Smoking and reproduction: gene damage to human gametes and embryos

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Assisted conception is a useful methodology for detecting disturbances in clinical outcome, meiotic maturation, and genetic integrity of human gametes. Germinal cells are vulnerable to genetic damage from smoking, but can repair damage during meiosis. In ejaculated spermatozoa, repair capacity declines drastically. Smoking alters the meiotic spindle of oocytes and spermatozoa, leading to chromosome errors which affect reproductive outcomes. Smoking is associated with reduced numbers of retrieved oocytes, leading to early age of menopause. Oocyte elimination occurs preferentially during meiosis I, a period sensitive to genetic damage. Smoking inhibits embryo fragmentation; inhibition may confer survival advantage to embryos genetically altered. Smoking is associated with low sperm quality, but clinical effects are not recognized. Cadmium (a heavy metal), nicotine (a toxic alkaloid), and its metabolite cotinine, are detectable in gonadal tissues and fluids in association with smoking. Cotinine incorporates into ovarian granulosa–lutein cells, compromising the developmental potential of follicles. Benzo[a]pyrene is a carcinogenic polycyclic aromatic hydrocarbon resulting from cigarette combustion. Its reactive metabolite binds covalently to DNA, forming adducts. Smoking-related adducts were detectable in ovarian granulosa–lutein cells, oocytes, spermatozoa and preimplantation embryos. Transmission of altered DNA from smoking by spermatozoa was demonstrated in preimplantation embryos and in association with increased risk of childhood cancer.

Key words: adducts/embryos/oocytes/smoking/spermatozoa

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Introduction

Although tobacco smoking is a widely recognized health hazard and a major cause of preventable mortality, consumption of tobacco remains prevalent in human societies. Approximately one-third of the world’s population aged ≥15 years smokes (World Health Organization, 1997). Smoking also affects reproductive health. Epidemiological studies from the general population of reproductive age have provided evidence of dose-related effects of smoking resulting in a conception delay of ~2 months (Zenzes, 1995; Hughes and Brennan, 1996; Bolumar et al., 1996) and an advance in the age of menopause by ~2 years (Midgette and Baron, 1990).

This suggests that certain components in cigarette smoke interact, directly or indirectly, with the gamete cells affecting their function and viability; the mechanisms involved need to be elucidated. Because the reproductive system is complex, many sites, from the hypothalamic–pituitary–axis to the germinal cells, can be vulnerable to disruption of reproduction. Therefore, clinical and experimental designs for in-vivo and in-vitro testing can be useful for detecting such disruptions. In this context, assisted conception provides an ideal setting for using fluids and cells of the reproductive system, for providing clinical data on reproductive performance, and makes it possible to analyse disturbances of meiotic maturation and genetic integrity in gamete cells. These aspects are reviewed here, focusing primarily on the mechanisms of damage to the gametes.

Vulnerability and repair capacity of germinal cells

Gametogenesis, the process of meiotic maturation of germinal cells, involves two cell divisions: meiosis I and meiosis II. Meiosis I is reductional and results in oocytes and spermatocytes with haploid chromosome complements (23 instead of 46 chromosomes). Meiosis II is equational; the two sister chromatids of each chromosome...
separate, leading to four haploid cells. The female meiotic process (oogenesis) involves nuclear and cytoplasmic maturation; the final product is one haploid oocyte arrested at metaphase II, which completes meiosis at fertilization, and three polar bodies (the first polar body divides shortly after extrusion), which are dysfunctional. The male meiotic process (spermatogenesis) leads to four haploid spermatocytes which, in turn, transform during spermiogenesis into four mature haploid spermatozoa.

Gametogenesis is very sensitive to the risk of damage from cigarette smoking (Mattison, 1983). Female and male germinal cells have different periods of sensitivity to external factors during meiosis I and II, and have different capacities to repair damage. This is explained below.

**Oogenesis**

During oogenesis there is a long period of meiotic arrest of primary (meiosis I) oocytes which starts before birth and lasts until sexual maturity shortly before each preovulatory surge. Oocytes are vulnerable when they divide during meiosis I and II and become metabolically active: firstly, during fetal life before the block at dictyotene stage (e.g. diplotene) in prophase I; secondly, in mature life during the preovulatory stage of the menstrual cycle when oocytes resume meiotic maturation into metaphase II (Mattison, 1993). In mice, diplotene oocytes and, to a lesser extent, oocytes in metaphase I and II, have an efficient DNA repair system (Ka et al., 1975; Masui and Pedersen, 1975; Brazill and Masui, 1978; Russell, 1980; Generoso, 1984; Guli and Smith, 1989) so that damage is repaired before fertilization. Also, fertilized eggs of mice and hamsters repair genetic damage by pre- and post-replication mechanisms (Brandriff and Pedersen, 1981; Matzuda and Tobarì, 1989; Genescà et al., 1992).

