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REVIEW

Ambient air pollution exposure and damage to male gametes: human studies and *in situ* 'sentinel' animal experiments

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Globally there is concern that adverse reproductive outcomes and fertility impairment in humans may be caused by exposure to environmental contaminants. Air pollution in particular has been linked to DNA damage, abnormal sperm morphology, and reduced sperm performance in men. Experimental studies using model species (mice and rats) exposed *in situ* provide evidence that ambient air pollution can cause damage to the respiratory system and other tissues or organs. This can take the form of DNA damage and other genetic changes throughout the body, including induced mutations, DNA strand breaks, and altered methylation patterns in male germ cells. Human and animal studies together provide strong evidence that air pollution, especially airborne particulate matter, at commonly occurring ambient levels is genotoxic to male germ cells. The mechanistic link between air pollution exposure and induced genetic changes in male germ cells is currently unclear. 'Sentinel' animal experiments explicitly examining air pollution affects on sperm quality in laboratory rodents have not been conducted and would provide a critical link to observations in humans. The importance of air pollution compared to other factors affecting fertility and reproductive outcomes in humans is not clear and warrants further investigation.

Keywords air pollution, DNA damage, fertility, germ cells, germline mutation, sentinel animals

Abbreviations PM: particulate matter; ESTR: expanded simple tandem repeat; HEPA: high efficiency particulate air; SM-PCR: single molecule PCR.

Introduction

People in developed countries worldwide are concerned that infertility and adverse reproductive outcomes may in some cases be caused by environmental contaminant exposures (reviewed by [Bhatt 2000; Foster 2003; Foster et al. 2008; Jurewicz et al. 2009]). Air pollution is of particular concern, and there is growing evidence linking ambient levels of exposure with negative effects on reproductive health in both men and women [Sram 1999; Slama et al. 2008]. Human studies have linked maternal air pollution exposure with reduced fetal growth and increased frequency of visible birth defects (e.g., [Wang et al. 1997; Bobak 2000; Ritz et al. 2002; Gilboa et al. 2005]), and male exposure with reduced semen quality and sperm DNA integrity [Jurewicz et al. 2009]. Male-mediated effects and the genetic integrity of sperm are of particular concern because they could potentially be affected by air pollution at any point during adulthood and are a common cause for couples to seek fertility treatment [Oehninger 2001]. Therefore this review will focus on air pollution-induced effects in male germ cells.

Experimental studies in which model species are exposed *in situ* to ambient air pollution provide a powerful tool for studying induced general health effects and changes to reproductive parameters [Saldiva and Böhm 1998; Somers 2006; Somers and Cooper 2009]. This *in situ* 'sentinel' animal approach provides a remarkable parallel to human studies of air pollution effects, and enables air pollution, or particular fractions of air pollution, to be isolated as single variables affecting the endpoint of interest. Thus, sentinel animals can serve as an extremely valuable supplement to human studies by allowing us to move beyond correlative associations to experimental evidence for causation [Somers 2007]. In this review I discuss air pollution effects on human male gametes and illustrate how the *in situ* sentinel animal approach has been used to study health effects and genotoxicity of ambient air pollution, including germline mutations and measures of DNA damage in the sperm of mice.

Air pollution exposure and damage to human sperm

Despite widespread concern over the general health effects of air pollution (reviewed by [Chan-Yeung 2000; Brunekreef and Holgate 2002]) and environmental influences on semen quality and male fertility (reviewed by [Jurewicz

