REDUCING ENVIRONMENTALLY INDUCED BIRTH DEFECTS WITH PREGNATAL SUPPLEMENTATION

by

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A Senior Honors Project Presented to the
Honors College
East Carolina University
In Partial Fulfillment of the
Requirements for
Graduation with Honors
by
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May, 2019

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Abstract

Pollutant exposure during development has been shown to induce developmental malformations. For example, penis deformities are the most common birth defects occurring in boys born in the United States, and are associated with exposure to endocrine disrupting chemicals. Hypospadias occurs when the urethra does not open at the distal tip of the penis, but rather along the shaft, and is associated with decreased anogenital distance (AGD). The incidence rate of hypospadias has doubled over the past 50 years. Currently there are no known prenatal supplements to protect the fetus from hypospadias-inducing pollutants. However, if we can up-regulate endogenous detoxifying enzymes within the mother, fetus, or placenta we should reduce the effects of toxicants on the fetus. It has been proposed that sulforaphane, a derivative of broccoli, could be developed as such a preventative prenatal supplement because of its ability to increase detoxification and decrease oxidative stress. Removal of the pollutant from maternal and fetal circulation before it can impact development is a viable and general method to reduce pollutant-induced diseases. Indeed, sulforaphane supplementation to pregnant mice exposed to the anti-androgen vinclozolin at concentrations (125 mg/kg) that demasculinize genital development can increase AGD, and reduce hypospadias severity (most effective sulforaphane dose is 45 mg/kg). Here I test the hypothesis that sulforaphane at 45 mg/kg can decrease the potency of vinclozolin. To test this I conducted a dose response experiment were I dosed pregnant CD1 mice with either 0 or 45 mg/kg of sulforaphane and crossed those doses with either 0, 50, 75, 100, and 125 mg/kg of Vinclozolin and measured hypospadias incidence and severity, and proximal urethral opening size. Sulforaphane exposure significantly lowered the dose response to vinclozolin across all endpoints. This work shows that sulforaphane can reduce the severity and incidence of hypospadias in the mouse model, and takes the first step required to develop a prenatal supplement that will protect humans from pollutant-induced birth defects.

Introduction

Environmental pollutants are ubiquitously distributed in soil, water, crops, and the atmosphere. All humans are exposed to toxins throughout their life through ingestion, inhalation or dermal contact (Aris, 2014) (Sandau et al., 2000) (Thomas et al., 2006). Exposure to environmental pollutants occur inevitably but at varying levels based on individual lifestyle, geographic location, socioeconomic disparities, occupation, and many other factors. Exposure to these toxins can dysregulate several physiological processes systemically. Dysregulation can be especially impactful on the developing fetus (Fernandez, et al., 2007). Evidence indicates that embryonic exposure to environmental pollutants has more severe effects because fetuses have not yet developed protective detoxification mechanisms that is present in the adult (K. McCoy, unpublished data). The structures fetuses are developing also require hormones, whose concentrations and function are commonly affected by pollutant exposure (WHO/UNEP, 2013).
In addition, during the fetal stage systems and physiological pathways are developed that will be used throughout the individual’s lifetime, thus developmental alterations can have lifelong repercussions (Lee & Blumberg, 2019).

Several congenital malformations such as hypospadias and cryptorchidism, and adult diseases, such as cardiovascular disease, developmental immunotoxicity, and obesity, are linked to embryonic exposure to environmental pollutants such as endocrine disrupting chemicals (EDCs) (Xing & Bai, 2018) (Hanson & Gluckman, 2008) (Bernal & Jirtle, 2010) (DeWitt & Patisaul, 2018). These data also support the hypothesis that adult diseases have fetal origins (Lucas, Fewtrell, & Cole, 1999). Stimuli, such as environmental pollutant exposures, during critical fetal developmental windows have negative health implications that transcend into adulthood and across a lifespan (Barker & Osmond, 1986) (Lee & Blumberg, 2019).