**Spermatogenesis**

In contrast, in spermatogenesis there is continuous production of functional male germinal cells from puberty until old age; once germinal cells enter meiosis (I and II) they become vulnerable to exposure to environmental toxins (Wyrobek, 1986). Spermiogenesis, the process of transforming spermatids into spermatozoa (Russell and Sylords, 1963), is a second period of sensitivity when de-novo point mutations arise (Generoso et al., 1979, 1984; Chandley, 1991).

Spermatogonia and spermatocytes have the ability to repair DNA (Dixon and Lee, 1980), as well as having efficient screening mechanisms to eliminate aberrant cells with reduced viability (Brusick, 1978). During the final stages of sperm cell differentiation, however, spermatids have little if any repair capacity; once the chromatin has condensed they do not repair DNA damage (Bentley and Working, 1988). Having no repair capacity, ejaculated spermatozoa are at risk for transmission of gene damage.

Indeed, it has been postulated that the risk of damage to germinal cells from exposure to external factors is greater in males than in females (Lessels, 1997). Mutation rates should be higher in males than in females (Haldane, 1947) because of a greater number of meiotic and mitotic divisions when mutations can accumulate. In mice it was found that the number of divisions from spermatogenesis to zygote formation (~205), is six times greater than the number from oogenesis to the zygote (~33; Chang et al., 1994).

**Smoking and clinical reproductive outcomes—females**

The impact of female smoking on clinical outcomes of IVF/embryo transfer has been a matter of interest in assisted conception (Trapp et al., 1986; Augood et al., 1998). Some studies, however, have poor adjustment of confounding variables (e.g. age), small sample sizes with insufficient statistical power, and lack information on smoking habits or concentrations of cotinine, a marker for smoking (Benowitz et al., 1983), precluding analysis of a dose-related association. After adjusting for some of these deficiencies, the overall results clearly implicate two mechanisms: (i) production of fewer oocytes, and (ii) increased rates of abortion. These are discussed below.

**Reduction in number of oocytes**

A relationship in number of retrieved oocytes in female patients undergoing assisted conception in relation to smoking was analysed (Harrison et al., 1990; Elenbogen et al., 1991; Pattinson et al., 1991; Hughes et al., 1992, 1994; Van Voorhis et al., 1992, 1996; Sharara et al., 1994; Zenzes et al., 1995a; and El-Nemr et al., 1998). Data from these studies are summarized in Table I. The ratio of mean number of oocytes of smokers divided by the mean for non-smokers, which should be exactly one (except for random sampling error) when there is no effect of smoking, is less than one in nine of the 10 studies. The weighted overall mean ratio for nine studies (omitting Pattinson et al., 1991; see Table I) is 0.920; this corresponds to a mean reduction of number of oocytes of ~8.0%. Using only the heavier smokers of the three studies in which they are identified (Harrison et al., 1990, 11–30 cigarettes/day; Hughes et al., 1992; Zenzes et al., 1995a), the weighted mean ratio is 0.828; this corresponds to a mean 17.2% reduction in number of oocytes in these heavy smokers.

This overall very significant reduction in number of oocytes in association with smoking may have consequences for reproductive outcomes in assisted conception, but this has not been consistently demonstrated. Clinical recognition is suggestive from epidemiological studies in natural conception which found a consistent dose-related smoking effect resulting in a conception delay of ~2 months (Weinberg et al., 1989; Zenzes, 1995; Curtis et al., 1997), with a common odds ratio for conception in smokers of 0.57 (95% confidence interval 0.42–0.78; Hughes and Brennan, 1996).

With advancing age the decline in number of retrieved oocytes was found to be faster in smokers than non-smokers (Harrison et al., 1990; Hughes et al., 1994; Sharara et al., 1994; Van Voorhis et al., 1996; Zenzes et al., 1997a; Joesbury et al., 1998), indicating that the synergistic effects of age and smoking may accelerate the rate of oocyte destruction. Sharara et al. (1994) reports on a clinically detectable decreased ovarian response with increasing age of 12% in smokers, compared with 5% in non-smokers.

These studies are supported by the epidemiological evidence for a dose-related decrease of age of menopause in smokers, (Jick et al., 1977; Mudgette and Baron, 1990), a clinical manifestation of follicle and oocyte depletion. Studies in animals exposed to various cigarette smoke components have also shown increased rates of follicular depletion (Mattison et al., 1983; Shiromizu and Mattison, 1984).
Selection for better-quality oocytes

A mechanism for the dose-related reduction of oocytes from smoking is that oocytes are preferentially eliminated shortly after resumption of meiotic maturation into metaphase II. This is suggested from quantitative studies on oocyte maturity (Zenzes et al., 1995a, 1997a). The first study analysed 253 unfertilized oocytes for chromosome status in relation to smoking habits.