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et al. 2009]), few studies have attempted to link these two fields. A review of the published literature on ambient air pollution effects in human sperm reveals a small number of studies that vary in size and that were conducted in only three areas of the world: the Teplice District of the Czech Republic, western United States, and south-eastern United States (Table 1). These areas differ substantially in atmospheric air pollution levels (and likely composition), and thus the presumed exposure of the study subjects; it is perhaps not surprising that the number and type of sperm quality parameters affected varied by location. In the Czech Republic, several ambient air quality measures, including inhalable particulate matter known to carry toxic and genotoxic compounds, frequently exceeded maximum World Health Organization guideline values by a large margin [Selevan et al. 2000; Rubes et al. 2005; WHO 2005]. Young men (age 18) experiencing these high air pollution exposures showed an array of responses in sperm, ranging from morphological and performance impairments to abnormal chromatin structure and aneuploidy (Table 1). These findings, including the study design and confounding factors, have been more thoroughly reviewed by Jurewicz et al. [2009]. The data provide suggestive, though not fully consistent, evidence for air pollution effects in several aspects of testes function ranging from spermatogenesis to DNA damage and repair under high-exposure conditions. However, the specific contaminants that caused sperm damage and the potential impact on fertility or pregnancy outcomes of the subjects were undetermined.

In two of the three American studies only a single measured sperm quality endpoint was affected by air pollution, and in the third study no significant effects were detected (Table 1). Subjects in these studies experienced ambient air pollution levels that were on average well below those in the Czech Republic [Sokol et al. 2006; Hansen et al. 2010; Hammoud et al. 2010], so it is likely that exposure levels were insufficient to induce similar effects. For example, a high air pollution winter in Teplice (Czech Republic) had toxic and genotoxic particulate matter (PM_{10}) levels of 184.7±211.9 μg/m^3, compared to only 35.7±13.8 μg/m^3 in Los Angeles, USA [Selevan et al. 2000; Sokol et al. 2006]. It is worth noting that PM_{10} levels in both the Czech Republic and Los Angeles at the time of these studies well exceeded current guidelines of 20 μg/m^3 for annual means [WHO 2005]. Nevertheless, even relatively low levels of ozone and $PM_{2.5}$ in parts of the USA were associated with statistically significant reductions in sperm concentration and motility, respectively (Table 1). The fertility impacts of air pollution exposure were not examined directly in these studies, but the magnitude of the effects documented was small and unlikely to be clinically relevant [Sokol et al 2006; Hammoud et al. 2010]. However, these small effects contribute to the emerging evidence that air pollution somehow interacts with the male reproductive system and can affect the production and quality of sperm. The genetic integrity of sperm was only measured in one of the three American studies, which showed no effects of any kind [Hansen et al. 2010]. It would be of substantial interest to know whether the other two studies that found some sperm quality changes in the USA [Sokol et al. 2006; Hammoud et al. 2010] would also have detected elevated levels of DNA damage. It is premature to make any conclusions based solely on these findings about whether human male germ cells suffer genotoxic effects under common North American air pollution conditions.

In situ exposures of 'sentinel' lab animals: an exprimental approach to air pollution studies

Human studies of air pollution and health effects, regardless of the endpoint studied, are largely correlative in nature and have some important shortcomings that limit their strength of inference. Even well-designed ecologic studies (like those above) lack precise control over potential confounding factors in the populations assessed, such as lifestyle or underlying genetics. In addition, air pollution exposures are most often indirectly inferred from monitoring stations that are at some distance from study subjects, which can lead to misclassification of exposure. Finally, steps in the pathway leading from exposure to disease are often not examined, likely because they require invasive or intrusive sampling, making it difficult to establish mechanistic links between air pollution exposure and the measured endpoint [Somers 2007]. Human studies therefore provide us with evidence of air pollution-induced effects, but stop short of demonstrating causation.

The major limitations of human studies described above can be overcome experimentally using rodents exposed to common components of urban and industrial air pollution under controlled laboratory conditions (e.g., [Meng et al. 2002; Oberdörster et al. 2004; Sun et al. 2005]). This approach allows much more precise regulation and characterization of exposures, application of a suite of standard biomarkers, and invasive sampling. However, ambient air pollution is far too complex to be recreated in a laboratory setting, and experiments often involve high-dose, short-term exposure conditions. Laboratory studies are therefore of uncertain relevance in understanding the steps leading from air pollution exposure to human biomarker responses or health effects [Saldiva and Böhm 1998].

