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Scrambled and fried: Cigarette smoke exposure causes antral follicle destruction and oocyte dysfunction through oxidative stress

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ABSTRACT

Cigarette smoke is a reproductive hazard associated with pre-mature reproductive senescence and reduced clinical pregnancy rates in female smokers. Despite an increased awareness of the adverse effects of cigarette smoke exposure on systemic health, many women remain unaware of the adverse effects of cigarette smoke on female fertility. This issue is compounded by our limited understanding of the molecular mechanisms behind cigarette smoke induced infertility. In this study we used a direct nasal exposure mouse model of cigarette smoke-induced chronic obstructive pulmonary disease to characterise mechanisms of cigarette-smoke induced ovotoxicity. Cigarette smoke exposure caused increased levels of primordial follicle depletion, antral follicle oocyte apoptosis and oxidative stress in exposed ovaries, resulting in fewer follicles available for ovulation. Evidence of oxidative stress also persisted in ovulated oocytes which escaped destruction, with increased levels of mitochondrial ROS and lipid peroxidation resulting in reduced fertilisation potential. Microarray analysis of ovarian tissue correlated these insults with a complex mechanism of ovotoxicity involving genes associated with detoxification, inflammation, follicular activation, immune cell mediated apoptosis and membrane organisation. In particular, the phase I detoxifying enzyme cyp2e1 was found to be significantly up-regulated in developing oocytes; an enzyme known to cause molecular bioactivation resulting in oxidative stress. Our results provide a preliminary model of cigarette smoke induced sub-fertility through cyp2e1 bioactivation and oxidative stress, resulting in developing follicle depletion and oocyte dysfunction.

Introduction

Tobacco smoke is a potent inducer of airway inflammation and has widespread systemic effects (Keely et al., 2012). It is a documented reproductive hazard associated with reduced rates of conception, decreased fertilisation and implantation rates in couples undergoing assisted conception, and premature ovarian failure (or menopause) in women (Freour et al., 2008; Fuentes et al., 2010; Gruber et al., 2008; Neal et al., 2005; Parente et al., 2008; Sun et al., 2011). However, the molecular mechanisms underpinning the adverse effects of cigarette smoke exposure on female fertility are not fully understood. Over the last three decades smoking prevalence among young women has increased worldwide, with over 30% of young women in the United States, and 12–20% of women in Canada, being regular smokers (CDCP, 2008; Huisman et al., 2005; Sen and Wirjanto, 2010; WHO, 2011). Unfortunately, many women remain unaware of the adverse effects of cigarette smoke on female fertility (Anderson et al., 2010; Hughes et al., 2000; Roth and Taylor, 2001). Compounding this issue are studies that suggest smoking does not significantly alter fertility outcomes in women undergoing assisted conception (Fuentes et al., 2010; Petanovski et al., 2012; Wright et al., 2006). Therefore further understanding of the cellular mechanisms responsible for cigarette smoke induced ovotoxicity would assist in defining these clinical issues.

In animal studies, direct cigarette smoke exposure via the nose has been shown to cause premature ovarian failure through primordial follicle depletion (Tuttle et al., 2009). Evidence suggests that a similar mechanism of ovotoxicity occurs in humans, with female smokers having reduced levels of serum anti-Müllerian hormone (AMH), a recognised predictor of the primordial follicle reserve (Freour et al., 2010; Van Disseldorp et al., 2008). A recent study using a whole body (secondary smoke) exposure indicated that this loss may be partially due to autophagy, with autophagosomes being detected in the granulosa cells of smoke exposed mice (Gannon et al., 2012). In addition to follicle loss, we have shown that oocytes collected from nose-only smoke exposed mice also appear to be defective, with significantly thicker zona pellucida and higher incidences of chromosomal abnormalities (Jennings et al., 2011). Cigarette smoke itself is made up of over

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4000 chemical constituents, including cytotoxic and carcinogenic compounds (Rustemeier et al., 2002). One class of toxins found in abundance are the polycyclic aromatic hydrocarbons (PAHs) such as Benzo(a)pyrene (BaP) and 3-methylcholanthrene (3MC). Both BaP and 3MC cause toxicity through the formation of macromolecular adducts and reactive oxygen species (ROS), with BaP-DNA adducts being detected in the granulosa cells and follicular fluid of women who smoke (Gruber et al., 2008; Sobinoff et al., 2012a; Zenzes, 2000). We have demonstrated that both BaP and 3MC induce primordial follicle depletion through a homeostatic mechanism of primordial follicle recruitment, whereby these follicles are stimulated to develop to replace the growing follicles that are targeted for destruction (Sobinoff et al., 2012b, 2012c). Additionally, short-term exposure to BaP caused long term mitochondrial leakage resulting in reduced oolemma fluidity and impaired fertilisation. As both BaP and 3MC are major constituents of cigarette smoke, their mechanisms of ovotoxicity may be partially responsible for the chronic levels of sub-fertility observed in female smokers.

To further investigate the mechanisms of cigarette smoke-induced ovotoxicity, we utilised a novel direct nasal exposure mouse model of cigarette smoke-induced chronic obstructive pulmonary disease that we have recently developed (Beckett et al., 2013). This model is characterised by smoke-induced chronic airway inflammation, mucus hypersecretion, emphysema, and impaired lung function. We hypothesised that mouse direct nasal exposure to cigarette smoke would negatively impact the ovarian follicular pool, follicular development, oocyte quality and fertility. Microarray analysis of ovarian tissue revealed a complex mechanism of ovotoxicity involving genes associated with xenobiotic metabolism, cellular growth and development, cell death, free radical scavenging, and immune responses. In particular, the phase I detoxifying enzyme cyp2e1 was found to be significantly up-regulated in developing oocytes, suggesting it is a major enzyme involved in ameliorating smoke-induced ovotoxicity. Histomorphological analysis identified reduced levels of non-growing/preantral follicles in exposed ovaries, and increased caspase activity and DNA damage in antral follicles. Increased levels of oxidative stress were also detected in smoke-exposed ovaries, with raised mitochondrial ROS and lipid peroxidation levels leading to reduced fertilisation potential in oocytes. Smoke exposed females also had increased time to conception and significantly reduced litter sizes, confirming reduced fecundity.