Table I. Trends of decreasing numbers of oocytes with smoking

<table>
<thead>
<tr>
<th>Reference</th>
<th>Smoking status</th>
<th>No. of cigarettes/day</th>
<th>No. of women</th>
<th>Mean age (years)</th>
<th>Mean no. of oocytes</th>
<th>S/NS (oocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harrison et al. (1990)</td>
<td>S</td>
<td>–</td>
<td>108</td>
<td>&lt;40</td>
<td>5.0</td>
<td>0.980</td>
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<tr>
<td></td>
<td></td>
<td>1–10</td>
<td>72</td>
<td></td>
<td>5.4</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>11–20</td>
<td>31</td>
<td></td>
<td>4.4</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>21–30</td>
<td>5</td>
<td></td>
<td>3.8</td>
<td></td>
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<tr>
<td></td>
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<td>542</td>
<td></td>
<td>&lt;40</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>Elenbogen et al. (1991)</td>
<td>S</td>
<td>–</td>
<td>20</td>
<td>33.5</td>
<td>6.2</td>
<td>0.912</td>
</tr>
<tr>
<td>Pattinson et al. (1991)</td>
<td>S</td>
<td>–</td>
<td>21</td>
<td>32.6</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>?</td>
<td>33.1</td>
<td></td>
<td>5.1</td>
<td>0.944b</td>
<td></td>
</tr>
<tr>
<td>Van Voorhuis et al. (1992)</td>
<td>S</td>
<td>–</td>
<td>18</td>
<td>32.6</td>
<td>13.4*</td>
<td>0.817</td>
</tr>
<tr>
<td>NS</td>
<td>?</td>
<td>32.6</td>
<td></td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hughes et al. (1992)</td>
<td>S</td>
<td>–</td>
<td>59</td>
<td>32.8</td>
<td>5.54c</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1–14</td>
<td>36</td>
<td>31.8</td>
<td>5.95c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥15</td>
<td>23</td>
<td></td>
<td>34.4</td>
<td>4.90c</td>
<td></td>
</tr>
<tr>
<td>Hughes et al. (1994)</td>
<td>S</td>
<td>–</td>
<td>96</td>
<td>33.5</td>
<td>6.57c*</td>
<td>1.127</td>
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<tr>
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<td>119</td>
<td></td>
<td>34.3</td>
<td>5.83c</td>
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</tr>
<tr>
<td>Sharara et al. (1994)</td>
<td>S</td>
<td>–</td>
<td>29</td>
<td>37.8</td>
<td>8.4</td>
<td>0.977</td>
</tr>
<tr>
<td>NS</td>
<td></td>
<td>73</td>
<td></td>
<td>37.2</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Zenzes et al. (1995)</td>
<td>S</td>
<td>–</td>
<td>33</td>
<td>32.3</td>
<td>7.67</td>
<td>0.765</td>
</tr>
<tr>
<td></td>
<td>&lt;15</td>
<td>19</td>
<td></td>
<td>32.2</td>
<td>7.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥15</td>
<td>14</td>
<td></td>
<td>32.4</td>
<td>7.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>21</td>
<td></td>
<td>32.1</td>
<td>9.52d</td>
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<td></td>
<td>33.8</td>
<td>10.02</td>
<td></td>
</tr>
<tr>
<td>Van Voorhuis et al. (1996)</td>
<td>S</td>
<td>–</td>
<td>37</td>
<td>31.5</td>
<td>14.0d</td>
<td>0.909</td>
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<td>351</td>
<td></td>
<td>32.9</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>El-Nemr et al. (1998)</td>
<td>S</td>
<td>–</td>
<td>65</td>
<td>33.3</td>
<td>6.2</td>
<td>0.559</td>
</tr>
<tr>
<td>NS</td>
<td></td>
<td>108</td>
<td></td>
<td>33.2</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>

S = smokers; NS = non-smokers; PS = husband smokes but not wife; XS = ex-smokers.

aMean no. of oocytes from smokers divided by the mean for non-smokers. Calculated by present authors from means given by study authors.