Exposure of laboratory animals *in situ* to ambient air pollution permits control over genetic background, husbandry conditions (analogous to lifestyle factors in human studies), and exposure location and duration, while maintaining the relevance of direct exposure to ambient air pollution conditions. In addition, the use of model species makes available a suite of established bioassays for assessing air pollution effects, as well as invasive sampling procedures for examining mechanistic steps from exposure to endpoint. Thus, although this experimental approach has some potential shortcomings (see [Somers 2007]), it is a potentially very powerful tool for air pollution studies because it incorporates some of the more important features of both human and laboratory studies [Saldiva and Böhm 1998; Somers 2006; 2007; Somers and Cooper 2009]. In this review experiments of this kind are referred to as 'sentinel' animal studies, although there is debate about use of the terms 'indicator,'

Table 1. Summary of Published Studies Examining Associations Between Ambient Air Pollution and Human Sperm Quality and Genetic Integrity. Only Studies of Outdoor Pollution have been Considered; Outdoor Occupational Exposures and Attempted Interventions (e.g., [De Rosa et al. 2003; Guven et al. 2008; Paradisi et al. 2009]) are Beyond the Scope of this Review.

Reference	Location	Participants[a]	Endpoints measured[b]	Endpoints affected[c]	Correlated air[d] pollutants	Fertility impacts
Perreault et al. [2000]	Teplice District, Czech Republic	272	-Standard WHO -Motility (CASA) -Aneuploidy 8 and Y -DNA integrity (SCSA)	-Velocity -YY disomy	n/a	?
Selevan et al. [2000]	Teplice District, Czech Republic	272	-Standard WHO -Progression, vigor (CASA) -DNA integrity (SCSA)	-% motile - % with normal morphology / head - % with abnormal chromatin	PM_{10}, TSP, SO_2, CO, NO_x possible	?
Rubes et al. [2005]	Teplice District, Czech Republic	36	-Standard WHO -Motility (CASA) -Aneuploidy 8, X and Y -DNA integrity (SCSA)	-% with abnormal chromatin	PM_{10}, SO_2, NO_x, PAH possible	?
Sokol et al. [2006]	Los Angeles, California, USA	48	-Standard WHO without morphology - Motility	- Concentration	Ozone	?
Rubes et al. [2007]	Teplice District, Czech Republic	35	-GSTM1 genotype -DNA integrity (SCSA)	-% with abnormal chromatin	PM_{10}, SO_2, NO_x, PAH possible; interaction with GSTM1	?
Hammoud et al. [2010]	Salt Lake City, Utah, USA	1465[e]; 561	-Standard WHO -Motility	- Motility	$PM_{2.5}$	Unlikely
Hansen et al. [2010]	North Carolina, Tennessee, Texas, USA	228	-Standard WHO -DNA integrity (SCSA) -Chromatin maturity (CMA) -Cytoplasmic drops	-None	n/a	No

[a]The total sample size of the study is listed here; in some cases the number of participants for specific assays is considerably less than the study total. Perreault et al. [2000] and Selevan et al. [2000] both used the same cohort of men, and thus are not independent samples; the same is true for Rubes et al. [2005 and 2007].

[b]Standard WHO indicates that semen characteristics (volume, sperm concentration, and sperm morphology) were assessed according to the World Health Organization [WHO 2005] guidelines. SCSA is the sperm chromatin structure assay, which measures relative susceptibility of sperm DNA to denaturation; CASA is computer-assisted sperm analysis; CMA is chromomycin A3 staining.

[c]Only changes with strong statistical support are listed here; there are other trends that are of potential interest in each paper and readers are encouraged to consult the original publication for more specific information.

[d]PM = particulate matter with the maximum aerodynamic particle size indicated as a subscript; TSP = total suspended particulate matter; PAH = polycyclic aromatic hydrocarbons; in all studies air pollution exposures were inferred from regional monitoring programs, none used personal monitoring approaches.