Endocrine disrupting chemicals (EDCs) are environmental contaminants that disrupt many aspects of endocrine signaling and regulation. Many studies show that exposure to EDCs can induce malformations and various pathologies (Bergman, Heindel, Jobling, Kidd, & Zoeller, 2012) (Ormond, et al., 2009) (Fernandez, et al., 2007). One defect associated with prenatal exposure to EDCs is hypospadias, a congenital abnormality of the penis. Hypospadias occurs when the urethra does not open at the distal tip of the penis but rather ventrally along the shaft (Baskin & Ebbers, 2006). Pollutant-induced hypospadias can be caused by androgen receptor antagonists during critical developmental periods (Wilson, Blystone, Hotchkiss, Rider, & Gray Jr, 2008). This antiandrogenic exposure disrupts the androgen signaling pathway at the cellular level (Wilson, Blystone, Hotchkiss, Rider, & Gray Jr, 2008). Hypospadias is one of the most common birth defects in the United States occurring in up to 1% male newborns each year (State Birth Defects Surveillance Program). Data from the Metropolitan Atlanta Congenital Defects Program (MACDP) and nationwide Birth Defects Monitoring Program (BDMP) showed the incidence of hypospadias approximately doubled from the 1970s to the 1980s (Paulozzi, Erickson, & Jackson, 1997) with more severe cases increasing at a faster rate than mild cases. Hypospadias incidence has continued to rise and is now the most common birth defect in 20 of the 37 US states that were recently surveyed (State Birth Defects Surveillance Program).

Hypospadias occurs in a range of severities, with mild cases having an urethral that opens near the distal tip and severe cases having an urethral exit at or below the base of the penis (Amato & McCoy, 2016; Leung & Robson, 2007). Severe cases can result in painful erections, reduced fertility, difficulty in urination, and can even have lasting negative psychological effects (Baskin & Ebbers, 2006). Surgical correction of the external genitalia remains one of the most challenging procedures due to the amount of soft tissue and excessive scar tissue that interferes with urethral function (Barbagli, De Angelis, Palminteri, & Lazzeri, 2006). The option for surgical treatment is a difficult one for families to make and can cause high emotional strain. Even with treatment, pain, psychological symptoms and negative experiences persist into adulthood (Adams & Bracka, 2016).
Even though EDCs induce congenital defects, such as hypospadias, few studies have been conducted to develop treatments to decrease exposure to pollutants during development. Due to the wide prevalence of EDCs it is unrealistic to expect pregnant mothers to fully protect the fetus by preventing exposure. Therefore, identifying a prenatal supplement that protects the fetus from general environmental toxicant exposure is vital.

Environmentally induced birth defects have the potential to be preventable. For example, poor nutrition and reduced circulating foliate concentrations induce neural tube defects, and prenatal folic acid supplementation has significantly reduced these and other birth defects. Recently, it was proposed that sulforaphane, a nutrient derived from cruciferous vegetables, such as broccoli, could be developed as a general preventative agent to protect the fetus from toxicant exposure (Philbrook et al., 2014). Sulforaphane is known to activate Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-induced detoxification and antioxidant proteins (Houghton et al, 2016) (Jahan et al, 2014). Sulforaphane is seen as a promising bioactive phytochemical under preclinical evaluation and clinical trials as a preventative for diverse disorders including several types of cancer, cardiovascular disease, neurodegenerative diseases, and diabetes (Oliveri et al., 2018).

EDC induced hypospadias is an excellent model to study the protective nature of prenatal supplementation because it is a common birth defect, gives a consistent phenotype, and is inducible in the laboratory setting. The mouse has been extensively used to study the mechanisms through which EDCs induce hypospadias. For example, the dose and exposure time that induces 100% hypospadias has been determined for the model EDC vinclozolin (Amato et al. 2018). The certainty of inducing hypospadias allows fetal mice to be sampled before the abnormality develops so that the mechanisms that cause hypospadias can be investigated. Another strength of the mouse as a model to study EDC-induced hypospadias is that a validated protocol for scoring hypospadias severity has been established (Amato & McCoy, 2016). Also, penis development in the mouse has been well characterized (Harding et al., 2011) (McMahon et al.). Both male and female genitalia arise from a bipotential genital tubercle and during early urogenital development a proximal urethral opening (PUO) forms in both sexes. In females, the PUO is reconstructed into the vagina. In males the PUO is closed by mesenchymal infiltration which septates the urethral plate. In addition to closing the PUO, the mesenchymal infiltration extends to the distal tip of the penis and pushes the urethra into the middle of the penis. Vinclozolin exposure disrupts this process and results in reduction or absence of mesenchymal infiltration (Kurzrock et al., 1999), causing the PUO to remain open, and urethra to open proximally on the shaft of the penis.

In this manuscript, I test the hypothesis that sulforaphane will decrease vinclozolin potency on hypospadias incidence and severity, and PUO size by decreasing its affects across vinclozolin doses (e.g., sulforaphane will reduce the vinclozolin dose response).
Methodology

Pregnant CD-1 dams were gavaged from embryonic day (E) 13.5-E16.5 with vinclozolin only, vinclozolin+sulforaphane, sulforaphane only, or a corn oil (CO) solvent control (N=5 dams per treatment). On E18.5, the dams were humanely sacrificed. Embryos were individually weighed and measured for total body length, anogenital distance, and genitalia were preserved in 10% neutral buffered formalin until they were evaluated for hypospadias incidence and severity. To score hypospadias severity genitalia of each pup was photographed in a standard position and evaluated using the standardized scoring system (M.O.U.S.E, (Amato & McCoy, 2016)). After photographs were taken genitalia samples were processed for histological evaluation of the PUO.