Weighting the individual study ratios by the numbers of smoker women or cycles, the overall weighted mean ratio for nine studies (omitting that of Pattinson et al., 1991, which did not give number of women or cycles) is 0.920 ± 0.008, significantly different from one (no smoking effect) at P ≤ 0.001. Using only heavier smokers (Harrison et al., 1990, 11–30 cigarettes/day; Hughes et al., 1992, Zenzes et al., 1995), the weighted mean is 0.828 ± 0.006, significantly different from one at P ≤ 0.001.

bNot used in the calculation of the overall S/NS ratio.

cUsing no. of cycles.

Dose-dependent correlation with pack-years of cigarette smoking (packs/day × years of smoking).

*Significant difference between smokers and non-smokers (P < 0.05).

**Significantly different (P ≤ 0.0001).
Smokers had proportionally more (\(P = 0.03\)) analysable oocytes (i.e. metaphase II oocytes of good quality for cytotogenetic analysis) than non-smokers. This observation was extended and confirmed in a larger study using 2020 oocytes retrieved in relation to concentrations of cotinine in follicular fluids. Oocytes were assessed for maturity stage (e.g. immature, intermediate, mature or post-mature) according to conventional morphological parameters (Veeck, 1986). After correcting for age, a consistent trend \((P = 0.003)\) for an increasing proportion of mature oocytes with increasing cotinine concentrations was found concomitantly with a consistent trend for decreasing proportions of oocytes of intermediate maturity (Zenzes et al., 1997a). This suggests that oocytes are sensitive to damage shortly after they resume meiosis; this would lead proportionally to deficiency in intermediary oocytes and excess of mature oocytes. This is supported by a study in human oocytes (Racowsky and Kaufman, 1992), in which degenerative changes in chromatins were found predominantly at resumption of meiosis I (diakinesis), a stage vulnerable to external insults.

In addition to the morphological evaluations of maturity status in oocytes, Zenzes et al. (1997a) used the fertilization rate (number of embryos/number of oocytes retrieved) as an independent and objective measure of oocyte maturity, since mature oocytes have better chances of fertilization than immature oocytes (Van Blerkom et al., 1994). The results also showed a strong positive correlation between cotinine and the rate of IVF (Zenzes et al., 1997a). This higher rate of fertilization from smoking reflects an increased incidence of mature, fertilizable oocytes. Higher rates of fertilization in smokers, compared with non-smokers, were also reported in several studies, also using relatively large numbers of patients \((n = 54-650)\); Trapp et al., 1986; Harrison et al., 1990; Pattinson et al., 1991; Hughes et al., 1992; Van Voorhis et al., 1992; Sterzik et al., 1996; Zenzes and Reed, 1999), but not in studies with smaller sample size \((n = 41-71)\); Elenbogen et al., 1991; Rosevar et al., 1992; Rowlands et al., 1992). Possibly, the much smaller sample sizes in the latter studies do not provide sufficient statistical power.

**Selection for better-quality embryos**

Apoptosis (programmed cell death) is a physiological process of cell death that controls normality of cell populations during embryogenesis and tissue homeostasis. Interestingly, nicotine and cotinine were found to inhibit apoptosis in different cell lines (Wright et al., 1993; Marana et al., 1998), suggesting that this inhibition confers survival advantage to neoplastic cells and contributes to the pathogenesis of tobacco-related cancer. Although apoptosis has not been investigated in this context, two different studies investigated whether fragmentation in human preimplantation embryos is associated with smoking (Zenzes and Reed, 1996, 1999). Morphological assessments in 1094 embryos (Zenzes and Reed, 1996), and in 1682 embryos (Zenzes and Reed, 1999) at 2–8-cell stage, graded for quality from 1 to 4 (grades 1 and 2; with even-size blastomeres and no fragments; 0–25% fragments, and grades 3 and 4; 25–45% fragments; >40% fragments) were analysed in relation to follicular fluid cotinine. The proportion of embryos of grades 1 and 2 increased with increasing cotinine concentrations \((P = 0.0001)\), without age effect. These results suggest a cotinine-mediated inhibition of apoptosis in developing embryos of smokers. Acquisition of resistance to fragmentation from smoking may confer survival advantage to embryos with genetic alterations.

**Spontaneous abortion**

A relationship between smoking and increased risk of spontaneous abortion in assisted conception has been investigated by several authors (Harrison et al., 1990; Pattinson et al., 1991; Hughes et al., 1992, 1994; Maximovich and Beyler, 1995). Data from these studies are summarized in Table II. The table shows, for each study, the probabilities from Fisher’s exact \(2 \times 2\) test for the proportion of pregnancies terminating in abortion for all smokers and for non-smokers. Two studies, Harrison et al. (1990) \((P = 0.028)\) and Maximovich and Beyler (1995) \((P = 0.0006)\) have significant probabilities (smokers have greater risk of abortion), and Pattinson et al. (1991) has borderline significance \((P = 0.074)\). The two studies with clear non-significance (Hughes et al., 1992, 1994) are also those with the smallest number of abortions, both in smokers and non-smokers. Comparison of the Hughes studies with the others in Table II is difficult, since the former studies are based on cycles and the latter on pregnancies.

