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Paternal smoking and germ cell death: a mechanistic link to the effects of cigarette smoke on spermatogenesis and possible long-term sequelae in offspring

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Abstract

Paternal exposure to constituents of cigarette smoke (CS) is reportedly associated with infertility, birth defects and childhood cancers even though the mechanism behind this relationship is still unclear. Chronic cigarette smoking by men leads to poor sperm quality and quantity mainly through oxidative stress and also direct assault by CS metabolites. Among several carcinogenic and teratogenic components of cigarette smoke condensate (CSC), polycyclic aromatic hydrocarbons (PAHs) display a preeminent role in accelerating germ cell death via the cytoplasmic transcription factor, aryl hydrocarbon receptor (AHR) that is present across all stages of spermatogenesis. Activation of AHR by growth factors though benefits normal cellular functions, its mediation by CSC in a spermatocyte cell line [Gc2(spd)ts] adversely affects the expression of a battery of genes associated with antioxidant mechanisms, cell proliferation and apoptosis, and cell cycle progress. Besides, the CSC-mediated cross talk either between AHR and NRF2 or AHR-NRF2 and MAPKs pathways inhibits normal proliferation of the spermatogenic GC-2spd(ts) cells *in vitro* and cell death of spermatocytes *in vivo*. Pharmacological inactivation of CSC-induced AHR but not its genetic manipulation seems preventing DNA and cell membrane damage in Gc2(spd)ts. Data from recent reports suggest that the cigarette smoke affects both the genomic and epigenomic components of the sperm and attributes any associated changes to developmental defects in the offspring. Thus, the studies discussed here in this review shed light on possible mechanistic factors that could probably be responsible for the paternally mediated birth defects in the offspring following exposure to the toxic constituents of cigarette smoke.

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Introduction

Tobacco use is the single largest preventable cause of death and disease for both men and women. Tobacco causes nearly six million deaths per year worldwide. In the US, smoking and second-hand smoke cause one in every five deaths and incur almost $300 billion annually in total economic costs (USDHHS, 2014). Approximately 30% of women and 35% men of reproductive age smoke cigarettes, affecting not just themselves but also the environment and their progeny (ASRM, 2012). Cigarette smoke (CS) contains more than 7000 chemicals, including at least 539 polycyclic aromatic hydrocarbons (PAHs), of which 69 are proven carcinogens (IARC, 2004; Rodgman and Perfetti, 2006) and mutagens (DeMarini, 2004). Additionally, CS is comprised of the entire top ten hazardous substances listed in section 204 of the Comprehensive Environmental Response, Compensation, and Liability Act. Cigarette smoke condensate (CSC), the particulate, or tar, phase of CS, consists mainly of dioxins (TCDD) and halogenated and nonhalogenated PAHs including benzo(a)pyrene (B[a]P) and pro-oxidants such as lipophilic semiquinones (Smith and Hansch, 2000; Ding et al. 2007). This review on paternal smoking and its impacts on offspring will summarize the current state of research in this area and describe possible mechanisms by which paternal smoking causes poor reproductive outcomes and developmental defects. Moreover, the molecular mechanisms we tried to understand by using a spermatogenic cell line [GC-2spd(ts)] may or may not reflect upon the actual events happening in the spermatocytes *in vivo*.

Paternal smoking and developmental defects

Maternal smoking and *in utero* exposure during pregnancy has so far been believed to be associated with reduced sperm quality, count, and testis size in adults (Jensen et al. 2004; Virtanen et al. 2010). However, several epidemiological and case control studies in humans have reported that children born to male smokers are at increased risk of childhood cancers (Ji et al. 1997; Chang et al. 2006; Vine, 1996) and the existence of a significant correlation between paternal smoking and childhood cancer (Liu et al. 2011) with the emphasis on the need to focus on underlying toxicological mechanisms, such as genotoxic, transcriptomic, or epigenomic effects on sperm or cord blood. Similarly, several other reports have highlighted close connection between paternal smoking and childhood leukemia (Pang et al. 2003; Lee et al. 2009). Birth defects such as anorectal malformations (Zwink et al. 2011), cardiovascular anomalies, congenital heart disease (Cresci et al. 2011), cleft palate, hydrocephalus, urethral stenosis (Savitz et al. 1991), spina bifida (Zhang et al. 1992), and reduced kidney volume (Kooijman et al. 2015) were some of the developmental defects observed in the offspring of paternal exposure. Additionally, paternal smoking has been reported to cause implantation failure (Janny & Menezo, 1994; Sofikitis et al. 1995; Ubaldi et al. 1999). However, only a few studies have correlated these outcomes to the intratesticular levels of harmful and potentially harmful constituents of cigarette smoke. Godschalk et al. (2015) recently showed that B[a]P is able to induce hypomethylation in testicular DNA, that leads to heritable mutations in the offspring. However, the data are not very conclusive on the effects of male smoking on *in vitro* fertilization (IVF) outcomes. (Pattinson et al. 1991; Hughes et al. 1994; Joesbury et al. 1998) even though the sperm numbers were found decreased in young men prenatally exposed to paternal smoking