To begin histological processing embryo genitalia were dehydrated, and embedded in paraffin wax using standard procedures (Presnell & Schreibman, 1997). Samples were then cross sectioned at 10 μm and stained with hematoxylin and eosin. Sections were microscopically evaluated using a Leica DME light microscope. Measures of masculinization including genitalia height, urethral length, and septation and PUO height, were collected for analysis to analyze internal morphological structures. To analyze these length measurements, the base of the penis was defined as the first section where the genitalia was disconnected from the perineum, establishing the first section for data collection. The height of the genitalia was calculated as the sum of all the sections between the base of the penis and the distal tip multiplied by the section thickness of 10 μm. Likewise, the urethral length was determined by counting the number of sections, from the base to the first section to the urethral opening (indicated by black arrow in figure 1) multiplied by section thickness. Septation height was collected as the

![Figure 1](image)

Figure 1. Normal penis development results in the urethral opening (black arrow) that occurs at the distal tip of the penis, while in hypospadias the urethral opening (black arrow) occurs on the shaft of the penis. (A) The base of the penis is collected as the first section where the genitalia was disconnected from the perineum. Septation (solid line) is indicated by mesenchymal cells (lightly stained) that split the epithelial cells (more darkly stained). (B) PUO (dotted arrow) height was collected from the base to the section where it closed.
number of sections that include a separated (septated) urethral and epithelial seam epidermis starting at the base of the penis ad multiplied by the section thickness (10 µm)(shown in Normal panel of figure 1 by the black bar). PUO height (reported here) was determined as the number of sections between the PUO start and end multiplied by the section thickness (10 µm). An example of the PUO start section can be seen in the Hypospadias panel of Figure 1, with the dash line indicating the location the section was taken from and the bottom of the panel displaying the histological section.

All data were analyzed in R statistical programming environment v. 3.4.3 (R Core Team, 2016) using lme4 package (Bates et al., 2005). Assumptions of homoscedasticity, normality, and dispersion were tested by interpreting residual plots, qqplots, and dispersion coefficients, respectively. Hypospadias incidence was analyzed using a binomial generalized linear mixed model (glmm). Hypospadias severity was analyzed using a Gaussian glmm. PUO height data were analyzed as raw counts of 10 µm histological sections using a Poisson glmm. To avoid pseudoreplication, dam was treated as a random effect in all models with pups being nested within dam (Bolker et al, 2009).

Results
For all endpoints there was an increase in effect with vinclozolin dose. However, a significant interaction between sulforaphane presence and vinclozolin dose occurred where the slope of the vinclozolin dose response was shallower (reduced) in the presence of sulforaphane. Both hypospadias incidence (FIGURE 2A, df=1, $\chi^2=7.6019$, LRT $p=0.005381$) and hypospadias severity (FIGURE 2B, df=1, $\chi^2=4.8313$, LRT $p=0.027948$) were reduced across all doses of vinclozolin in the presence of sulforaphane supplementation. Males

Figure 2. Sulforaphane (SFN) rescues penis development. Embryos exposed to vinclozolin and no sulforaphane (circles) reliably develop hypospadias (A). Supplementing VCZ exposed dams with 45mg/kg sulforaphane (triangles) reduces hypospadias incidence (A) and severity (B). Sulforaphane supplementation decreased incidence and height of PUO.
exposed to vinclozolin in this study had high incidences and large PUOs when compared to males exposed to both vinclozolin and sulforaphane (Figure 2C, Figure 3, df=1, \( \chi^2=17.4581 \), LRT \( p=2.937 \times 10^{-5} \)). In the presence of sulforaphane at 45 mg/kg the presence of a PUO was completely eliminated in all but the highest vinclozolin dose, while 50% of the 50 mg/kg and 100% of the 75 and 100 mg/kg vinclozolin only exposed embryos had PUOs. Additionally, in the cases where vinclozolin + sulforaphane exposed animals had PUOs, the PUO height was reduced (Figure 2C) relative to vinclozolin and no sulforaphane supplementation.

**Figure 3.** Prenatal sulforaphane supplementation rescues genital morphology in the presence of the anti-androgenic EDC vinclozolin. Control embryos exhibit normal penis development, with the urethral opening (red arrow) being present at the distal tip (A). In Vinclozolin treated animals, the embryos display hypospadias, with the urethral opening (red arrow) being present on the ventral shaft of the penis. Males exposed to vinclozolin also had a high incidence of PUO (white arrow), a structure seen in female morphology (B). Vinclozolin+Sulforaphane treated individuals showed a rescued phenotype, with the urethral exit being returned closer to the distal tip and no PUO, except in extremely high doses of vinclozolin (C).