This evidence from a relatively small number of studies is supported by epidemiological evaluations from \(
\approx 100,000\) women using natural reproduction, in which a small, but significant, increase in abortion rate with a dose–response effect was found (for review see Hughes and Brennan, 1996).

**Smoking and clinical reproductive outcomes—males**

Research is sparse on effects of paternal smoking in assisted conception because reproductive problems (e.g. pregnancy loss) have been traditionally associated with women. The available data are not conclusive that male smoking affects IVF outcomes (Pattinson et al., 1991; Hughes et al., 1994; Hughes and Brennan, 1996; Joesbury et al., 1998). The latter authors, addressing paternal smoking effects in IVF outcomes, report reduced pregnancy rates associated with male smoking and with increasing age of male smoking partners; age is considered here a surrogate for duration of smoking exposure (Joesbury et al., 1998). A limitation of this study is that information on number of cigarettes smoked per day was incomplete, precluding analysis of other variables.

Studies on the effect of smoking on conventional seminal parameters (sperm density, motility, and morphology) give some indication whether smoking impairs sperm function sufficiently to be clinically apparent. Studies which adjusted smoke exposure to cigarettes/year (Chia et al., 1994; Vine et al., 1996), or cotinine concentrations in seminal plasma (Saaranen et al., 1989; Vine et al., 1996; Pacifici et al., 1993), demonstrated a dose-related association of smoking with low quality of spermatozoa. A study in young healthy teenagers, to avoid a confounding age effect, also found a trend towards poorer semen quality among smokers in association with urine cotinine (Rubes et al., 1998). A meta-analysis found that, in smokers, the sperm concentration is on average 13% lower than that of non-smokers (Vine et al., 1994, 1996).

The standard parameters of semen analysis, however, cannot predict the outcome of IVF (Toda et al., 1992). Low correlation coefficients between sperm parameters and fertilization rates in assisted conception (Sofikitis et al., 1993) may also be related to a female factor which cannot be excluded in the analysis. On these grounds, sperm function tests are more informative. Using the outcomes of the zona-free hamster oocyte penetration test,
Changes in temperature (Pickering et al. 1996), oocyte ageing (Eichenlaub-Ritter et al. 1992), and male smoking lead to disturbances of normal meiotic spindle function. It is known that alkaloids bind to tubulin, a protein in the microtubules of spindles (Singer and Himes, 1992; Jordan et al., 1998). By a similar mechanism of disruption of meiotic spindle function in spermatozoa, male smoking leads to disturbances of normal chromosome number. A recent cohort study done in 25 teenage males, to exclude a possible age effect, found increased rates of disomic spermatozoa, with disomy for chromosomes Y, X, X-Y, and X in association with urine cotinine concentration (Rubes et al., 1998). A study of 45 healthy male volunteers aged 19–45 years found a suggestive (P = 0.07) but not consistent association between smoking ≥20 cigarettes per day and XX,18 aneuploidy (Robbins et al., 1997).

### Effects of smoking on meiotic spindle function

Disturbances in microtubule polymerization and assembly affect the pulling of chromosomes from the equator to the poles, leading to errors in chromosome number (aneuploidies) in the resulting daughter cells. Because aneuploidy can be induced by chemicals (for reviews see Dellarce et al., 1985; Tarín, 1996), cytochemical analyses of the spindle and chromosomes provide useful models for studying the action of environmental chemicals.

The meiotic spindle in human oocytes can be disrupted by external factors such as advanced maternal age (Battaglia et al., 1996), oocyte ageing (Eichenlaub-Ritter et al., 1988), subtle changes in temperature (Pickering et al., 1990), and cigarette smoking (Zenzes et al., 1995a). The latter study found increased proportions of oocytes with diploid chromosome complements (46 instead of 23) in association with number of cigarettes smoked per day (P = 0.0006), and an increased proportion of triploid zygotes. Diploid (digynic) oocytes arise from lack of extrusion of first polar body (Tarín et al., 1991), indicating failure of meiotic spindle function. It is known that alkaloids bind to tubulin, a protein in the tubules of spindles (Singer and Himes, 1992; Jordan et al., 1998).