(Axelsson et al. 2013). So far in humans, the studies on the association between paternal smoking and congenital anomalies among offspring have yielded mixed results. They are either poorly understood mechanistically, and or very limited data are available on the germ cell and reproductive effects of paternal exposure even though maternal exposure to second-hand smoke during pregnancy is known to cause adverse fetal outcomes (Olshan AF, Faustman EM, 1993; Leonardi-Bee, and Britton, 2011). In case of rodents, CS-induced mutations in sperm DNA (Yauk et al. 2007) cause lack of pregnancy after fertilization, disrupted blastocyst implantation, impaired embryonic development, and IVF failures (Kapawa et al. 2004).

Cigarette smoking and male infertility

Even though several studies have indicated the harmful effects of *in utero* exposure to CS on male fertility (Jensen et al. 2004; Mackenzie and Angevine, 1981), chronic cigarette smoking by men also leads to male infertility; the available biologic, experimental, and epidemiological data indicate that 13% of male infertility is attributed to cigarette smoking (ASRM, 2012), and the time to pregnancy is extended in cases in which the man smokes more than 15 cigarettes per day (Ford et al. 2000). A comprehensive review by Ramlau-Hansen et al. (2007) and Mostafa (2010) have provided a thorough overview on cigarette smoking by men and associated abnormalities in sperm count, motility, and morphology, as well as other qualitative and quantitative measures of sperm characteristics. Male smokers exhibit several seminal anomalies including increased levels of oxidative DNA damage (Fraga et al. 1996; Shen et al. 1997), sperm DNA strand breaks (Potts et al. 1999), DNA adducts (Horak et al. 2003), chromosomal abnormalities (Robbins et al. 1997; Rubes et al. 1998), and decreased viability, and fertility (Kunzle et al. 2003). Exposure to cigarette smoke (CS) results in decreased sperm membrane permeability and activity of acrosin (Sofikitis et al. 2000). The testicular endocrine and spermatogenic functions, and epididymal functions are also reportedly reduced in rats upon exposure to B[a]P (Ramesh et al. 2008). Nicotine causes testicular toxicity by degenerating germ cells (Jana et al. 2010) and cigarette smoke metabolites such as cotinine drastically affect seminal parameters such as sperm membrane damage, reduced motility, capacitation and hyperactivation (Pacifici et al. 1993; 1995) that correlates to low sperm count (Chia et al. 1994; Vine et al. 1996). Vine et al. (1994, 1996) reported that sperm concentration is 13% lower in smokers than non-smokers. Meanwhile, there is a modest reduction (10–17%) in sperm counts reported in adult men who smoke heavily and these reductions in sperm quality and quantity are directly proportional to the number of cigarettes smoked daily (Ramlau-Hansen et al. 2007). Such adverse effects of male smoking are thought to be due to absorption of constituents of CS and its metabolites into the systemic circulation and accumulation, either by diffusion or active transport, into seminal plasma (Zavos and Zarmakoupis-Zavos, 1999).

Several environmental and food contaminants are known to reach the testis in significant concentrations (Gaspari, et al. 2003; Bjorge et al. 1996). However, little is known about the molecular mechanisms by which the components of CS damage male germ cells during spermatogenesis. One likely candidate is the oxidative stress caused by the generation of excess reactive oxygen species (ROS) or free oxygen radicals by the toxic constituents of CS (Saleh et al. 2002; Aitken and Baker, 2004). Additionally, the sperm of smokers have

increased levels of oxidized unsaturated fatty acids (Jones et al. 1979). Although ROS are required for sperm maturation, capacitation, and the acrosome reaction (de Lamirande et al. 1993), mature male gametes are highly susceptible to oxidative damage because they express low levels of antioxidant enzymes and have high concentrations of polyunsaturated fatty acids in their plasma membrane (Aitken and Roman, 2008). Cigarette smokers with high levels of ROS in their seminal plasma capable of causing DNA damage mediate oxidative male infertility (Potts et al. 1999; Tremellen, 2008). Meanwhile, infertile men who smoke cigarettes have higher levels of seminal OS than infertile nonsmokers and significantly low sperm count (Saleh et al. 2002; Collodel et al. 2010; Zenses, 2000). Therefore, the oxidative imbalance could be, in part, responsible for CS-mediated male infertility. Genetically, smoking has been known to be associated with sperm disomy in teenage men (Rubes et al. 1998). Smoking also affects morphology and ultrastructure of the flagellum and, more specifically, the axoneme of the human spermatozoon (Evans et al. 1981; Hoidas et al. 1985; Zavos et al. 1998). In addition, the change mediated by CS in sperm mRNA profile can serve as the marker of gene–environmental toxicants interactions in human germ cells (Linschooten et al. 2009). In contrast, studies by Mocarelli et al. (2011) showed that only *in utero* and lactational exposure of children to low doses of TCDD could permanently reduce sperm quality. Meanwhile, a meta-analysis by Li et al. (2011) have highlighted that the smoking seems to degrade semen volume and total sperm count in heavy smokers.