[e]This study had two components with different sample sizes. The first portion of the study does not report the total number of men analyzed, but indicates 1,699 semen samples, with an average of 1.16 samples per subject; the total number of participants indicated here was calculated as 1,699/1.16 = 1,465.

'monitor,' and 'sentinel' species in the literature (reviewed by [O'Brien et al. 1993]).

Sentinel animal studies of air pollution and pathology/genotoxicity

Experimental exposures of sentinel animals have been employed to investigate a wide variety of general health and genotoxicity effects in areas that have high levels of air pollution (Table 2). The goal in briefly summarizing this body of literature, despite the fact that it does not focus on reproductive effects, is two-fold: (i) to illustrate the effectiveness of sentinel animal experiments in air pollution studies, and (ii) to present further rationale for assessing changes to germ cells by providing experimental evidence that air pollution can affect virtually any organ system. Each of the sentinel animal studies in Table 2 was prompted by concern over local human health issues that were linked to air pollution, but lacked definitive evidence of causation or mechanistic explanation as described above. Sentinel animal studies have been conducted in a limited number of geographic locations to date, but cover the continents of South America, Europe, and Asia, and so are likely representative of exposure conditions for many human populations globally (Table 2; additional sentinel animal studies on the germline conducted in North America are summarized below). Several themes emerge from these sentinel animal studies that give us insight into the human epidemiology studies that prompted the research.

Sentinel animal experiments clearly demonstrate that exposure to ambient air pollution for even a relatively short period causes damage to the respiratory tract from the upper airway to the lungs (Table 2; one exception, see [Moss et al. 2001]). It is likely that similar changes in humans cause the increases in respiratory illnesses and related deaths that have previously been correlated with air

Table 2. Sentinel Animal Studies Experimentally Examining the Effects of Ambient Air Pollution on Endpoints Related to General Health and Genotoxicity. Only Studies of Ambient Outdoor Pollution have been Considered; Laboratory Studies and Mobile Lab Studies that Regulate Animal Exposures to Particular Doses are Beyond the Scope of this Review.

Reference	Location[a]	Organism	Sample size[b]	Exposure duration	Effect documented	Important air pollutants
Böhm et al. [1989]	Sao Paulo, Brazil	Rats	120	6 months	Lesions and other damage to upper and distal airways	Diesel exhaust; industrial emissions
Saldiva et al. [1992]	Sao Paulo, Brazil	Rats	54	6 months	Respiratory lesions; clearance impairment	PM, SO_2, CO, O_3; no specific links possible
Lemos et al. [1994]	Sao Paulo, Brazil	Rats	30	6 months	Damage to nasal epithelium; acidic mucus; ciliary damage	PM, SO_2, CO, O_3; no specific links possible
Reichrtova et al. [1995]	Slovakia	Rabbits	24	6 months	Hg in respiratory tract, bone, brain, kidney, and heart; altered trachea	Siderite and tetraedrite ore particulates
Reymão et al. [1997]	Sao Paulo, Brazil	Mice	550	2 months	Lung tumor promotion (urethane initiated); altered tumor phenotype	PM, SO_2, CO, O_3; no specific links possible
Moss et al. [2001]	Mexico City, Mexico	Rats[c]	172	21–49 days	No differences between exposed and filtered-air reference groups	Many types of urban and industrial pollutants
Soares et al. [2003]	Sao Paulo, Brazil	Mice	40	120 days	Elevated micronucleus frequency in peripheral erythrocytes	PM_{10}, CO
Sato et al. [2003]	Kawasaki City, Japan	Rats	76	4–60 weeks	Elevated DNA adduct levels in nasal mucosa, lungs, and liver	Carbon black; $PM_{2.5}$
Mohallem et al. [2005]	Sao Paulo, Brazil	Mice	134	4 months	Reduced female fertility; smaller litters, more failed implantations	PM_{10}, NO_2 likely; no specific links possible
Lemos et al. [2006]	Sao Paulo, Brazil	Mice	40	4 months	Lung inflammation; thickening of pulmonary/coronary artery walls	Diesel exhaust; industrial emissions
Pires-Neto et al. [2006]	Sao Paulo, Brazil	Mice	40	5 months	Damage to nasal epithelium; acidic mucous	PM_{10}, NO_2 likely; no specific links possible

