Increased zona pellucida thickness and meiotic spindle disruption in oocytes from cigarette smoking mice

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BACKGROUND: The precise effects of cigarette smoking on female fertility have not yet been clearly defined. We have used a mouse model that mimics human smoking and is able to control for variables that may confound clinical studies to assess the impact of chronic smoking on the quality of mouse oocytes.

METHODS: Mice received cigarette smoke directly to their lungs for 12 weeks. Lung tissue was analyzed for emphysematous changes and cumulus enclosed oocytes (CEOs) were recovered to study their quality. CEOs were in vitro matured, fixed and stained for chromatin and tubulin. Meiotic spindles, chromatin and the zona pellucida were all examined using confocal microscopy.

RESULTS: After 12 weeks of cigarette smoking, mice developed alveolar tissue damage that was determined by an increase in destructive index of the lung parenchyma. The numbers of oocytes recovered and the rates of oocyte maturation were not significantly different from non-smoking mice. However, oocytes from smoking mice had a significantly thicker zona pellucida along with shorter and wider meiotic spindles. Furthermore in total, almost a quarter of oocytes from smoking mice were abnormal as assessed by either errors in chromosomal congression or spindle shape.

CONCLUSIONS: We have used a novel model of inhalational cigarette smoking to show that chronic smoking has a detrimental effect on oocyte quality, and this can be observed even though oocytes are removed from the ovary and cultured in vitro.

Key words: oocyte quality / zona pellucida / meiosis / environmental effects / smoking

Introduction

Cigarette smoking is a recognized risk factor for more than 30 diseases that have a high rate of morbidity and mortality (Schelling, 1992). In addition, smoking has a negative impact on fertility, essentially compromising every aspect of the reproductive process (Winter et al., 2002; Wright et al., 2006; Cooper and Moley, 2008; Soares and Melo, 2008). In women, cigarette smoking has been linked with lower fertilization rates (Elenbogen et al., 1991; Rosevear et al., 1992; El-Nemr et al., 1998), lower pregnancy rates (Van Voorhis et al., 1988; Hughes et al., 1992; Maximovich and Beyler, 1995; Hughes and Brennan, 1996), zona pellucida thickening (Shiloh et al., 2004), impaired oviduct function (Talbot and Riveles, 2005) and increased rates of miscarriage (Fuentes et al., 2010). Given its association with lower fecundity rates, it is concerning that the prevalence of smoking among women of reproductive age has increased over the past few decades (Dechanet et al., 2011).

Despite knowledge that smoking is detrimental, the mechanisms of the effects at the cellular or molecular level remain poorly defined. This is due to at least two reasons. Firstly, human ART data are uncontrolled for other factors that may themselves contribute to diminished fertility (Wright et al., 2006). Secondly, animal models of smoking have not been able to replicate accurately chronic exposure through the normal mode of smoking via the airways. Such animal models would be able to control for variables that may confound clinical studies. In the past, animal studies have used uncontrolled whole-body smoke exposure (Hassa et al., 2007; Esposito et al., 2008; Braber et al., 2010), which cannot replicate the concentrations achieved orally, although they do illustrate the deleterious effects of smoking. Alternatively, animal studies have investigated the effects of a single cigarette toxin, such as nicotine or benzo[a]pyrene on cultured oocytes in vitro (Zenzes and Bielecki, 2004; Liu et al., 2007; Liu et al., 2008; Tuttle et al., 2009). This is a limited approach given that tobacco smoke contains >4000 identifiable chemicals, including...
numerous toxins and carcinogens (American Cancer Society, Revised October 2006).

Here we report the use of a mouse model of cigarette smoking that is characterized by induced alveolar tissue destruction (emphysema), the major characteristic pathological feature of lung changes in chronic obstructive pulmonary disease (COPD; Vlahos et al., 2006). We have used this smoking model to investigate the quality of oocytes recovered from such mice and to examine the zona pellicula thickness, chromosome congression and meiotic spindle structure.