Polycyclic aromatic hydrocarbons (PAHs) act as testicular toxicants through aryl hydrocarbon receptor (AHR)

Treating adult rodents with PAHs such as TCDD, B[a]P, and 3-Methylchloranthrene increases the number of abnormal sperm and immature germ cells (Viczian, 1968; Wyrobeck and Bruce, 1975), blocks spermatogenesis, and causes testicular atrophy (Mattison, 1982), decreased testis weight, and increased apoptosis in seminiferous tubules (Denison and Heath-Pagliuso, 1998; Revel et al. 2001; Coutts et al. 2007). PAHs have a strong effect on germ cells because the pre-and post-meiotic germ cells, particularly the spermatocytes (Georgellis et al. 1990; Essenberg et al. 1951) in the seminiferous epithelium (Schultz et al. 2003; Coutts et al. 2007), highly express the aryl hydrocarbon receptor (AHR). AHR is a ligand-activated transcription factor that, upon activation by a natural or synthetic compound, translocates to the nucleus, binds to the AHR nuclear transporter, and regulates several downstream targets (Thackaberry et al 2005). AHR is best known for induction of the cytochrome P450 superfamily of genes (*Cyp1a1* and *Cyp1a2*) in response to detoxification of endogenous and exogenous ligands (Barouki et al. 2007). AHR binds with high affinity and specificity to dioxin-like compounds (Grassman et al. 1998). Given that various environmental toxicants including TCDD have been found in human seminal fluid (Schecter et al. 1996), the presence of AHR may make the sperm highly vulnerable to PAHs of CSC. For example, adult exposure to TCDD decreased daily sperm production in rat and the critical period for TCDD effects on testis weight suggested to occur before puberty (Simanainen et al. 2004a; 2004b). However, *in utero* exposure of male rats on different days of gestation adversely affected the ejaculated sperm counts with lesser effect on testicular sperm production (Rider et al. 2010). A cross species comparative study further revealed that

the prenatal maternal exposure to TCDD at low dose causes functional alterations rather structural malformations (Peterson et al. 1993).

AHR is essential for spermatogenesis and post-testicular sperm maturation

Despite its capacity to mediate the damaging effects of PAHs in sperm and the controversies surrounding the direct impact of AHR activation, several lines of evidence indicated that AHR is essential for germ cell development, differentiation, and maturation during its epididymal transit. First, earlier findings collectively suggested that either the absence of AHR or its activation would lead to inflammation, apoptosis, and oxidative stress-mediated DNA damage in sperm (Aitken and Roman, 2008; Matsumura, 2009). Expression of AHR is tissue-, and developmental stage-specific and regulates normal cellular processes, such as cell cycle, stem cell proliferation, and tissue differentiation (Puga et al. 2002; Gasiewicz et al. 2012). In rat, AHR expression in the seminiferous tubule is restricted to the primary pachytene spermatocytes, whereas in humans, AHR is expressed across all stages of spermatogenesis (Roman et al. 1998; Schultz et al. 2003). In mice, male fertility, sperm count, seminal vesicle weight, and dorsolateral prostate weights are all decreased in *Ahr*–deficient mice (Karman et al. 2012; Baba et al. 2008; Lin et al. 2001). On the other hand, *in utero* and lactational exposure of male rats to TCDD alone significantly decreased the weight of testis, epididymis and daily sperm production (Mably et al. 1992). Likewise, even though AHR appears to be critical for post-testicular sperm survival and maturation in the epididymis, the testicular production of germ cells remain unaffected due to TCDD treatment in adult mice (Foster et al. 2010). Using an AHR knockout mouse model, we recently showed that AHR is required for the structure and function of the seminiferous tubules (Hansen et al. 2014). Histologically, the testes from $Ahr^{-/-}$ mice have significant structural disorientations in the seminiferous epithelium suggestive of germ cell degeneration and compromise in Sertoli cell function (Boekelheide, 2005). Additionally, we noted drastic down regulation of several germ cell-associated marker genes such as *Magea4, HspA2, Prm1,* and *Prm2* in $Ahr^{-/-}$ mice. Finally, we found that AHR protein was highly expressed across different germ cell stages and on the acrosome and principal piece of the sperm tail. This expression was reflected in AHR function of the mature sperm, as we noticed that $Ahr^{-/-}$ sperm were less efficient than wild-type sperm in fertilizing oocytes. Therefore, the role of AHR in spermatogenesis though appears promising remains inconclusive and warrants further attention.