Methods

Animals. All animal experimental procedures were performed with the approval of the University of Newcastle's Animal Care and Ethics Committee (ACEC). Specific pathogen-free adult female (5-week-old) C57BL/6 mice were obtained from the animal services unit and maintained according to the recommendations prescribed by the ACEC. Mice were housed under a controlled lighting regime (16L:8D) at 21–22 °C and supplied with food and water ad libitum.

Smoke exposure. Cigarette smoke exposure was carried out as descried previously (Beckett et al., 2013). Briefly, C57BL/6 5 week-old female mice were exposed via the nose-only to cigarette smoke [twelve 3R4F reference cigarettes (University of Kentucky, USA) twice/day, five times per week, for 12–18 weeks]. Each exposure lasted 60 min. Control mice received room air. In total, 27 mice underwent cigarette smoke exposure.

Fertility trial. C57BL/6 11 week-old female mice exposed to cigarette smoke for 6 weeks were separated into groups of three and housed with a single control stud male aged 7–8 weeks with proven fertility for a maximum of 12 weeks. Females were monitored every second day for post-coital plugs and pregnancy. Pregnant females were separated into single cages and litter sizes/pup weights were recorded. Smoke exposure continued throughout mating/pregnancy/lactation until weaning of pups at 21 days post birth.

Histomorphological evaluation of follicles. Ovaries obtained from 12 week control and smoke exposed animals were placed in Bouin's fixative for 6 h, washed in 70% ethanol, paraffin embedded and serially sectioned (4 μm thick) throughout the entire ovary, with every 20th section counterstained with hematoxylin and eosin. Healthy oocyte containing follicles were then counted in every section as described previously (Sobinoff et al., 2010).

Immunohistochemistry. Ovaries obtained from 12 week control and smoke exposed animals were fixed in Bouin's fixative and sectioned 4 μm thick. Antibodies specific for Proliferating Cell Nuclear Antigen (PCNA, NA03T, Merck KGaA), tumour protein 53 (phospho S315) (p53, ab131352, abcam), active Caspase2 (Casp2, ab2251, abcam), active Caspase3 (Casp3, ab13847, abcam), Cytochrome P450 2e1 (Cyp2e1, ab73878, abcam) and Cytokine-inducible SH2-containing protein (Cis, ab5923, abcam) were used to probe ovarian tissue sections using the same protocol with the exception of the primary antibody. Slides were deparaffinised in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 3 × 3 min in Tris buffer (50 mM, pH 10.6). Sections were then blocked in 3% BSA/TBS for 1.5 h at room temperature. The following solutions were diluted in TBS containing 1% BSA. Sections were incubated with either anti-PCNA (1:80), anti-p53 (1:200), anti-Casp2 (1:200), anti-Casp3 (1:200), anti-Cyp2e1 (1:200), or anti-CIS (1:100) for 1 h at room temperature. After washing in TBS containing 0.1% Triton X-100, sections were incubated with the appropriate fluorescent conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG (A11012, Invitrogen), Alexa Fluor 594 goat anti-mouse IgG (A11005, Invitrogen); 1:200 dilution) for 1 h. Slides were then counter-stained with 4′-6-Diamidino-2-phenylindole (DAPI) for 5 min, mounted in Mowiol and observed on an Axio Imager A1 epifluorescent microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) under fluorescent optics and pictures taken using a Olympus DP70 microscope camera (Olympus America, Center Valley, PA). Experiments were performed in triplicate.

TUNEL analysis. Bouin's fixed ovarian sections from 12 week control and smoke exposed animals were deparaffinised and rehydrated (Sobinoff et al., 2010). Sections were then boiled in Tris buffer (50 mM, pH 10.6) for 20 min and treated with 20 μg/ml Proteinase K (Promega) for 15 min in a humidified chamber. TUNEL analysis was then performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Pty Ltd.; Dee Why, NSW) according to the manufacturer's instructions. Slides were then counter-stained with DAPI for 5 min, mounted in Mowiol and observed using an Axio Imager A1 epifluorescent microscope (Carl Zeiss) and images captured using an Olympus DP70 microscope camera. Experiments were performed in triplicate.

RNA extraction. Total RNA was isolated from ovaries obtained from 12 week control and smoke exposed animals using two rounds of a modified acid guanidinium thiocyanate–phenol–chloroform protocol followed by isopropanol precipitation (Chomczynski and Sacchi, 1987; Sobinoff et al., 2010).

Microarray analysis. Total RNA (approximately 3 μg) was isolated from 3 adult ovaries after 12 weeks of smoke/control exposure using a QIAGEN RNeasy Mini Kit (Hilden, Germany) in accordance with the manufacturer's recommendations. RNA was prepared for microarray analysis at the Australian Genome Research Facility (AGRF) using an Illumina Sentrix Mouse ref8v2 Beadchip. Labelling, hybridising, washing and array scanning were performed by the AGRF using the Illumina manual on an Illumina BeadArray Reader, and normalised according to the quantile normalisation method using GenomeStudio version 1.6.0 (Illumina, Inc., San Diego, CA). All experiments were performed in