By a similar mechanism of disruption of meiotic spindle function in spermatozoa, male smoking leads to disturbances of normal chromosome number. A recent cohort study done in 25 teenage males, to exclude a possible age effect, found increased rates of disomic spermatozoa, with disomy for chromosomes Y, X, X-Y, and X in association with urine cotinine concentration (Rubes et al., 1998). A study of 45 healthy male volunteers aged 19–45 years found a suggestive (P = 0.07) but not consistent association between smoking ≥20 cigarettes per day and XX,18 aneuploidy (Robbins et al., 1997).

### Components of cigarette smoke in gonadal fluids and cells

Cigarette tobacco contains several thousand compounds (Dube and Green, 1982). The bulk of the tobacco consists of carbohydrates and proteins (~50%). Other significant constituents are alkaloids (0.5–5%) with nicotine the predominant compound (90–95% of total alkaloids), polyphenols (0.5–4.5%), phytoteroles (0.1–2.5%), carboxylic acids (0.1–0.7%), alkanes (0.1–0.4%), aromatic hydrocarbons, aldehydes, ketones, amines, nitriles, N- and O-heterocyclic compounds, pesticides, alkali nitrates (0.01–5%) and at least 30 metallic compounds (Wynder and Hoffmann, 1967; International Agency for Research on Cancer, 1986). More than 40 compounds are known to be chemical carcinogens, most of which are also considered mutagenic to humans (IARC, 1986). Three components of cigarette tobacco cadmium, cotinine, and benz[a]pyrene are discussed here in relation to their interactions with human gametic cells.

### Cadmium

This heavy metal is present in water, soil, air, foods, and in cigarettes; smoking constitutes a major source of inhaled cadmium (Norman, 1977; Saaranen et al., 1989; Staessen et al., 1990). Cadmium occurs in amounts of 1.0–2.0 µg per cigarette (Nandi et al., 1969). Up to 70% of this amount is passed through burning into smoke, and up to 10% of cadmium in a cigarette (0.1–0.2 µg) is...
inhaled (Schröeder and Balassa, 1961; Nandi et al., 1969; Szadkowski et al., 1969).

Cadmium accumulates over time in different organs, and in blood (Lewis et al., 1972; Watanabe et al., 1983), with a substantial increase in blood cadmium concentrations in heavy smokers of ≥20 cigarettes per day (Nandi et al., 1969; Lewis et al., 1972; Brockhaus et al., 1983; Watanabe et al., 1983; Ulander and Axelsson, 1984; Staessen et al., 1990; Chia et al., 1994). In ovarian and testicular tissue, epididymides, and seminal vesicles, removed at necropsy from individuals who died suddenly, a linear age-independent accumulation of cadmium from smoking was found (Oldereld et al., 1993; Varga et al., 1993). Cadmium was detected in follicular fluids of women undergoing assisted conception, with a significant dose effect in relation to smoking (Zenzes et al., 1995b). In seminal plasma the concentration of cadmium in heavy smokers is ~1.8 times greater than in non-smokers (Saarinen et al., 1989; Oldereld et al., 1993, 1994; Chia et al., 1994; Telisman et al., 1997).

Nicotine/cotinine

The principal alkaloid in tobacco, nicotine, is present in cigarettes in amounts varying from 0.8 to 1.8 mg per cigarette, according to the brand and size of cigarettes (Benowitz et al., 1983; Rosa et al., 1992). As much as 1 mg of nicotine can be absorbed by smoking a single cigarette (Barbieri et al., 1986). Nicotine is a very toxic alkaloid, and is quickly absorbed through the respiratory track, mouth mucosa and skin (Gandini et al., 1997). About 80–90% of nicotine is metabolized mainly by the liver, but also by the kidney and lung (Armitage et al., 1975).

Cotinine, its major metabolite, is a more stable compound than nicotine, with a longer half-life of ~20 h (nicotine is 2 h), and its concentration is not influenced by confounding factors, e.g. diet (Isaac and Rand, 1972; Matsukara et al., 1979; Rosenberg et al., 1980; Benowitz et al., 1983; Pojer et al., 1984; Darby et al., 1984; Pirkle et al., 1996; Davis et al., 1991; Rosa et al., 1992). Cotinine is therefore used as a marker for recent cigarette smoke exposure and dose (Benowitz et al., 1983).

Cotinine has been detected in follicular fluids of women undergoing assisted conception (Weiss and Eckert, 1989; Rosevear et al., 1992), and in dose-related association with cigarette consumption (Sterzik et al., 1996; Zenzes et al., 1996). On the male side, nicotine and/or cotinine were also detected in seminal plasma of male subjects in dose-related association with cigarette consumption (Pacifici et al., 1993, 1995; Vine et al., 1993; Zenzes et al., 1999b). This indicates passage of the metabolite through the blood–testis barrier.