[a]The location indicated is that of the exposed group where air pollution was suspected to have health effects. For most studies a reference (control) group for comparison was simultaneously housed under similar conditions at a more pristine site removed from major sources of air pollution. Moss et al. [2001] and Sato et al. [2003] used animals simultaneously exposed inside of filtration chambers at polluted sites as control groups.
[b]The sample size reported is the total number of animals included in the study.
[c]Studies of free-ranging dogs in Mexico City provide evidence for dramatic damage and health effects caused by air pollution exposure (see [Calderón-Garcidueñas et al. 2001a; 2001b; 2002; 2003]); it is unclear why rats did not respond to this exposure.

pollution exposure (e.g., [Pope et al. 1995; 2002]). In addition, urban air pollution caused lung tumor promotion in sentinel rodents and provides a potential explanation for elevated rates of lung cancer observed in humans exposed to particulate matter (e.g., [Pope et al. 2002; Chiu et al. 2006]). The experimental nature of the sentinel animal studies rules out other factors that could have caused these changes to respiratory pathology, and provides mechanistic insight into the steps leading from exposure to clinical presentation in humans. Respiratory tract changes are perhaps not surprising given the intuitive relationship between the respiratory system and air pollution exposure; however, there is much more to these studies than airway effects.

Sentinel animal studies also begin to reveal that the effects of air pollution can extend well beyond the respiratory system to almost any part of the body (Table 2). Airborne contaminants clearly reach systems remote from the respiratory tract [Reichrtova et al. 1995], and can cause changes to the circulatory system [Lemos et al. 2006] that might be important for explaining elevated rates of human cardiovascular events associated with air pollution (reviewed by [Vermylen et al. 2005; Franchini and Mannucci 2009]). In addition, DNA damage and pre-mutational lesions can be induced in a variety of tissues, including liver and blood [Sato et al. 2003; Soares et al. 2003], providing further evidence that body-wide effects are possible. Body-wide exposure and effects set the context for possible damage to the reproductive system and effects on fertility. Only a single study has addressed this issue from the female perspective using a sentinel animal approach; it demonstrated that female mice exposed chronically to ambient urban air pollution shortly after birth had reduced fertility as adults [Mohallem et al. 2005]. Male fertility parameters have not yet been explicitly studied using an experimental sentinel animal approach.

Sentinel animal studies of air pollution and germline mutations

Several of the sentinel animal studies in Table 2 have shown accumulation of contaminants in body tissues, pre-mutational lesions in somatic cells, and tumor development, but few have attempted to determine if these effects extend to male germ cells. Wild rodents provide additional justification for addressing this issue, as feral mice living in high air pollution areas near heavy automobile traffic were observed to have elevated frequencies of head and tail deformities in their sperm [Ieradi et al. 1996]. In addition, wild herring gulls living in industrial areas with high levels of air pollution were shown to have elevated rates of germline mutations at minisatellite loci (tandem repetitive DNA loci; [Yauk and Quinn 1996; Yauk et al. 2000]), suggesting a potential interaction between air pollution and germ cells. However, herring gulls were potentially exposed to environmental mutagens through multiple routes, so air

pollution could not be specifically identified as the causative agent.