CSC deregulates gene expression in testis

Only a few studies have investigated the influence of cigarette smoking on the development and function of testis. In other tissue types, CS affects the expression of genes involved in antioxidant mechanisms, metabolism of PAHs, and cell cycle progress (Georgellis et al. 1990; Narayan et al. 2004). For example, the expression of several genes that are associated with PAH metabolism were up regulated in oral cancer cells upon exposure to CSC (Nagaraj et al. 2006). Bosio et al. (2002) and others have attributed the strong oxidative potential of CSC as a likely mechanism by which CSC influences gene expression in various cell and tissue types (Fields et al. 2005; Van Leeuwen et al. 2005; Han et al. 2008). Therefore,

determining the mechanisms by which CS impairs spermatogenesis requires evaluation of the gene expression changes that occur in response to CS-induced oxidative stress.

In a study using a mouse spermatogenic GC-2spd(ts) cell line, we explored the effects of CSC on the expression of several antioxidants both *in vitro* and *in vivo* (Esakky et al. 2012). We reported that exposure to CSC leads to oxidative stress, which in turn affects normal cellular functions by modifying the expression of several antioxidant genes such as *Hsp90, Nrf2, Sod1, Sod2, Ahr, Arnt, Cyp1a1 Gpx4,* and *Ucp2* through both AHR-dependent and -independent manners. The *in vivo* data further indicated that the constituents of CSC mediate cell death in the testis and suggested that this may occur both directly, by genotoxicity of CSC constituents, and indirectly through generation of ROS. Notably, we observed profound AHR-dependent up-regulation of *Cyp1a1* even with low concentrations of CSC. CYP1A1 contributes to detoxification of PAHs, but its induction may be harmful due to the generation of mutagenic metabolites like benzopyrene diol epoxide (BPDE). Consistent with this, we observed germ cell death in the testis and BPDE-intercalated DNA in the apoptotic spermatocytes. This study also revealed that exposure to CSC induced *Ahr* gene expression but did not increase the levels of AHR protein similar to an earlier outcome (Song and Pollenz, 2003). Thus, this report was an illustration of how CSC could cause germ cell death by inducing AHR mediated oxidative stress and thereby altering gene expression in the testis.

CSC modulates cell cycle in spermatogenic GC-2spd(ts) cells via AHR-NRF2 pathway

The constituents of CSC such as TCDD and other PAHs exert their growth-modifying effects primarily through AHR even though it exhibited direct detrimental impact (Gu et al. 2000). Similarly, ROS activate *Nrf2*, which regulates genes that possess antioxidant response elements (AREs) in their promoters (Moi et al. 1994; Venugopal and Jaiswal, 1996). The regulation of *Ahr* target genes by *Nrf2* in liver in response to TCDD indicated the convergence of *Ahr* and *Nrf2* pathways (Yeager et al. 2009). Though the precise mechanism still remains obscure, a large body of evidence implicated *Ahr* in cell cycle control including the TCDD-induced thymic atrophy (Kremer et al. 1994; Puga et al. 2000; Marlowe et al. 2004). For instance, Ma and Whitlock (1996) showed that *Ahr*-defective Hepa-1 cells exhibit growth arrest at G1 phase, while mouse embryonic fibroblasts from *Ahr*-null mice show delayed S-phase progress (Tohkin et al. 2000) and accumulation at the G2/M phase (Elizondo et al. 2000). Abdelrahim et al. (2003) showed that *Ahr* silencing accelerates MCF-7 cells to S phase, whereas it works opposite in HepG2 cells. Therefore, a comprehensive model that illustrates the contradictory roles of *Ahr* in cell cycle control particularly in germ cells is still evolving.

