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Paternal exposure to cigarette smoke condensate leads to reproductive sequelae and developmental abnormalities in the offspring of mice

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ABSTRACT

Paternal smoking is associated with infertility, birth defects and childhood cancers. Our earlier studies using cigarette smoke condensate (CSC) demonstrated several deleterious changes in male germ cells. Here, we hypothesize that chronic paternal exposure to CSC causes molecular and phenotypic changes in the sire and the offspring, respectively. In this mouse study, CSC caused DNA damage and cytotoxicity in testes via accumulation of benzo(a)pyrene (B[a]P) and cotinine. Decreased expression of growth arrest and DNA damage inducible alpha (*Gadd45a*), aryl hydrocarbon receptor (*Ahr*), and cyclin-dependent kinase inhibitor 1A (*P21*) was seen in CSC exposed testes. Apoptotic germ cell death was detected by induction of Fas, FasL, and activated caspase-3. The CSC-exposed males displayed reduction in sperm motility and fertilizing ability and sired pups with reduced body weight and crown-rump length, and smaller litter size with higher numbers of resorption. This model of CSC exposure demonstrates testicular toxicity and developmental defects in the offspring.

1. Introduction

Tobacco is responsible for nearly six million deaths per year worldwide; in the US, one in every five deaths is attributed to cigarette smoking, which incurs more than $300 billion annually in total economic costs [1]. Approximately, 35% of men of reproductive age in the US smoke cigarettes, affecting not only themselves but also the environment and their progeny [2]. Paternal smoking is a known cause of erectile dysfunction and has been implicated in several birth defects including childhood cancers [3]. Cigarette smoke (CS) contains more than 7000 chemicals that include 69 proven carcinogens [4]. Chronic male smokers exhibit several seminal abnormalities including decreased concentration, volume, motility, viability, and fertilizing ability of ejaculated spermatozoa [5] proportionate to the number of cigarettes smoked daily [6]. Despite these facts, there is ambiguity in understanding how the long term exposure to cigarette smoke and its shared constituents in other environmental pollutants such as incompletely burned fossil fuels [7] are responsible for causing such devastating consequences in the exposed fathers and their offspring.

Chronic exposure to CS in rodents abruptly blocks spermatogenesis and strongly inhibits meiotic spermatocytes [8], induced testicular atrophy [9], and decreased testis weight, increased apoptosis in seminiferous tubules [10], and genetic mutations in sperm [11]. This has been primarily attributed to two important factors; one, the heightened sensitivity of testis due to greater susceptibility of germ cells to polycyclic aromatic hydrocarbons that act via the predominantly distributed aryl hydrocarbon receptor (AHR) [12,13], second; the accumulation of constituents of CS and

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their metabolites in systemic circulation and seminal plasma [14]. CSC, representing the mainstream smoke has been reportedly consisting of mainly dioxins and halogenated and nonhalogenated PAHs including benzo(a)pyrene (B[a]P) and pro-oxidants such as lipophilic semiquinones [15,16]. CSC exhibits its pernicious effects both directly via oxidative stress and indirectly by altering the expression of a battery of genes involved in antioxidant mechanisms, metabolism of PAHs, cell cycle progress and cell death [17–19]. During spermatogenesis, Fas, a transmembrane receptor protein and its ligand (FasL) meticulously maintains germ cell homeostasis through apoptotic signaling [20] in the testis. In addition, several cell types such as thymic stromal cells and endothelial cells remain highly prone targets of CS exposed apoptosis that is mediated through Fas/FasL interaction [21,22].

Given that the previous reports on the effects of cigarette smoking on male fertility remain inconclusive, and mice are obligate nose breathers that do not realistically model human exposure to CS, there is a continuing need to develop and improve an *in vivo* mouse model first to understand human exposure to CS, and second; to reliably establish the reproductive toxicity thresholds of cigarette smoke on male fertility and the development of offspring. Our recent studies using CSC both *in vitro* and *in vivo* by a short-term intraperitoneal administration (2 mg/kg/day every 12 h for 7 days) has unearthed several molecular changes in spermatocytes [13]. Nonetheless, it failed to extrapolate these effects on to sperm motility, fertility, or further downstream events. In the present study, we propose using a mouse model of chronic paternal CSC exposure to examine phenotypic changes in both adult and the offspring. The administration of CSC resulted in restricted body weight and crown-rump length of pups as well as smaller litter sizes and higher levels of resorption. These developmental changes were preceded by altered gene expression in the spermatocytes and testis, and the exposed males appeared to have activated both extrinsic and intrinsic pathways of apoptosis in testis, and induced increased expression of antioxidants, structural proteins and downstream targets of *Ahr* in the mature sperm. In addition, the observed accumulation of CSC metabolites and its constituents, cotinine and benzo(a)pyrene respectively, in systemic circulation might have caused DNA damage and cytotoxicity and finally, such upstream events could be responsible for the marked reduction in sperm fertility and motility. Thus, this current model of chronic CSC exposure encompassing one complete cycle of spermatogenesis recapitulates its detrimental effects of testicular damage in the adult and transduces developmental abnormalities in the offspring.

