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Assessing reproductive toxicity of two environmental toxicants with a novel in vitro human spermatogenic model

Charles A. Easley IV [a,*], Joshua M. Bradner [b,d], Amber Moser [a], Chelsea A. Rickman [a], Zachary T. McEachin [a,e], Megan M. Merritt [a], Jason M. Hansen [c], W. Michael Caudle [b,d]

[a] *Laboratory of Translational Cell Biology, Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA*
[b] *Department of Environmental Health, Rollins School of Public Heath, Emory University, Atlanta, GA 30322, USA*
[c] *Division of Pulmonology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA*
[d] *Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA 30322, USA*
[e] *Wallace H. Coulter Department of Biomedical Engineering, Georgia Tech College of Engineering, Atlanta, GA 30332, USA*

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Abstract
Environmental influences and insults by reproductive toxicant exposure can lead to impaired spermatogenesis or infertility. Understanding how toxicants disrupt spermatogenesis is critical for determining how environmental factors contribute to impaired fertility. While current animal models are available, understanding of the reproductive toxic effects on human fertility requires a more robust model system. We recently demonstrated that human pluripotent stem cells can differentiate into spermatogonial stem cells/spermatogonia, primary and secondary spermatocytes, and haploid spermatids; a model that mimics many aspects of human spermatogenesis. Here, using this model system, we examine the effects of 2-bromopropane (2-BP) and 1,2,dibromo-3-chloropropane (DBCP) on *in vitro* human spermatogenesis. 2-BP and DBCP are non-endocrine disrupting toxicants that are known to impact male fertility. We show that acute treatment with either 2-BP or DBCP induces a reduction in germ cell viability through apoptosis. 2-BP and DBCP affect viability of different cell populations as 2-BP primarily reduces spermatocyte viability, whereas DBCP exerts a much greater effect on spermatogonia. Acute treatment with 2-BP or DBCP also reduces the percentage of haploid spermatids. Both 2-BP and DBCP induce reactive oxygen species (ROS) formation leading to an oxidized cellular environment. Taken together, these results suggest that acute exposure with 2-BP or DBCP causes human germ cell death *in vitro* by inducing ROS formation. This system represents a unique platform for assessing human reproductive toxicity potential of various environmental toxicants in a rapid, efficient, and unbiased format.

* Corresponding author at: Department of Cell Biology, Emory University School of Medicine, Rm 405H Whitehead Biomedical Research Building, 615 Michael St., Atlanta, GA 30322, USA.
E-mail address: caeasle@emory.edu (C.A. Easley).

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Introduction

To date, most of the pioneering work in understanding the effects of environmental toxicants on male reproduction in pubertal and post-pubertal exposures has been done in mice and rats (for review, Campion et al., 2012). These rodent models, while very informative, possess challenges and limitations (for review, Campion et al., 2012) and typically focus on morphological/structural defects using histopathological studies to assess exposure-related damage. Because of the limitations of these models, sub-cellular effects of toxicant exposure are only now just being studied and are difficult to assess in the testis *in vivo*. Furthermore, these animal studies are laborious, time consuming, and expensive and may not replicate what happens in humans as rodent spermatogenesis is distinctly different from human spermatogenesis, with rodents possessing several spermatogonial amplifying events that humans do not have (Ehmcke et al., 2006). As such, human studies on the effects of toxicant exposure are largely relegated to clinical and epidemiological studies examining testis tissue defects or sperm quality. Thus, for the case of human spermatogenesis, the underlying mechanisms by which environmental toxicants impair spermatogenesis are understudied and mainly extrapolated from work in rodents.