Materials and Methods

Animals

Specific pathogen-free adult female (6-week-old) BALB/c mice were obtained from the animal services unit and all procedures were performed with approval from the animal ethics committee of The University of Newcastle. Mice (n = 8) were exposed via the nose only to mainstream cigarette smoke for 1 h, twice daily, for a total of 12 weeks (custom built, based on Jaeger System Technologies, Inc., NJ, USA). Control mice (n = 4) received room air.

Lung histology

Lungs from six exposed and six control mice were perfused, inflated, fixed, sectioned and stained with hematoxylin and eosin as previously described (Horvat et al., 2010). To assess the effects of smoking on alveoli, alveolar tissue destruction was determined using the alveolar tissue destructive index. A series of 40 random images were captured and the first 10 that did not contain airways or blood vessels were overlayed with a 69-point template. Points were scored for the presence of parenchymal tissue and the percentage of points not overlaying parenchymal tissue was determined (counts absent of tissue/total count × 100%; Robbesom et al., 2003). Therefore, the higher the score, the lower the parenchymal tissue, and the higher the destructive index.

Collection and culture of CEOs

CEOs were collected by puncturing both left and right ovaries from 18-week-old BALB/c mice using a 30-G needle. One hundred and two CEOs were collected from 16 smoke exposed ovaries, and 69 CEOs were collected from 8 control ovaries. All ovaries and intact CEOs were collected and handled in M2 medium at 37°C with 1–1.2 mg/ml of MEM taxol (Zenzes and Bielecki, 2004). Oocytes were briefly stained with Hoechst (20 μg/ml) to label chromatin before mounting on glass slides with Citifluor (Citifluor Ltd, UK).

Confocal imaging

Confocal microscopy was performed using an Olympus FV1000 equipped with a 60x/1.2 NA UPLSAPO oil immersion objective lens. All confocal images are representative equatorial scans on individual oocytes, presented as 8-bit (0–255 arbitrary units) images. Z-stacks were compiled with 1–1.2 μm intervals. It is important to note that all confocal images were analysed using 3D rendering, so that the metaphase plate could be assessed regardless of its orientation with respect to the stage. Analysis was performed with FV10-ASW 2.0 Viewer software (Olympus). Spindle integrity was assessed by comparing tubulin and Hoechst staining in each Z-plane through a stack of planes encompassing the entire spindle.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 software. To calculate the P value a Student’s t-test was used unless otherwise stated.

Results

COPD in a chronic smoking mouse model

Lungs of cigarette smoking mice were collected and examined by histological analysis to confirm the development of COPD. Mice exposed to cigarette smoke for 12 weeks via the airways suffered substantial levels of alveolar destruction, measured as a reduction in alveolar tissue and presented as a destructive index compared with non-smoking mice (Fig. 1; 42.46 ± 1.44% in non-smoking mice versus 56.66 ± 1.34% in smoking mice; P < 0.001). Alveolar destruction is the major hallmark feature of COPD and this confirmed that cigarette exposed mice had developed this smoking-induced disease.

Oocyte fixation and immunofluorescence

Oocytes were fixed for 20 min in a microtubule-stabilizing buffer containing 2% formaldehyde, 0.5% Triton-X100 and 1 μM taxol (Zenzes and Bielecki, 2004). Immunofluorescence was performed as described previously (Holt et al., 2010) using a monoclonal anti-tubulin antibody (1:400, Molecular Probes) supplemented with 1% bovine serum albumin and 0.2% Tween 20 for 2 h at room temperature. Secondary antibodies were conjugated with Alexa555 or Alexa633 (Molecular Probes).
Smoking increases zona pellucida thickness

Ovaries were collected from mice in which COPD had developed at 12 weeks, in order to examine the effects of smoking on oocytes. We noted that all fully grown oocytes, enclosed within antral follicles, recovered from smoking and non-smoking mice were of a similar diameter as measured with respect to the plasma membrane. Oocytes from non-smoking mice had a diameter of 70.5 ± 0.46 μm as compared with 71.7 ± 0.47 μm for those from smoking mice; these values were not statistically different (P = 0.09). However, we did observe differences when we compared the thickness of the zona pellucida, which was measured for thickness at four different locations to obtain a mean thickness per oocyte (Fig. 2A–C). The zona was 22% thicker in smoking mice (7.22 ± 0.06 μm in non-smoking mice versus 8.78 ± 0.04 μm in smoking mice; mean ± SEM; Fig. 2D), an increase that was highly statistically significant (P < 0.0001).