To understand the molecular conundrum behind the CSC mediated AHR role in cell cycle, we examined the effect of CSC on the *Ahr-Nrf2* pathway by using the spermatogenic GC-2spd(ts) cells (Esakky et al. 2014). This study provided evidence that, as it does in other cell types (Jeffy et al. 2000; Khan and Dipple, 2000; Hamouchene et al. 2011), CSC blocks GC-2spd(ts) cell cycle progress at the S-G2/M phase and deregulates expression of cell

cycle regulators such as *cyclin D1*, *P21*, and *Gadd45a*. We found that cyclin D1 expression requires *Ahr* and growth factors for its basal expression under normal condition, but is down regulated by CSC either in presence or absence of AHR. Our finding that CSC caused AHR-dependent upregulation of NRF2 suggested that NRF2 might protect spermatogenic GC-2spd(ts) cells from oxidative stress (Rangasamy et al. 2004; Nakamura et al. 2010) based on its nuclear translocation, which was found both complementary (Niture et al. 2010) and contradictory to earlier findings (Nguyen et al. 2005). As has been reviewed in other cellular systems (Hayes et al. 2009), there exists an autoregulatory loop between *Ahr* and *Nrf2* in spermatogenic GC-2spd(ts) cells. Moreover, the absence of CSC-induced *Cyp1a1* expression in absence of *Ahr* or *Nrf2* in spermatogenic GC-2spd(ts) cells corroborates TCDD action on NAD(P)H:quinone oxidoreductase 1 (Yeager et al. 2009). Furthermore, the CSC induced growth arrest in spermatogenic GC-2spd(ts) cells at G2-M checkpoint parallels the induction of DNA-damage-inducible *Gadd45a* since it has been shown earlier as a molecular sensor of DNA damage and adducts formation in response to genotoxic agents such as benzo[a]pyrene (Schackelford et al. 1999; Wan et al. 2000; Akerman et al. 2004). Thus, this body of evidence supported the fact that CSC mediates the formation of an autoregulatory loop between AHR and NRF2 in the spermatogenic GC-2spd(ts) cells during S-/G2-M phase.

CSC facilitates crosstalk between AHR-NRF2 and MAPK in spermatogenic GC-2spd(ts) cells

AHR agonists in CSC such as TCDD and B[a]P activate multiple cell signaling including MAPKs (Henklová et al. 2008), implicating MAPKs in connecting AHR with various physiological processes (Tan et al. 2002; Long et al. 1998). However, the mechanistic link between these toxicants to their effects on a particular signaling pathway have not been sufficiently established. AHR ligands activate MAP kinases in a cell or tissue specific manner, and that the kinase in turn mediates AHR activation, facilitating the transactivation of target genes (Weiss et al. 2005). We demonstrated that growth arrest of the spermatogenic GC-2spd(ts) cells by CSC involves a bidirectional crosstalk between AHR and MAPKs and regulation of a cascade of downstream targets (Esakky et al. 2015a). As demonstrated in this study, CSC induced accumulation of spermatogenic GC-2spd(ts) cells at S-phase and downregulation of cyclins. We further showed that CSC activates p38 and ERK MAPKs through AHR, and pharmacological inhibition of these pathways prevented CSC-mediated cell cycle arrest. When examined for the influence of MAPKs-mediated *Ahr-Nrf2* role in cell cycle progress, the accumulation of the spermatogenic GC-2spd(ts) cells at G2-M implicated *Ahr* and *Nrf2* in the activation of G2/M kinases (Elizondo et al. 2000), and DNA lesions (Reddy et al. 2008), respectively. Taking into account the cell type-specific functional dependency of MAPKs on AHR, the MAPKs activation in the spermatogenic GC-2spd(ts) cells suggested that the CSC constituents that activate p38- and ERK-MAPKs might also be the ligands of AHR. This was later confirmed by using the MAPK specific inhibitors.

Activating transcription factor 3 (*Atf3*) has been shown earlier to be induced by the CSC constituent, benzo(a)pyrene diolepoxide (Hai et al. 1999) and its regulation by MAPK here

corroborated previous studies (Inoue et al. 2004; Lu et al. 2007). Corresponding to the *Ahr-Nrf2* pathway, CSC facilitated cross talk between *Nrf2* and *Atf3* through AREs (Kim et al. 2010) even though the primary regulation of *Atf3* by *Nrf2* indicated the interplay of other upstream mediators of oxidative stress. Several lines of evidence suggested that CSC ligands like TCDD (Puga et al. 2002; Marlowe and Puga, 2005) cause cell cycle arrest (Ge and Elferink, 1998; Puga et al. 2000) by catalyzing the interaction between the transformed AHR and the retinoblastoma (RB)/E2F complex. In conjunction with earlier reports, this study has proposed a model for E2F4 action that the CSC-induced triad of AHR-RB-E2F4 inhibitory complex could be responsible for inhibiting E2F4 target. Thus, this complex CSC elicited intracellular *in vitro* signaling in the spermatogenic GC-2spd(ts) cells added greater strength by complementing *in vivo* TCDD-mediated MAPK activation (Jin et al. 2008).