In 1996, the US Food Quality Protection Act ordered the EPA to implement a reproductive toxicant screening program to begin assessing the reproductive toxic potential for the large number of chemicals humans encounter (Tomerlin, 2000). The purpose of this Act was risk assessment for chemicals that potentially could contribute to human infertility (Tomerlin, 2000). To date, current models have been burdened by a lack of sensitivity leading to inconsistent or non-responses to toxicant exposures, which in turn confound outcomes and lead to several false negative results. Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs), represent a unique system by which investigators can examine the effects of toxicants as these cells can be differentiated into any cell type in the adult organism, including germ cells (Bucay et al., 2009; Easley et al., 2012; Eguizabal et al., 2011; Fukunaga et al., 2010; Geijsen et al., 2004; Kee et al., 2009; Panula et al., 2011; Park et al., 2009; Teramura et al., 2007; Tilgner et al., 2008; Yamauchi et al., 2009; West et al., 2011). Recently, we demonstrated that male human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) are directly differentiated into adult-type spermatogonial stem cells/spermatogonia, pre-meiotic and post-meiotic spermatocytes, and post-meiotic spermatids (Easley et al., 2012). Our work demonstrates the utility of hPSCs to be used to model several key aspects of human spermatogenesis *in vitro* and maintain critical windows of susceptibility necessary to evaluate the effects of reproductive toxicants on spermatogenic cell lineages. Likewise, this *in vitro* model system would permit determinations of sub-cellular mechanisms by which reproductive toxicants negatively impact human spermatogenesis and would allow investigators to examine whether mechanisms observed in rodents apply to human spermatogenic defects in a high throughput/high content approach.

1,2-Dibromo-3-chloropropane (DBCP) is a banned nematicide that has been shown to cause male infertility (Amann and Berndtson, 1986; Bjorge et al., 1995; Holme et al., 1991; Potashnik and Porath, 1995; Slutsky et al., 1999; Whorton et al., 1979). In exposed workers, DBCP is gonadotoxic resulting in a loss of germ cells, including spermatogonia, causing oligo- or azoospermia, without affecting the somatic Leydig and Sertoli cells (Amann and Berndtson, 1986; Bjorge et al., 1995; Holme et al., 1991; Potashnik and Porath, 1995; Slutsky et al., 1999; Whorton et al., 1979). Clinical studies also indicate that DBCP interferes with meiosis as exposed male workers with observed sperm counts frequently generate aneuploid sperm (Kapp et al., 1979). Rodent studies on DBCP have been erratic as some models have shown gonadotoxicity, but others have not. Specifically, rats demonstrate less tolerance and higher lethality to lower doses of DBCP compared to their mouse counterparts (Teramoto et al., 1980). Mouse models have indicated that DBCP does not induce spermatogonia cell death but instead blocks differentiation (Meistrich et al., 2003), a phenotype that to date has not been observed in human exposure cases. Likewise, mouse models indicate that Leydig cell loss plays a major role in DBCP-mediated infertility (Kelce et al., 1990). Thus, a model that mimics human exposure phenotypes is needed to fully investigate the effects of DBCP on human spermatogenesis.

2-Bromopropane (2-BP) was an alternative to ozone-depleting cleaning solvents and is also used in organic synthesis to add isopropyl groups to compounds (Anon, 2003). Recently, the National Toxicology Program (NTP) concluded that there was sufficient evidence to suggest that 2-BP negatively impacts fertility in exposed males (Boekelheide et al., 2004). Occupational exposure to high levels of 2-BP has resulted in oligo- or azoospermia, and observations from clinical samples suggest that 2-BP reduces the number of pre-meiotic spermatocytes by affecting viability of both spermatogonia and spermatocytes by inducing germ cell apoptosis (Ichihara et al., 1999; Kim et al., 1996, 1999; Son et al., 1999; Wu et al., 1999; Yu et al., 1997). The effects of 2-BP exposure in humans appear to be on germ cell viability and not on somatic Leydig and Sertoli cell function or viability. Studies using mouse models indicated that 2-BP exposure in mice, like humans, shows a loss of spermatogonia and spermatocytes; but some studies implicate Leydig cell defects as the main factor behind 2-BP-mediated germ cell death in mice. This does not appear to be the case in humans. Even in the case of 2-BP, where rodent models mostly mimic human exposure phenotypes, a new model that simulates many aspects of human spermatogenesis would be of importance to examining the exact mechanism of how 2-BP interferes with human spermatogenesis, especially given that 2-BP is still used.

Here, we demonstrate that we can adapt our recently described human spermatogenic differentiation model to examine the effects of 2-BP and DBCP on *in vitro* human spermatogenesis. We demonstrate that our model mimics phenotypes observed in human exposure cases and that both 2-BP and DBCP directly affect germ cell viability in culture. Furthermore, we are able to utilize this system to examine one potential cellular mechanism by which 2-BP and DBCP affect germ cell survival. The work presented here also validates our model system as an appropriate platform for evaluating other environmental toxicants on human spermatogenic toxicity.