Smoking does not reduce in vitro maturation rates

The mean number of CEOs collected per mouse, which varied between 10 and 24 per mouse, was not found to be statistically affected by smoking (P = 0.36). To establish if there was any detrimental effect of smoking on the ability of the recovered oocytes to undergo meiotic maturation, we cultured CEOs for 15–16 h, a time period which allowed full maturation to metaphase II. At the end of the maturation period, oocytes were denuded and their maturation status was assessed morphologically. In vitro culture of CEOs produced similar, high rates of maturation that were not statistically different between the exposed and non-exposed groups. First polar body extrusion was observed in 71% of oocytes from non-smoking mice and in 78% of those from smoking animals (P = 0.27; Fig. 3).

Smoking affects spindle morphology and chromosome alignment

Gross morphological assessment of oocytes undergoing meiotic maturation cannot be expected to uncover more subtle changes in the quality of oocytes. Therefore, we examined in more detail the nuclear maturation status of those oocytes that had been classified as fully mature due to polar body extrusion. Oocytes from both smoking and non-smoking mice were fixed, permeabilized and fluorescently labeled for chromatin and tubulin. Confocal imaging was used to image the spindle structure, which during metaphase II arrest should display fully congressed, biorientated chromatin on a barrel-shaped spindle.

We did not observe any differences in the orientation and location of the spindle within the majority of oocytes between the two groups of mice. The spindle axis was often tangential to the plasma membrane and was located in the cortex of the oocyte, and we conclude that the location of the spindle was not influenced by smoking (data...
However, we did find that both the pole-to-pole spindle length and the equatorial spindle diameter were both significantly altered by smoking (Fig. 4). Oocytes from smoking mice had a shorter pole-to-pole spindle length (24.5 ± 0.53 μm versus 28 ± 0.75 μm; \( P < 0.0001 \); Fig. 4A and B) but wider spindle equators (11 ± 0.22 versus 9.6 ± 0.50 μm; \( P < 0.0001 \); Fig. 4C and D) compared with non-smoking mice. As spindle size is associated approximately with cell size (Goshima and Scholey, 2010), it is important to note that these differences were not due to differences in cell size, which was not statistically significant between groups.

In addition to changes in the size and shape of the meiotic spindle of oocytes from smoking mice, we also observed errors in chromosome alignment, principally where chromosomes were not bi-oriented at the spindle equator. Severe chromosome non-congression (in which the majority of chromosomes were non-congressed; Fig. 5A) was observed only in oocytes from smoking mice (\( n = 7/79 \)), while non-congression of one or two chromosomes (Fig. 5B) was observed in a further eight oocytes. In contrast only 1 of the 47 oocytes from the non-smoking group displayed any of these errors. Thus oocytes from smoking mice had significantly higher congression defects; with 19% (\( n = 15/79 \)) of all oocytes from smoking mice having one or more sister chromatids non-aligned on the metaphase plate (Fig. 5A and B), compared with only 2% (\( n = 1/47 \)) of oocytes in non-smoking mice (\( P < 0.05 \), Fisher’s exact test). A further defect found only in oocytes from smoking mice was the loss of bipolar spindle integrity, which occurred in 5% of these oocytes (\( n = 4/79 \)). Such oocytes developed a multi-polar spindle structure in meiosis II following extrusion of the first polar body (Fig. 5C and D). Thus, in total, 24% of oocytes from the smoking group displayed abnormalities in the spindle structure or chromosome congression, compared with just 2% in the non-smoking group (Fig. 5E).

