a redox-sensitive transcription factor that is highly involved in regulating proliferation, differentiation, and apoptosis in developing blood cells. Similar to Pim-1, dysregulation of NF-κB is often involved in the development of myeloid and lymphoid leukemias as well as lymphomas [23–25]. In resting cells, NF-κB resides predominantly in the cytoplasm bound to inhibitor of κB (IκB) proteins, which act to inhibit NF-κB DNA binding activity. After cells are exposed to a variety of stimuli including oxidative stress, cytokines, infections, or carcinogens, IκB is phosphorylated and, as a consequence, becomes polyubiquitinated and targeted for degradation via the proteasome pathway [26]. Degradation of IκB liberates NF-κB which then translocates to the nucleus and regulates transcription of a variety

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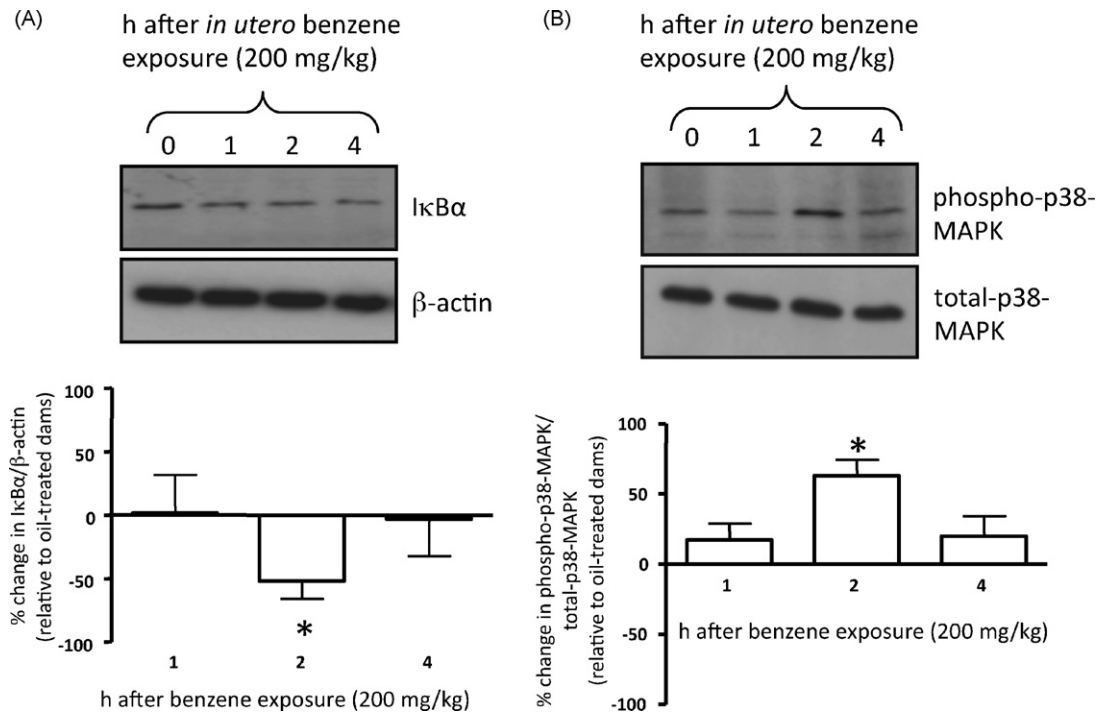


**Fig. 4.** The effects of *in utero* benzene exposure on IκBα and activated p38-MAPK levels in male CD-1 mouse fetal liver tissue. Pregnant CD-1 mice were injected with either corn oil (vehicle) or 200 mg/kg benzene i.p. on GD 8, 10, 12, and 14. Fetal liver tissues were lysed at 0 h (corn oil), 1 h, 2 h, and 4 h after the last injection on GD 14, and analyzed by Western blot. (A) IκBα protein expression. β-Actin levels were used as loading controls. (B) Phosphorylated (activated) p38-MAPK. Total p38-MAPK levels were used as loading controls. Densitometry was performed on at least 4 fetal liver tissues, and results are presented as the percent change in IκBα/β-actin (A) or phospho-p38-MAPK/total p38-MAPK (B) relative to oil-treated dams. Values significantly different from controls are denoted with an asterisk (*) ($n = 4$, $p < 0.05$).

