Sensitivity of mouse oocytes to nicotine-induced perturbations during oocyte meiotic maturation and aneuploidy in vivo and in vitro

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Oocyte meiosis is sensitive to endogenous and exogenous perturbations that upset the temporal sequence of biochemical reactions during oocyte maturation (OM) and predispose oocytes to aneuploidy. Nicotine is an alkaloid that has been reported to disrupt the rate of OM, reduce ovulation and fertilization rates, and increase diploidy. The objective of this study was to test the hypothesis that nicotine perturbs the rate of OM and induces aneuploidy in mouse oocytes in vivo and in vitro. Female mice were given 7.5 IU pregnant mare’s serum and either 0, 5.0, 7.5, or 10 mg/kg nicotine in vivo at –3, 0, and +3 h relative to a 5 IU injection of HCG. Oocytes were also cultured in vitro in the presence of 0, 1.0, 5.0, or 10.0 mmol/l nicotine. In vivo, significant (P < 0.05) differences in the proportions of oocytes with premature centromere separation and premature anaphase were found at 10.0 mg/kg nicotine suggesting that the rate of OM was advanced. Also, at this dose the proportion of ovulated oocytes was reduced by ~50% relative to controls. In vitro, only non-significant differences were found among the parameters measured. Although nicotine reduced the ovulation rate and perturbed the rate of OM in vivo, these data show that the rate of aneuploidy was not significantly elevated.

Key words: aneuploidy/chromosome/nicotine/oocyte maturation.

Introduction

Of the different types of genetic abnormalities found in humans, aneuploidy is the most prevalent. Approximately 0.3% of human liveborns and 40–60% of early spontaneous abortions are aneuploid or polyplod (Bond and Chandley, 1983; Hansmann, 1983; Hook, 1985). Although a paucity of information exists about the numerous potential causes and mechanisms of abnormal chromosome segregation, it is known that a diverse group of chemicals to which humans are exposed can induce aneuploidy in the oocytes of experimental mammals (Mailhes, 1987; Mailhes and Marchetti, 1994). Based upon the known and suspected mechanisms whereby these compounds can interact with certain cellular organelles and biochemical processes, information about the mechanisms of aneuploidy is gradually being acquired.

Oocyte meiosis is sensitive to endogenous and exogenous perturbations that alter the rate of oocyte maturation (OM). Intra- and extrafollicular changes (pH, perifollicular microvasculature, oxidative stress, toxins) can disturb the orderly sequence of OM and predispose oocytes to faulty chromosome segregation (Hansmann and Pabst, 1992; Eichenlaub-Ritter et al., 1996; Mailhes et al., 1997). Most compounds that induce aneuploidy in mammalian oocytes alter microtubular assembly and function, and a large proportion are alkaloids such as colchicine, vinblastine (Mailhes and Marchetti, 1994), and cocaine (J.B.Mailhes et al., unpublished data). Both in-vivo and in-vitro models are used for evaluating the aneugenicity of chemicals. Generally, in-vitro studies yield data relevant to mechanisms; whereas, in-vivo studies may provide information about genetic risk. However, there is a paucity of intra-laboratory comparative in-vivo and in-vitro aneuploidy data, and we have recently found that the results between treatment modes may differ (London et al., 1999a,b). This is not necessarily unexpected due to differences in pharmacokinetics, catabolism, and the influence of follicular cells. The present study offers additional insight into the role of induced perturbations during OM and aneuploidy and also points out that the mode of treatment has a significant influence on OM.

Nicotine is an alkaloid that has been reported to interfere with OM and chromosome disjunction (Racowsky et al., 1989), retard embryonic development (Baldwin and Racowsky, 1988), and induce aneuploidy and polyploidy in bone marrow cells of mice (Bishun et al., 1972). These findings coupled with its chemical class suggest that nicotine may be aneugenic in oocytes. When hamster oocytes were exposed to 5 mmol/l nicotine in vitro, the proportions of oocytes blocked in metaphase I (MI) and of oocytes with non-disjoined bivalents at anaphase I were increased (Racowsky et al., 1989). These investigators also reported a dose-dependent decrease in the proportion of mouse embryos reaching the blastocyst stage following in-vitro exposure to 0.5–5.0 mmol/l nicotine (Baldwin and Racowsky, 1988). Other studies in rodents (Risenfeld and Oliva, 1987; Blackburn et al., 1994) and humans (Rosevear et al., 1992; Zenzes et al., 1995, 1997) have shown that nicotine decreased the number of oocytes retrieved.

Additional data from humans showed that the proportion of
Materials and methods

Animals
Female ICR (Institute of Cancer Research) (Harlan Sprague–Dawley; Indianapolis, IN, USA) mice 8–12 weeks of age (weight 25–34 g) were used in all experiments. They were housed under a 12 light:12 dark photoperiod, ambient temperature of 21–23°C, and relative humidity of 50 ± 5%. Food and water were provided ad libitum.