AHR inhibition is anti-apoptotic in GC-2spd(ts) cells

Germ cell apoptosis is an indispensable evil to maintain cellular homeostasis during normal spermatogenesis. Since germ cells are highly sensitive to environmental toxicants such as the various harmful constituents of CS, it is indeed necessary to develop a protective mechanism that would help safeguarding the process of spermatogenesis by preventing unwanted germ cell death. With this objective, we have showed in a recent study, that treating spermatogenic GC-2spd(ts) cells with an AHR-specific pharmacological inhibitor, CH223191, (Kim et al. 2006) significantly reduced the proapoptotic actions of CSC by reducing DNA and membrane damage and caspase activation (Esakky et al. 2015b). Apoptosis is regulated by interaction between pro- and anti-apoptotic genes, and our work revealed that AHR coordinates the two. We found that CSC treatment of spermatogenic GC-2spd(ts) cells elevated the levels of both BCL2L1 and BCL2 prosurvival proteins and increased the numbers of apoptotic BAX- and BAD-positive cells. On the other hand, the activation of this intrinsic mitochondrial apoptotic signaling underlined the adaptive ability of the cells against growth-inhibitory CSC, which generates excessive oxidative stress in AHR-deficient cells. One of the key findings of this report was the inhibition of CSC induced caspase-3/7 by CH223191 while its elevation in *Ahr* silenced Spermatogenic GC-2spd(ts) cells and AHR-KO MEF. This divergence in caspase-3 response under both basal and CSC-induced conditions has been attributed to its heightened sensitivity to ROS.

Cigarette smoking destroys sperm ultrastructure and plasma membrane integrity (Belcheva et al. 2004). We found here that the spermatogenic GC-2spd(ts) cells treated with CSC exhibited externalization of phosphatidylserine on the plasma membrane, a hallmark feature of apoptosis. However, pretreatment with CH223191 prevented this membrane damage phenomenon. Meanwhile, the significant rise in apoptotic spermatogenic GC-2spd(ts) cells in the *Ahr*-deficient state while reiterating the cytoprotective role of AHR (Esakky et al. 2015a), appeared increasingly sensitive to oxidative stress due to reduced expression of super oxide dismutase. Moreover, the apoptosis regulating ability of AHR seems not uncommon as AHR-suppressed spermatogenic GC-2spd(ts) cells also displayed greater sensitivity to smoke-induced apoptosis. Therefore, the outcome of this particular study suggested that the hyperinducibility of AHR by environmental toxicants such as CSC is an undesirable event and the activation of such pathways need to be blocked / prevented to avoid the accumulation of unwanted metabolites such as free oxygen radicals that may lead

to complete germ cell loss. Specific AHR antagonists such as CH223191 can be employed under these circumstances to prevent undesirable excess germ cell death. Others have adapted similar approach to prevent unwanted AHR activation by using natural antagonists such as resveratrol (Revel et al. 2001; Ciolino and Yeh, 1999). Thus, this study inferred that AHR could be a viable therapeutic target to prevent germ cell death induced by environmental toxicants such as CSC.

The need for improved animal models of paternal cigarette smoking

Our data indicate that mouse pups sired by males subjected to long-term CSC exposure have phenotypic defects (unpublished observation) at the early days of development. One challenge with such studies is that mice are obligate nose breathers that do not realistically model human exposure to CS, and the contribution of components of CS to overall cytotoxicity remains ambiguous. However, the animal data cannot be ignored as it provides an incontrovertible link between DNA damage in spermatozoa and defects in embryonic development. Therefore, there is a continuing need to develop an *in vivo* rodent model first to mimic human exposure to cigarette smoke and second, to reliably establish the reproductive toxicity thresholds of constituents of cigarette smoke on male fertility and the development of offspring.

Timing of paternal exposure and transfer of effects to progeny

Fabia and Thuy (1974) reported that paternal exposure to chemical substances could affect the integrity of spermatogenesis and result in the transmission of carcinogenic effects to children. Later, Wilkins and Sinks (1984) demonstrated that children born to painters are six times more likely to develop Wilms' tumor than children from other fathers. Paternal exposures to PAHs of cigarette smoke are related to poor sperm quality and childhood leukemia (Castro-Jimenez and Orozco-Vargas, 2011; Jeng et al. 2013; Ji et al. 2013). An overview by Friedler in the year 1996 suggested that the paternal exposures to a variety of toxicants could induce a broad spectrum of deleterious effects on the normal course of offspring development. The Avon Longitudinal Study of Parents and Children (ALSPAC), led by Pembrey (2014), showed that adolescent sons of fathers who were smoking before puberty are at greater risk of becoming obese and further suggested that cigarette smoke metabolites may induce epigenetic changes in the spermatogonial stem cells during prepubertal stage. Several recent reviews on epidemiological and experimental studies suggest that paternal nutritional, and toxicological exposures can lead to several male-mediated developmental toxicity in the following generations (Curley et al. 2010; Nanassy and Carrell, 2008).