In response to the findings from herring gull studies [Yauk and Quinn 1996; Yauk et al. 2000], Somers et al. [2002] conducted a sentinel animal experiment with laboratory mice that examined germline mutation rates in two groups exposed to ambient air pollution in Ontario, Canada. In this scenario: (i) 20 out-bred males and females (housed separately by sex) were exposed at an industrial site near two integrated steel mills and a major highway, and (ii) the same number of animals were exposed at a rural reference location removed from point sources of air pollution. They exposed mice for 10 weeks in a special housing facility at each location, and then returned them to the laboratory for 6 weeks prior to breeding pairs within treatment groups. The delay in breeding was designed to ensure that offspring in the study were derived from sperm that matured from diploid spermatogonial stem cells that underwent the entire 10-week air pollution exposure. Family groups were then assayed for germline mutation events resulting in size changes at expanded simple tandem repeat (ESTR) DNA loci [Kelly et al. 1989; Gibbs et al. 1993; Bois et al. 1998], a class of repetitive DNA that is unstable in the mouse germline and well-suited for mutation induction studies (reviewed by [Yauk 1998; 2004; Dubrova 2003; 2005; Somers 2006; Somers and Cooper 2009]).

Somers et al. [2002] found a significant 1.5- to 2-fold elevation in mutation frequency in the offspring of animals exposed at the polluted site, primarily through ESTR mutation events in the paternal germline. In addition, litter sizes in the polluted group were reduced in size by on average 1.7 pups compared to the reference group. This change was not statistically significant, but provided some preliminary evidence for an effect of air pollution on fertility. Both male and female mice were exposed, so either sex could have contributed to the litter size reduction. In general, this sentinel animal experiment provided proof-of-principle that air pollution can cause genetic changes in the male germline; however, the components of air pollution contributing to induced ESTR mutations were unknown and therefore difficult to set in context in terms of human exposure guidelines. For Canada, the polluted site had relatively high levels of airborne particulate matter and associated contaminants, as well as many gas-phase pollutants, but these levels were low to moderate on a global scale. The authors proposed that mutagenic polycyclic aromatic hydrocarbons (PAHs) associated with particulate matter might be important, but this was largely speculation.

To confirm their original findings and to learn more about the components of air pollution that might be important for inducing elevated germline ESTR mutation rates in mice, Somers et al. [2004] performed a second sentinel animal experiment at the same locations described above. In addition to exposing 20 male and 20 female mice to whole ambient air, they also included similar sized groups exposed inside of chambers that received only high efficiency particulate air (HEPA)-filtered ambient air at each location. This experimental design enabled a comparison of germline mutation rates between sites, and also within sites between groups exposed to ambient air and HEPA-filtered air. HEPA filtration removes only particulate matter (at least 99.97% of all particles >0.3 μm in diameter; [Ettinger et al. 1969]), so mice inside of HEPA chambers were exposed to ambient air that was essentially particulate-free, but still contained gas-phase constituents. Animals were exposed for 10 weeks *in situ*, and then bred within groups after a 9-week delay in the laboratory for the reasons described above. The frequency of ESTR size-change mutation events was compared among treatment groups.

Somers et al. [2004] noted that ESTR mutation frequencies were elevated 1.9 to 2.1-fold in the paternal germline of mice exposed to whole ambient air at the polluted industrial site compared to the other treatment groups. Maternal ESTR mutation frequencies were similar in all treatment groups, and therefore unaffected by air pollution exposure. These findings confirmed that air pollution exposure could induce germline mutations in the paternal germline via exposure of spermatogonial stem cells in sentinel mice. In addition, the fact that mutation frequencies were elevated in the whole air-exposed group, but not the HEPA-filtered group at the polluted site, showed that removal of particulate matter essentially negated excess mutation induction. Thus, airborne particulate matter was an important causal factor in germline ESTR mutation induction. Litter sizes did not differ among treatment groups in this experiment as they did in the first study [Somers et al. 2002], providing no further evidence of fertility impacts. The mechanism leading from particulate matter exposure via the respiratory tract to ESTR mutation induction in spermatogonial stem cells, and the identity of any specific contaminants or groups of contaminants causing the effect were entirely unknown. In addition, the health relevance of elevated size-change mutation frequencies at neutral, hyper-variable ESTR loci was uncertain (discussed by [Samet et al. 2004; Somers and Cooper 2009]).