**Figure 4** Cigarette smoking alters meiotic spindle morphology. (A) Meiotic spindle demonstrating the measurement of spindle pole-to-pole length. (B) Pole-to-pole lengths for in vitro matured oocytes from non-smoking and smoking mice as indicated. (C) Meiotic spindle demonstrating the measurement of spindle equator length. (D) Equator lengths for in vitro matured oocytes from non-smoking and smoking mice as indicated. The number of oocytes in parenthesis; *** \( P < 0.0001 \). Tubulin stained in green, chromatin in blue. Scale bar, 10 μm.

**Figure 5** Chromatin and spindle pole defects in oocytes from smoking mice (A–D). Following in vitro maturation, oocytes from smoking mice were stained for tubulin (green) and chromatin (blue). Examples of chromatin defects: (A) severe chromosome non-congression in which the majority of chromosomes were non-congressed and (B) non-congression of one or two chromosomes (arrowhead). Some of the oocytes displayed multipolar shaped spindles (C, D), with a cluster of microtubules evident in the cytoplasm (circle). Arrows indicate the spindle poles, scale bar, 10 μm. (E) Percentage of abnormalities in oocytes exposed to smoke (24%) compared with non-exposed oocytes (2%) \( P < 0.005 \). The number of oocytes in parenthesis with abnormalities versus the total number of oocytes analysed.
Disci11us

The present study was designed to examine the quality of oocytes in mice exposed to cigarette smoke via the airways, which accurately mimics human exposure. By making direct comparisons between two groups of mice from the same strain that differ only in exposure to cigarette smoke, we have controlled other variables in a way that is not possible in human studies. One of the most common confounding variables, age, is not a factor here as all mice are 18 weeks at the time of analysis, and all mice have been exposed to the same dose of cigarette smoke.

There were a couple of parameters examined that did not appear to be affected by the smoking regimen used in this study. Firstly, oocyte numbers retrieved and, secondly, maturation rates of CEOs following in vitro culture. It remains possible that smoking has a detrimental effect on numbers but differences are small and require a larger cohort or a longer period of exposure to achieve statistical significance. We suggest this as a possibility because in human studies there is some support for a reduction in oocyte numbers, which is not universally observed (Harrison et al., 1990; Elenbogen et al., 1991; Hughes et al., 1992; Sharara et al., 1994; Sterzik et al., 1994; Zenzes et al., 1997; Lutterodt et al., 2009; Mamsen et al., 2010). For the purposes of this study, CEOs were in vitro matured following collection. Oocytes were left cumulus cell intact because it was not necessary to observe maturation status during the culture period and the maintenance of oocyte–granulosa cell connection is generally regarded as beneficial for maturation (Schroeder and Eppig, 1984; Barret and Albertini, 2010). Overall rates of in vitro maturation judged by the numbers of oocytes extruding a first polar body were the same in smoking and non-smoking groups. This observation is supported by clinical observations that show human oocyte maturation status is not affected by smoking (Wright et al., 2006).

Studies in IVF patients show a correlation between female smoking and increased zona pellucida thickness (Shiloh et al., 2004). Here we have demonstrated that this correlation is indeed causative, i.e. smoking does cause zona thickening. In our study, the zona increased in thickness from 7.22 to 8.78 μm, which represents an increase of 22%, remarkably similar to the 31% calculated in humans (Shiloh et al., 2004). As human oocytes are larger than those of the mouse (the human zona is ~14 μm), the difference in percentage increase is not surprising. Furthermore, the human studies have confounding variables, with age and follicular serum FSH levels also correlated with zona pellucida thickness (Shiloh and Dirnfeld, 2000; Nawroth et al., 2001; Shiloh et al., 2004; Kilani et al., 2006). This increase in thickness is likely to be of clinical relevance, given that the zona plays important roles during fertilization and subsequent embryonic development, such as in sperm binding, blocking polyspermy and protecting the preimplantation embryo in the female reproductive tract. Importantly, dissolution of the zona has to occur for implantation at the blastocyst stage and its thickness is proposed to be one of the primary factors influencing the success of this process for further normal development (Cohen et al., 1989; Roux et al., 1995). Indeed, many clinical interventions, collectively labeled as ‘assisted hatching’, have been used to reduce zona thickness in an attempt to improve implantation rates and aid post-implantation development (Practice Committee, 2008; Das et al., 2009). In mice the zona is composed of three glycoproteins (Litscher et al., 2009), which polymerize to form an extracellular matrix (Jovine et al., 2007). These proteins undergo extensive post-translational modifications including glycosylation and sulfation (Prasad et al., 2000). Smoking may also affect the zona pellucida thickness through interference with the redox status of thiols and consequently protein–protein interactions in the glycoprotein matrix. Alternatively, the high NO₂ content of smoke may induce formation of sulfur-free radicals, which could also influence the formation of disulfide bridges (Bergmark et al., 1997).