**Discussion**

Sulforaphane reduces vinclozolin-induced hypospadias severity and incidence, and increases mesenchymal infiltration to fill in the PUO in our model system. Sulforaphane significantly lowered the potency of vinclozolin by the reducing the dose response relationship in the sulforaphane supplemented embryos relative to the vinclozolin only treatment. Sulforaphane, therefore, is a strong candidate for use as a prenatal supplement to protect developing embryos from contaminants that induce hypospadias, and it should be evaluated in more detail for use in humans.

More research must be conducted into the underlying mechanism through which this rescue effect occurs. In this investigation it is unclear how and where this hypospadias rescue is originating. For example, we have no evidence that sulforaphane is directly affecting genitalia development. However, a new study showed that rats with hypospadias have reduced expression of Nrf2 the transcription factor through which sulforaphane is thought to work. It is possible that sulforaphane is mediating detoxification of vinclozolin. This detoxification could be occurring in the liver or in the placenta. The other possibility for how this rescue is occurring is sulforaphane mediated reduction of oxidative stress. This could be occurring in the testis, and
thereby increase testosterone synthesis, which would decrease the ability of vinclozolin to compete for the androgen receptor. To truly understand the mechanisms inducing the sulforaphane mediated rescue of vinclozolin-induced hypospadias, tissue level analyses need to be conducted.

Hypospadias is the most common birth defect in the United States and occurs up to one percent of males born each year (State Birth Defect Surveillance Program, 2016). Genetic mutations cannot account for the recent rise in hypospadias occurrence in the population. For example, one study showed that after removing patients with known genetic causes of hypospadias, the remaining 74% of cases were exposed to higher toxicants (Kalfa, et al., 2015). In fact, mothers with occupational exposures to phthalates, pesticides, and other xenoestrogens have a higher risk of their children having hypospadias (Ormond, et al., 2009) (Baskin et al., 2001) (Aho et al., 2003). Here we show that sulforaphane protects the fetus from the pesticide vinclozolin, we believe, however, that sulforaphane will be generally protective.

Although it is not currently used as a prenatal supplement, sulforaphane is known to increase detoxification efficiency (Egner et al., 2014) (Houghton et al, 2016) (Kensler, et al., 2012) (Abiko et al., 2018). The ability of sulforaphane to upregulate antioxidant and detoxifying enzymes is being clinically investigated in a variety of contexts. For example, sulforaphane is being investigated for treatment in autism (Bent et al., 2018) and in the protection of cardiac muscle in ischemia–reperfusion (Piao et al., 2010). Sulforaphane has also been used to increase the metabolism of air pollution in China (Kensler, et al., 2012), to reduce DNA damage induced by fried meat (Shaughnessy, et al., 2011), and is a general dietary supplement that can be purchased at local drug retails. Therefore, sulforaphane is generally useful and available. As a test of its general use as a protective prenatal supplement, future work should test its effectiveness to protect developing fetuses from other contaminants and mixtures that are known to induce congenital abnormalities.

Environmental EDCs are responsible for many cases of hypospadias and lifestyle and occupation can increase susceptibility (Ormond, et al., 2009). My data suggests that if we can augment natural detoxification pathways we can reduce contaminant exposure and protect the fetus during development. The development of a prenatal therapy to reduce the incidence of this common birth defect will be exciting and extremely beneficial.

This study combined with past sulforaphane studies provide important steps toward the development of targeted prenatal therapeutics to reduce environmental toxicant induced birth defects such as hypospadias. In a world were environmental pollutants are increasing, being able to reduce environmental toxicant loads will also have implications far beyond decreasing one of the most common birth defects. This potential prenatal therapy could allow us to protect future generations from the harmful programing induced by pollutant exposure. We are especially hopeful that sulforaphane will protect against a multitude of contaminants and will
empower women to protect their developing babies from the ubiquitous contaminants that we are unavoidably exposed.

References


Amato, C. M., & McCoy, K. A. (2016). Amato, Ciro MA validated protocol to quantify severity of male urogenital feminization using the MOUSE (Mouse objective urethral severity evaluation). Pediatric research, 880-885.


Sandau et al., C. D. (2000). Analysis of hydroxylated metabolites of PCBs (OH-PCBs) and other chlorinated phenolic compounds in whole blood from Canadian inuit. *Enviornmental Health Perspectives*, 611-616.