Yauk et al. [2008] performed a third sentinel animal experiment using 15 male mice exposed to either whole ambient air or HEPA-filtered ambient air (as in [Somers et al. 2004]) at the polluted site described above. In this case, ESTR mutations were detected using single molecule PCR (SM-PCR) of ESTR locus Ms6-hm directly in sperm [Yauk et al. 2002], which eliminates the need to produce offspring as part of the experiment, and makes a much larger number of gametes available for mutation screening. SM-PCR identifies size-change mutations in ESTR loci similar to the approach used in family groups above. However, SM-PCR is performed directly on gamete DNA, so it detects only mutation events that occur during spermatogenesis and become fixed in sperm. This was important because the authors wanted to determine whether air pollution exposure induced true germline mutations, or sperm lesions that induced somatic instability post-fertilization, which is common at ESTR loci in mice [Kelly et al. 1989; Gibbs et al. 1993]. Bulky DNA adduct

formation in lung and testes tissue, strand breaks in sperm DNA, and global genomic methylation patterns in sperm were also quantified in these mice. All assays were performed after 3 and 10 weeks of ambient air or HEPA-filtered air exposure, and also after 10 weeks of exposure followed by 6 weeks in the laboratory. This experimental design enabled assessment of germ cell response following exposures of different durations, but it also allowed evaluation of induced effects in different germ cell stages ranging from spermatids (3 weeks of exposure) to spermatogonial stem cells (10 weeks of exposure plus 6 weeks in the laboratory).

Yauk et al. [2008] found that the germline ESTR mutation frequency measured directly in sperm was significantly elevated by 1.6-fold in mice exposed to whole ambient air compared to HEPA-filtered air after 10 weeks of exposure followed by 6 weeks in the laboratory. ESTR mutation frequency was similar in both treatment groups after 3 and 10 weeks of exposure. This finding demonstrated that particulate air pollution induces true germline ESTR mutations in spermatogonial cells that become fixed during gametogenesis. In addition, the authors found significantly more bulky DNA adducts in the lungs of mice exposed to ambient air compared to HEPA-filtered air after 3 weeks, thereby confirming exposure of the animals to DNA-reactive contaminants associated with particulate matter. However, DNA adducts were not detected in testes tissues at any time point in the study, suggesting that ESTR mutations were unlikely to be directly induced by the presence of PAHs or their metabolites in the gonads. DNA strand breaks in sperm were significantly elevated in the whole air-exposed group after 3 weeks, and marginally so after 10 weeks, indicating that physical DNA damage was being induced in germ cells despite the absence of bulky adducts. In addition, sperm DNA in mice exposed to whole ambient air was globally hypermethylated compared to those exposed in the HEPA-filtration chamber. These methylation changes appeared early in the environmental exposure and were still present after 6 weeks in the laboratory. Persistent changes in the methylation status of genes may have health implications for the next generation through altered gene expression (reviewed by [Reamon-Buettner and Borlak 2007; Dolinoy and Jirtle 2008]). The mechanistic steps leading from particulate matter exposure in the respiratory system to DNA strand breaks, methylation changes, and ESTR mutations in sperm have yet to be determined. Nevertheless, this work clearly demonstrates that particulate air pollution can damage male germ cells.

Linking germ cell studies of humans and sentinel animals

Human studies raise the possibility that ambient air pollution exposure can have a negative impact on sperm quality that likely stems from impaired spermatogenesis and elevated levels of DNA damage in germ cells (Table 1). These studies, while concerning, are limited in their ability to identify air pollution as a causative factor. Sentinel animal studies enable experimental isolation of ambient air pollution as essentially a single variable, and therefore provide clear causal links between exposure and endpoint. A suite of sentinel animal experiments has provided evidence for the damaging effects of air pollution in many parts of the body (Table 2), strongly suggesting that the reproductive system is also at risk. A series of sentinel animal experiments has subsequently shown that particulate matter causes induced germline mutations, as well as physical DNA damage and epigenetic changes in the sperm of mice [Somers et al. 2002; 2004; Yauk et al. 2008]. Thus, there is now substantial evidence of potentially important changes to male germ cells caused by air pollution exposure. Unfortunately, human and sentinel animal studies have not measured identical endpoints in air pollution studies of germ cell effects (a situation that should be rectified), so it is difficult to draw direct comparisons.