In rodents, males do not interact with their offspring and thus transmit information only via germ cells. If the offspring are programmed differently as a result of paternal exposures to cigarette smoke, then the father's sperm must carry that information. Both genomic and epigenetic pathways can be evoked to explain the transmissible effects of environmental toxicants. For example, plastic derived endocrine disruptors can promote epigenetic transgenerational inheritance of adult onset disease and sperm DNA methylation regions can serve as potential epigenetic biomarkers for transgenerational disease and/or ancestral

environmental exposures (Manikkam et al. 2013). Its known from animal models that high ROS in testis is related to epigenetic changes in sperm (Tremellen, 2008; Kumar et al. 2013). Therefore, paternal exposure to cigarette smoke might affect both genetic and epigenetic characteristics of the sperm through altered ROS, which ultimately increases the risk for disorders in the offspring. For example, males exposed to chronic stress either during puberty or adult age may reprogram the sperm to generate male and female offspring with a hypo functioning HPA stress axis (Rodgers et al. 2013). The increasing number of reports on associations between paternal environmental exposures and risk of disease in the next generation evokes the serious question of how and when the effects of CS exposures are transferred to the male gamete, and whether these effects are sustained through developmental processes.

Possible means of transferring paternal CSC exposure from germ cells to developing embryos may include DNA methylation, histone modifications, mRNAs, and non-coding RNAs. Several periods during the life span of a male may be “windows of susceptibility” when these epigenetic marks are programmed. These include spermatogenesis in the testis and sperm maturation in the epididymis (Nixon et al. 2015). Therefore, studies are needed to determine the point in spermatogenesis at which the germ line is susceptible to constituents of cigarette smoke and the types of epigenetic marks, if any, are established (Bale, 2014). Several small RNAs have been detected in sperm, suggesting that any change in non-coding RNAs due to paternal smoking would be inheritable (Peng et al. 2012; Kiani and Rassoulzadegan, 2013), and their delivery into the oocyte may alter essential functions during early embryogenesis (Sendler et al. 2013). Sperm RNAs reportedly have the ability to direct histone modifications and DNA methylation, for instance in response to paternal smoking (Marczylo et al. 2012), whereas chromatin structure and DNA modifications in turn affect transcription of RNAs. Therefore, exposure to cigarette smoke toxicants might influence this epigenetic crosstalk (Rando, 2012). Male gametes are consistently at enormous risk of epigenetic damage during epigenetic reprogramming, and paternal smoking could change the fidelity of this process. Therefore, research on human sperm is necessary to obtain better insights into the epigenetic mechanisms underlying transmission of environmental effects through the paternal lineage (Soubry et al. 2014). Such work will also lay the foundation for identification of potential biomarkers in predicting disease risk.

Conclusions and future perspectives

As summarized here, the paternal smoking causes generation of ROS, alteration of gene expression, activation of xenobiotic metabolism and MAPK pathways, and apoptosis of germ cells. These mechanisms, which have mainly been identified *in vitro,* accompanied by other unknown *in vivo* changes, could cause deleterious outcomes in smokers and their offspring. However, the development of a more suitable animal model with the potential to truly express the effects of paternal exposure to CS and transgenerational transmission of such impact would be an ideal milestone in the field of research. Studies on humans can be improved by establishment of a biomarker of exposure, such as cotinine, B[a]P, and determination of its pharmacokinetics in the offspring would be of added value. As suggested by others, the change in protamine 1 / protamine 2 could serve as an accurate predictor and important marker in better understanding the key regulatory signaling during

spermatogenesis (Carrell et al. 2007). In addition to the current most plausible Mendelian concept of disease etiology normally involves DNA sequence mutations, environmentally induced epigenetic inheritance of disease should likely be an equally important consideration. Work such as is described in this review is likely to have impacts beyond cigarette smoking, as PAHs are also released as the unintentional byproducts of industrial processes such as incineration, burning treated wood, and the incomplete combustion of fossil fuels such as diesel truck exhaust (Evans et al. 1993; Douben et al. 2003). Men exposed to these environmental toxicants in occupational, industrial, and military settings experience several negative reproductive effects. For example, the children of firefighters (Olshan et al. 1990) and veterans of Gulf War I (Araneta et al. 2003) and the Vietnam war (IOM, 2000) have higher rates of cardiac defects, cleft palate, renal agenesis, and neural tube defects than the children of men in other professions. Thus, the mechanistic lessons learned in studies of the effects of paternal smoking will apply to many other environmental exposures that affect offspring health.