Cellular components of ovarian follicles are readily permeable to endogenous and exogenous chemicals (Fabro, 1978). Indeed, cotinine was found incorporated into ovarian granulosa–lutein cells in dose–relationship with follicular fluid cotinine (Zenzes et al., 1997b). Using antiserum to cotinine and immunoperoxidase, a positive reaction was visualized in the nuclei and cytoplasm of granulosa–lutein cells. Binding of this alkaloid to nuclear and cytoplasmic proteins could affect the developmental potential of maturing follicles and could lead to perturbations in meiotic maturation of oocytes. When female hamsters were injected with nicotine, Racowsky et al. (1989) found in their oocytes a significant delay in meiotic maturation and meiotic arrest, but only at doses higher than those in heavy smokers of cigarette tobacco.

In-vitro studies of smoking on the physiology of human granulosa–lutein cells are controversial. In-vitro exposure to low molecular weight constituents of aqueous tobacco smoke extracts inhibited granulosa cell aromatase; nicotine inhibited steroid aromatization (Barbieri et al., 1986). In contrast, Weiss and Eckert (1989) found that neither nicotine nor cotinine altered progesterone or oestradiol secretion by granulosa cells in vitro in the presence of serum, while in serum-free medium Bódis et al. (1992) found dose-dependent increase in the secretion of oestradiol and decrease in the secretion of progesterone in serum-free medium. This study speculated that early abortion in pregnant women who smoke may be due to nicotine-related corpus luteum insufficiency; however, other factors may also be involved.

Benzo[a]pyrene

Benzo[a]pyrene belongs to a group of environmental pollutants, polycyclic aromatic hydrocarbons (PAHs), produced mainly by combustion of fossil fuels (IARC, 1986). PAHs have the ability to bind covalently to DNA, giving rise to adducts (Koreda et al., 1978). Benzo[a]pyrene occurs in cigarettes by combustion in amounts of 6–40 ng per cigarette; a pack–a–day smoker who consumed 20 cigarettes in 8 h could be expected to inhale from 0.067 to 0.568 µg of benzo[a]pyrene (Kaiserman and Rickert, 1992).

Benzo[a]pyrene is highly mutagenic and carcinogenic (IARC, 1986). The carcinogenic metabolite (8a-dihydroxy-9a,10a-epoxy-7,8,10-tetrahydro-benzo[a]pyrene) is a diol epoxide derivative of benzo[a]pyrene (BPDE-I) which binds predominantly to the 2-amino group of DNA guanosine, forming adducts (Jeffrey et al., 1977). These adducts are premutagenic lesions in guanosine nucleosides, which, if not repaired, constitute a potential source of carcinogenic damage (Denissenko et al., 1996).

Immunocytochemical techniques using monoclonal or polyclonal antibodies directed against PAH–DNA adducts are useful for quantitative analysis of adducts in different target cells of smoking individuals. In ovarian tissue, BPDE–DNA adducts were detected in oocytes, luteal cells, and stromal arteries of postmortem ovaries (Shamsudin and Ghan, 1988), but no quantitative data on adduct values were given, and no detailed information on the smokers status was provided. In granulosa–lutein cells, the mean BPDE–DNA adduct values were detected in dose-related association with follicular fluid cotinine (Zenzes et al., 1998). Granulosa–lutein cells treated in vitro with anti-BPDE monoclonal antibody, which recognizes and is structurally related to BPDE–DNA adducts and other PAH-derived DNA adducts (Santella et al., 1970, 1984), showed positive reactivity confined to the nuclei. A 3-fold and a 2-fold increase of adduct concentrations was found respectively, in active and passive smokers, relative to non-smokers (Zenzes et al., 1998). Mooney et al. (1995) also found that passive exposure to smoke at home was significantly associated with PAH–DNA adducts in leukocytes.

The presence of BPDE–DNA adducts in the ovarian cells of smokers (Shamsudin and Ghan, 1988; Zenzes et al., 1998) indicates a potential risk for DNA damage. In hamster cell lines benzo[a]pyrene induced cytogenetic damage including structural and numerical chromosomal aberrations and spindle disturbances (Sbrana et al., 1995; Matzuoka et al., 1997). Administration in high doses into mice resulted in destruction of primordial oocytes (Mattison and Thorgerison, 1979; Mattison et al., 1983; 1989),
depending on genetic differences in mice of benzo[a]pyrene metabolism (Felton et al., 1978).