Nevertheless, even though precisely identical endpoints were not measured, human studies in the Czech Republic [Selevan et al. 2000; Rubes et al. 2005] and mouse studies in Ontario, Canada [Somers et al. 2002; 2004; Yauk et al. 2008], have each documented elevated rates of DNA damage and/or mutation induction in male germ cells following air pollution exposure. The human studies [Selevan et al. 2000; Rubes et al. 2005] employed the sperm chromatin structure assay to quantify chromatin integrity, a test that is also used as a marker for fertility [Evenson and Wixon 2005; 2006]. Sperm DNA fragmentation indices were considered moderate in exposed men, and thus likely not clinically relevant for infertility [Rubes et al. 2005]. However, the study subjects were healthy men of 18 years of age. It would be of significant interest to know how air pollution exposure may interact with age or pre-existing conditions to cause more severe DNA damage, thereby contributing to increased rates of clinical infertility. As a case in point, Rubes et al. [2007] showed that the GSTM1 genotype was a significant factor affecting the DNA fragmentation index of study subjects, suggesting that genotype should also be considered when grouping subjects for assessment of air pollution-induced effects. Perhaps just as important, it will be critical to assess whether increased DNA fragmentation rates, even to moderate levels, are reflective of elevated germline mutation frequencies and epigenetic changes (as in mouse studies), and therefore adverse reproductive outcomes not necessarily associated with fertility *per se*. Recognizing infertility and adverse reproductive outcomes associated with air pollution (or other environmental factors) will be a significant challenge for clinicians and a potential barrier to further insight into these important issues.

Missing sentinel animal studies: sperm quality and fertility

Given the precedent provided by human studies (Table 1), and the relatively long history of the sentinel animal approach (Table 2), it is surprising that no sentinel animal

experiments have been conducted to explicitly examine air pollution affects on sperm quality. Appropriate study designs have been described in detail (Table 2; [Somers et al. 2002; 2004; Mohallem et al. 2005; Yauk et al. 2008]) and in principle require only an exposed and reference group comparison. Careful consideration should be given to the location and air pollution profiles of candidate exposure sites, and a comprehensive study should also conduct simultaneous laboratory exposures as a form of positive control. Many of the same sperm quality endpoints measured in humans can also be applied to rodents, including computer-assisted sperm analysis for performance (e.g., [Neill and Olds-Clarke 1989]) and the sperm chromatin structure assay for chromatin integrity (e.g., [Paul et al. 2008]). The ability to directly compare changes to identical endpoints in rodents and humans will substantially improve our ability to identify important common changes to sperm induced by air pollution. Finally, *in situ* animal experiments could be used in a more truly sentinel context (as in [O'Brien et al. 1993]) if human monitoring and animal exposures took place at the same general locations simultaneously. This approach has been used to examine sperm quality in wild rodents and humans for exposures not related to air pollution (e.g., [Multigner et al. 2008]), but has not been extended to the experimentally-exposed laboratory animals as described here.

Conclusions

Human and sentinel animal studies provide strong evidence for damage to male germ cells caused by ambient air pollution exposure. In particular, there is a strong parallel between the two types of studies suggesting that air pollution is genotoxic to sperm. Air pollution exposure conditions that cause genotoxic effects in the germ cells of humans and model species are unknown. To date, the general observation that particulate matter is important is the only insight we have into this issue. The clinical relevance of air pollution-induced effects in male germ cells is unclear and requires further investigation. Diagnosis of environmentally induced DNA damage or male infertility by clinicians is likely to remain a significant challenge.

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