2. Materials and methods

2.1. Animals and organ sampling

Eight- to twelve-week-old C57BL/6J sexually mature male and female mice were housed and the experimental procedures were conducted in accordance with the guidelines for the humane and ethical treatment of animals. All studies were approved by the Animal Studies Committees at Washington University School of Medicine and the St. Louis VA Medical Center. Testis and epididymis collected from the exposed males following treatment were processed for immunohistochemistry, laser microdissection, and caudal sperm isolation. Blood and body fluids collected were processed for ELISA.

2.2. A mouse model of CSC exposure

Based on our earlier results [13] and a previous report [8], we obtained a stock of 80 mg/ml of CSC in 100% dimethyl sulfoxide (DMSO) from Murty Pharmaceuticals Inc. (Lexington, KY) for the present study and exposed the adult male mice to CSC for 40 consecutive days by intraperitoneal (IP) injection at a daily dose of 10 mg/kg/day. Equal volume of DMSO at 100% served as the vehicle control. This CSC dosage regimen was designed to encompass one complete cycle of spermatogenesis in order to determine its effect on the mature sperm.

2.3. RNA isolation, cDNA synthesis, and quantitative real-time reverse transcription PCR (q-RTPCR)

At the end of CSC exposure, the caudal sperm were teased out and the exposed testes were dissected out as reported earlier [23]. The total RNA from caudal sperm, whole testis or CSC-treated spermatocytes isolated by laser microdissection (LMD) were extracted using either Trizol reagent (GIBCO BRL, MD) or PicoPure kit according to the manufacturer's directions (Arcturus # PN: KIT0204) and used for cDNA synthesis and q-RTPCR as previously described [24]. Q-RTPCR was performed based on relative quantification of gene expression by using TaqMan gene expression assays (Applied Biosystems) with the ABI 7500 FAST (Applied Biosystems) as per MIQE guidelines [25]. The data were normalized against β-actin and converted to relative fold difference (CT). The ΔCT of desired genes was calculated by subtracting the ΔCT of the DMSO group from the ΔCT of CSC-treated groups. The $2^{-\Delta\Delta CT}$ method [26] was used to analyze gene expression data, which reflects the relative fold difference in the expression of candidate genes (*Nrf2*, *Atf3*, *E2f4*, *Gadd45a*, and *P21* for whole testis; *Ahr*, *Hsp90*, and *Gadd45a* in LMD spermatocytes; and *Sod1*, *Sod2*, *Tnp1*, *Prm1*, and *Prm2* for caudal sperm) between the control and CSC-treated groups. These candidate genes were selected based on their role in oxidative stress, xenobiotic metabolism, and germ cell development as per our earlier reports [13,19].

2.4. Evaluation of health outcome in CSC exposed males and phenotypic changes to their pups

At the cession of CSC treatment, the CSC and DMSO exposed males were analyzed for their general viability, and body weight. To evaluate the phenotypic changes in pups, one group of pregnant dams was sacrificed on e16.5 and the resorption sites were counted and recorded. The other group of females was allowed to deliver and the body weight and crown–rump length of pups on PND 0.5 were measured as previously shown [27]. Each group consists of 4–16 females and equal number of males.

2.5. Computer assisted sperm analysis (CASA) of CSC-exposed caudal sperm motility

The mature sperm from the cauda epididymis of DMSO- and CSC-injected mice were collected as reported earlier [23]. The sperm suspension *in vitro* fert medium (Cook Medical, Bloomington, IN) at a concentration of ~0.2–0.4 million/ml was analyzed for motility parameters by CASA using the HTM-IVOS Vs12 integrated visual optical system (IVOS) motility analyzer (Hamilton-Thorne Research, Beverly, MA) as previously studied [28].

2.6. Determination of CSC-modulated membrane potential of caudal spermatozoa

The cauda epididymal sperm were collected from both CSC-, and DMSO-treated males as reported earlier [23] in HS medium (in mM): 135 NaCl, 5 KCl, 2 CaCl2, 1 MgSO4, 20 HEPES, 5 glucose, 10 lactic acid, 1 Na-pyruvate, supplemented with 15 mM of NaHCO3 and 5 mg/ml of bovine serum albumin at 37 °C. The swim up sperm with >90% motility were capacitated at 37 °C for 40 min