The size of the metaphase II spindle was also altered in response to cigarette smoking. The spindle was both shorter, with a ~15% decrease in pole-to-pole length and wider, with a ~15% increase in equatorial length. Such changes were not due to the mistaken inclusion of meiosis I spindles, which do have different dimensions to meiosis II spindles, because we examined only in vitro matured oocytes in which chromatin was clearly visible in the first polar body. At the molecular level, the effect of smoking in compressing the spindle may be mediated through changes in microtubule dynamics given that tobacco alkaloids, such as nicotine, are known to bind tubulin directly (Singer and Himes, 1992; Jordan et al., 1998). Alternatively, the actions of cigarette smoke on spindle structure may be indirect for example through oxidative damage, which is substantially increased following smoking (Church and Pryor, 1990; Stone et al., 1994) and has been observed in ovarian follicles of smoking female IVF patients (Paszkowski et al., 2002). Experimentally, when oxidative stress is induced in mouse oocytes, errors in chromosome segregation are detected (Tarin et al., 1996).

There is some similarity in the present observations of spindle defects and chromosome misalignments with those made previously following nicotine addition to cultured bovine, mouse and hamster oocytes (Racowsky et al., 1989; Zenzes and Bielecki, 2004; Liu et al., 2008). For example, in mouse oocytes dispersed chromosomes and multi-polar spindles have been observed (Zenzes and Bielecki, 2004). In that study, mouse oocytes were analysed following maturation in 10-nM nicotine in vitro without their cumulus cells attached. They classified 33% of oocytes as abnormal, assessed by either a lack of a bipolar spindle or possessing chromosomes not aligned at the spindle equator. Although it is tempting to conclude therefore that in vitro nicotine addition is a good mimic for female smoking, it is important to stress that to achieve these in vitro effects, the nicotine concentration used would be at least 1000-fold higher than blood nicotine levels measured in smokers (0.1–1 μM; Langone et al., 1973; Benowitz et al., 1982). Independent of the precise mechanism that leads to the change in the spindle shape, we find here that tobacco smoking is also associated with misaligned chromosomes on the metaphase II plate. Such misalignment, although not tested here, presents the possibility of non-disjunction of sister chromatids following fertilization and the creation of an aneuploid embryo. The association of smoking with aneuploidy has been reported previously in a number of cell types including human sperm (Shi et al., 2001; Robbins et al., 2005; Perry, 2008), while in human oocytes, maternal smoking has been correlated with a rise in trisomy 18 (Miron et al., 2008) and higher rates of aneuploidy in spontaneously aborted fetuses (Kline et al., 1983).

In summary, this report directly establishes that changes in oocyte quality accompany exposure of the maternal airways to cigarette smoke. Importantly, these changes occur over the same time window as the development of hallmark features of COPD, one of
the classical lung conditions of habitual smoking. Future studies using this mouse model system would be useful in characterizing the impact of changes in oocytes on fertilization rate and embryo development, as well as in assessing the impact of smoking on other components of the reproductive system, notably the uterus. This model could also be used in determining the reversibility of smoking cessation on female reproductive health. Such findings would be useful to help inform the public about the consequences of female smoking and may facilitate quit smoking campaigns for young women.

Authors’ roles

P.J., J.M., P.H. conceived and designed the project. P.J., J.M. and E.B. acquired the data. P.J., J.M., K.J. and P.H. interpreted the data. Drafting the article was by P.J. and K.J., with J.M., E.B. and P.H. providing critical revision. All authors were involved in final approval of the version to be published.

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