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Highlights

- Paternal smoking is both directly and indirectly associated with poor sperm quality and quantity.
- Constituents of cigarette smoke appear to block spermatogenesis and cause testicular atrophy in animal models.
- Cigarette smoke condensate (CSC) is mutagenic, carcinogenic, and teratogenic.
- Polycyclic aromatic hydrocarbons (PAHs) of CSC act as germ cell toxicants via AHR.
- CSC generates oxidative stress and modulates gene expression in testis.
- CSC mediates AHR-NRF2 crosstalk in spermatogenic GC-2spd(ts) cells.
- CSC mediates interaction between AHR-NRF2 and MAPK pathways during cell cycle arrest in spermatogenic GC-2spd(ts) cells.
- AHR in germ cells can be targeted for therapeutic purposes by pharmacological inhibition.
- Paternal smoking may mediate epigenetic changes in the offspring through spermatogonial stem cells.

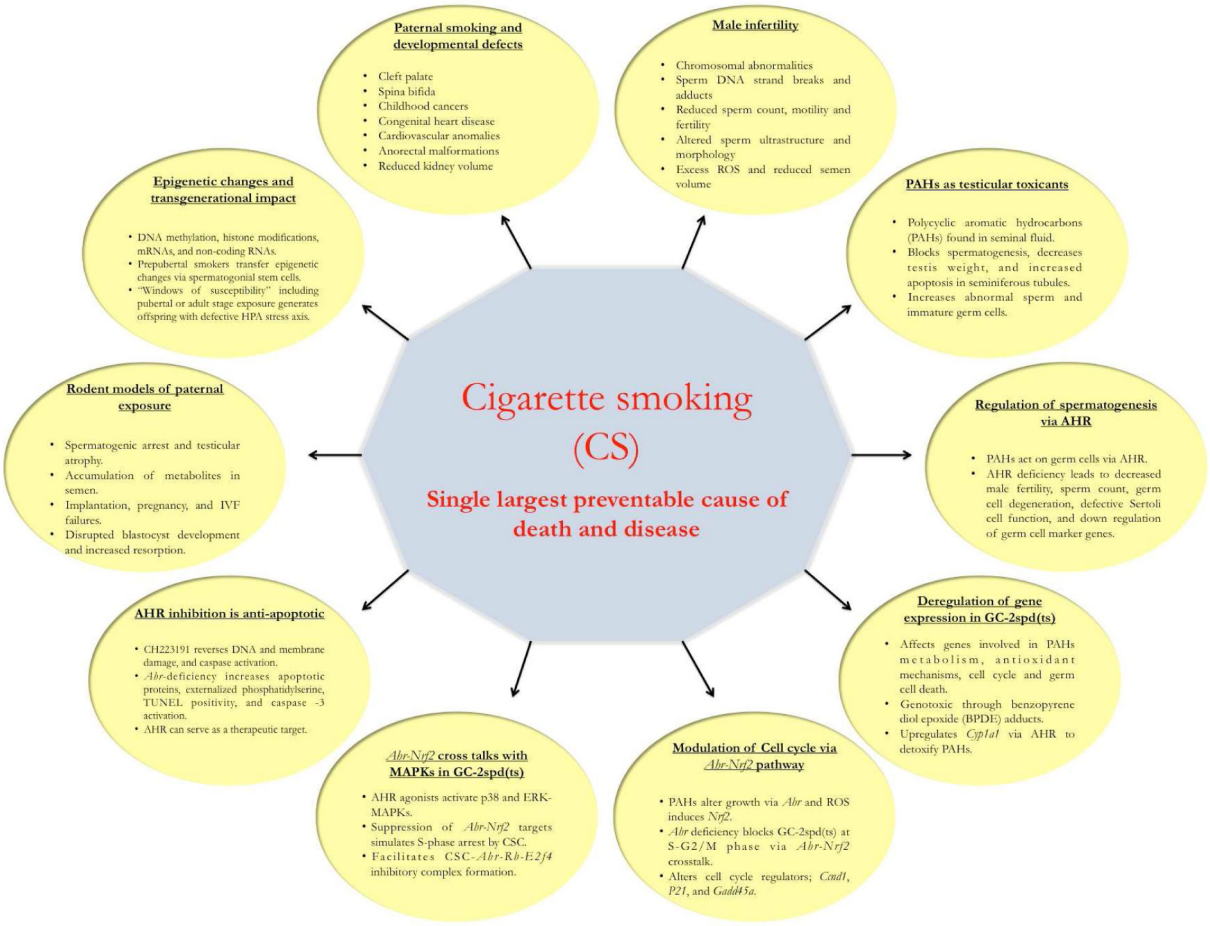

Figure 1.