The impact of smoking on ovarian function is not only through interactions between gonadal cells and constituents in cigarette smoke, but also from decreasing oestrogen secretion (Schulman et al., 1990). Women smokers excrete only about one-third as much oestrogen during the luteal phase of the ovulatory cycle than non-smokers or ex-smokers (MacMahon et al., 1982). Cigarette smoking appears to interfere with biosynthesis of oestrogens, suggested by lower serum oestrogen concentrations in oestrogen-treated women smokers after treatment, compared with their non-oestrogen treated smoker counterparts (Jensen et al., 1985). This oestrogen deficiency in smokers is linked to an earlier onset of menopause (Jick et al., 1977).

**Gene damage to spermatozoa from tobacco smoke**

Tobacco smoke can cause DNA damage or chromosomal damage in human germinal cells, and in oocytes and spermatozoa (Little and Vainio, 1994; Zenzes et al., 1995a, 1999b; Fraga et al., 1996; Robbins et al., 1997; Shen et al., 1997; Rubes et al., 1998). Experimental work has shown that cigarette smoke constituents and/or their DNA-reactive metabolic intermediates react directly with spermatozoa (Fraga et al., 1996; Shen et al., 1997; Zenzes et al., 1999b). A major form of DNA damage found in lung cells of smokers, 8-hydroxydeoxyguanosine (8-OHdG), is an oxidative lesion of guanine (Asami et al., 1997). Concentrations of 8-OHdG also occur in spermatozoa in association with smoking and seminal plasma cotinine (Fraga et al., 1996; Shen et al., 1997).

Using [32P]-postlabelling autoradiograms of DNA isolated from sperm cells of heavy smokers, light smokers and non-smokers, Gallagher et al. (1993) did not detect smoking-related adducts. Using immunoperoxidase on sperm cells pre-treated with a disulphide reducing agent for accessibility to DNA binding sites, BPDE–DNA adducts were detected in spermatozoa in dose-dependent association with seminal plasma cotinine (Zenzes et al., 1999b). Inter-individual variations in adduct concentrations were found both in smokers and non-smokers. This reflects individual variations in biological response to exposure via life style, occupation and ambient air pollution, and to variations in metabolism and detoxification of carcinogens (Niebert, 1991; Mooney et al., 1997).

**Oxidative damage from smoking**

Occurrence in cigarette smoke of toxic oxygen reactive species, i.e. superoxide anion (O2[−]), hydrogen peroxide (H2O2) and free radical (OH[·]) has been widely demonstrated (Stone and Bermudes, 1986; Church and Pryor, 1990). These cause pro-oxidant/antioxidant imbalance, leading to oxidative stress (Fraga et al., 1996; Hulea et al., 1995). Accumulated oxidative stress induces mitochondrial and nuclear DNA damage (Ames et al., 1994; Ballinger et al., 1996). Also, a rise in oxidative stress is associated with in-vitro ageing (Tarín, 1995), and is bound to produce cytoskeletal alterations in oocytes and embryos leading to chromosomal aneuploidy and cellular fragmentation. Oxidative stress can also be induced experimentally in mice oocytes using oxidizing agents; this leads to errors in chromosome segregation in meiosis I and II (Tarín et al., 1996).

**Gametic transmission of gene damage**

Because ejaculated spermatozoa have minimal, if any, repair capacity (Bentley and Working, 1988), formation of smoking-related adducts in spermatozoa are a potential source of transmissible prezygotic DNA damage. Parental transmission of BPDE–DNA adducts was recently investigated in early human preimplantation embryos (Zenzes et al., 1999a). In this study of twenty-seven preimplantation embryos at 4–8-cell stage treated with monoclonal antibody against benzo[a]pyrene, reactivity was assessed individually in 83 blastomeres. The proportion of reactive blastomeres was higher (P = 0.007) in embryos of smoker couples (either both members smoked or only the husband smoked), than in embryos of non-smokers. A 3.7-fold increase in mean adduct concentrations of embryos from smokers was found to indicate transmission of modified DNA from parental smoking. Figure 1 depicts two such treated embryos. The embryo from a smoking couple shows reactivity in the nuclei in several blastomeres, in contrast to the embryo from a non-smoking couple where reactivity is negligible.

In this study, similar mean adduct concentrations were found in embryos from couples where both parents smoked, or where only the father smoked. This suggests preferential gametic transmission...
of modified DNA from smoking fathers (Zenzes et al., 1999a). Ideally, embryos of couples where only the wife smoked should have been included to confirm preferential paternal transmission, but these were not available.

Paternal transmission of altered DNA may compromise embryonic development in utero, resulting in failed implantation, early pregnancy loss, or disturbances of postnatal development. In a case control population study of 642 individuals of a Chinese community where female smoking is discouraged (Ji et al., 1997), paternal smoking during the preconception period was associated with increased risk of childhood cancer within the child’s first 5 years. This evidence of paternal gametic transmission of genetic damage from smoking has implications for male reproduction.

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