of genes encoding cell adhesion molecules, cytokines, chemokines, growth factors, as well as proteins promoting cell proliferation and survival [27]. Because NF-κB has been implicated in leukemogenesis, presumably due to its propensity to activate genes that promote survival and proliferation [24,28] and its significant role in regulating hematopoiesis, we are currently investigating its role in benzene-induced dysregulation of fetal hematopoiesis as a mechanism of *in utero*-initiated leukemogenesis. Using Western blot analysis we have shown that *in utero* exposure to 200 mg/kg benzene causes a significant decrease in IκBα protein levels within 2 h in male CD-1 fetal liver tissue (Fig. 4A). We have shown that this dose and route of exposure produces peak maternal benzene levels that are very similar to levels measured in adult C57Bl/6 mice that develop leukemia after long-term benzene exposure [29,30]. Furthermore, this decrease in IκBα protein levels correlates with the increased levels of ROS observed 2 h after maternal benzene exposure in male fetal liver tissue (Fig. 3). Whether IκBα degradation after *in utero* benzene exposure is a direct consequence of increased ROS generation is the subject of further investigation. Regardless, increased IκBα degradation in hematopoietic tissue after *in utero* benzene exposure and subsequent NF-κB nuclear translocation is consistent with our c-Myb/Pim-1 results and may play a significant role in dysregulated hematopoiesis during leukemogenesis.

In addition to dysregulated c-Myb/Pim-1 and IκB/NF-κB signaling, other redox-sensitive embryonic signaling pathways may be implicated in aberrant hematopoieisis during leukemogenesis. For example, it is well established that various cytokines and growth factors that regulate normal hematopoietic cell proliferation and differentiation activate mitogen activated protein kinase (MAPK) and AKT signaling pathways to exert their effects [31–33]. While these pathways are redox-sensitive, interestingly we did not observe any differences in the levels of extracellular regulated kinase (ERK)-MAPK or AKT in male CD-1 fetal liver tissue after *in utero* exposure to 200 mg/kg benzene, as determined by Western blotting (data not shown). ERK-MAPK and AKT are activated primarily in response to mitogens and induce the activation of genes that promote cell survival. Despite the fact that we did not observe differences in ERK-MAPK or AKT activation, they could still play an essential role in the promotion of *in utero* benzene-induced hematotoxicity as the activation of these molecules could be cell- or time-specific events that were not detected at the time-points analyzed.

Studies have indicated that activation of another member of the MAPK family, p38-MAPK, can mediate signals that regulate growth of lymphoma, leukemia, and multiple myeloma cells [34,35]. Activation of p38-MAPK is primarily mediated by cellular stresses such as excessive ROS, inflammatory stimuli, osmotic stress, or heat shock. We found a significant induction of phosphorylated p38-MAPK in liver tissue of male fetuses within 2 h after *in utero* exposure to 200 mg/kg benzene (Fig. 4B). This finding correlates with the increased ROS levels and enhanced IκBα degradation observed in male CD-1 mouse fetal liver tissue at this time point. The precise role that p38-MAPK activation plays during *in utero* benzene-induced hematotoxicity is not known, but studies have shown that chemical inhibition of p38-MAPK activation completely prevents NF-κB activation during periods of oxidative stress [36]. Therefore it is possible that aberrant activation of p38-MAPK after *in utero* benzene exposure may indirectly promote leukemogenesis by activating the NF-κB pathway in addition to its direct effects on hematopoietic cell survival and proliferation.

## 4. Conclusions and future directions

In summary, our studies clearly demonstrate that *in utero* exposure to benzene leads to increased ROS production, causing alterations in critical embryonic cell signaling pathways involved in normal hematopoiesis. These disruptions alter fetal progenitor cell numbers, which we believe increases the susceptibility to

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hematopoietic malignancies later on in life and is the focus of one of our on-going studies. The major question that still remains unanswered is how these early changes could lead to leukemia in early childhood. We believe that this involves the fact that developmental hematopoiesis is complex and perturbations in cell signaling pathways initiate a carcinogenic effect *in utero*, which then progresses to leukemia once the child is born. This is consistent with the early onset of the disease in childhood and the notion that cancer is a multiyear process. What remains to be investigated are the additional causative factors that lead to a differential response to benzene exposure and the eventual development of leukemias in certain individuals. Interestingly, we found in our studies that male fetuses appear to be more susceptible to benzene-induced ROS production than female fetuses. We are currently evaluating whether or not alterations in embryonic signaling pathways are also gender specific and whether gender differences in fetal benzene metabolism and repair capabilities exist.

Taken together our studies support the hypothesis that *in utero* exposure to environmental pollutants such as benzene may increase the risk of childhood cancers.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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