Hormones and chemicals
For both the in-vivo and in-vitro experiments, females received an i.p. injection of 7.5 IU pregnant mare’s serum (PMS) (Folligon; Intervet Ltd., Cambridge, UK) to increase the number of maturing follicles. After 48 h, animals in the in-vivo experiment received an i.p. injection of 5 IU HCG (Ayrest Inc, Philadelphia, PA, USA) to induce ovulation. The females in the in-vitro experiments did not receive HCG and their oocytes were obtained by follicular puncture 46 h post-PMS. Nicotine (lot no. 34H3310) was purchased from Sigma Chemical Co (no. N-5260).

In-vivo experiments
The mean LD50 for i.p. administration of nicotine to 8 week old (29.6 g) mice was reported as 12.5 mg/kg (Stalhandske et al., 1969). Preliminary experiments were conducted to determine a nicotine dose range and a treatment modality that were neither cell nor animal toxic. The acquisition of such information is a prerequisite for formulating an appropriate protocol for chemically-induced aneuploidy studies (Mackay and Elliott, 1992; Mailhes, 1995). When 10 females were given 12.5 mg/kg nicotine i.p. at –3, 0, and +3 h HCG, one mouse died within 4 h of the last injection, and two mice were non-ovulatory. The remaining seven females ovulated a total of 84 oocytes, which is considerably below the mean of 51 oocytes per mouse found in the controls (Table I). Thus, 12.5 mg/kg nicotine exceeded the acceptable limits of toxicity. To help alleviate the potential effect of a specific treatment time on the incidence of aneuploidy (Mailhes and Yuan, 1987; Hummiller and Hansmann, 1988; Mailhes and Marchetti, 1994), three doses of nicotine were administered over a period of 6 h. The working solutions of nicotine were prepared in sterile distilled water (Abbott Laboratories, Chicago IL, NDC 0074–4887–10) within 1 h prior to injections. Mice were given either distilled water (controls), 5, 7.5, or 10.0 mg/kg body weight of nicotine at –3, 0, and +3 h HCG. Thus, the total dosages were 15.0, 22.5, and 30.0 mg/kg. Ovulated oocytes were collected from oviducts 17 h after the last dose of nicotine and processed for cytogenetic analysis (Mailhes and Yuan, 1987a).

In-vitro experiments
A preliminary investigation involving eight experiments and 40 mice was conducted to determine a non-cell toxic nicotine dose range. When oocytes were cultured in medium containing 15.0 or 20.0 mmol/l nicotine, the oocytes did not respond to hypotonic treatment. This response is typical of degenerate cells and suggestive of cell toxicity (Mailhes, 1995). Thus, 10 mmol/l was chosen as the highest dose for the study.

At 46 h after PMS, cumulus–oocyte–complexes (COC) were collected from the antral follicles of five mice and placed into Waymouth medium (Gibco No. 11220–035) containing 100 μmol/l 3-isobutyl-1-methylxanthine/ml (3-IBMX; Sigma Chemical Co, no. I-5879) and maintained at 37°C until all COC were obtained (~2 h). Antral follicles contain fully grown, germinal vesicle-stage oocytes that are capable of undergoing spontaneous maturation in vitro (Eppig and Telfar, 1993). The COC were then washed three times in 3-IBMX-free Waymouth medium and between 150–250 oocytes were transferred to 2.5 ml of complete medium [Waymouth medium, 942.7 ml, Gibco No. 11220–035; fetal bovine serum, 50 ml, Gibco No. 16000–036; penicillin–streptomycin (10 000 IU/ml each of penicillin G sodium and streptomycin sulphate), 5 ml, Gibco No. I5140–122; and pyruvic acid, 2.3 ml, (Gibco No. 11360–070) FSH (1 μg/ml, NIDDK, lot No. AFP-7028D)] was added to the 2.5 ml of complete medium and the cells were incubated in a 5% CO2–5% O2–90% N2 gaseous atmosphere at 37°C for 16 h. A 20 mmol/l stock solution of nicotine was prepared in Waymouth culture medium and the pH adjusted to 7.5 within 1 h prior to placing oocytes into culture. The final concentration of nicotine in the culture medium was 0 (control), 1.0, 5.0, or 10.0 mmol/l. After 16 h of culture, oocytes were then processed for cytogenetic analysis in the same manner as those from the in-vivo experiments. These in-vivo

Table I. Nicotine-induced cytogenetic abnormalities in mouse oocytes in vivo. Values in parentheses are means

<table>
<thead>
<tr>
<th>Nicotine (mg/kg)</th>
<th>No. oocytes collected</th>
<th>No. oocytes analysed</th>
<th>No. MI</th>
<th>No. diploid</th>
<th>No. MII</th>
<th>PCS (%)</th>
<th>PA (%)</th>
<th>SC (%)</th>
<th>hypoploid (%)</th>
<th>haploid (%)</th>
<th>hyperploid (%)</th>
</tr>
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<tr>
<td>0.0</td>
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<td>302</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>1</td>
<td>6</td>
<td>296</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(51.0)</td>
<td>(46.6)</td>
<td></td>
<td></td>
<td>(4.6)</td>
<td>(0.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>964/20</td>
<td>404</td>
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<td>0</td>
<td>0</td>
<td>17</td>
<td>10**</td>
<td>3</td>
<td>19</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>(48.2)</td>
<td>(4.2)</td>
<td></td>
<td></td>
<td>(2.5)</td>
<td>(0.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>922/20</td>
<td>449</td>
<td>0</td>
<td>1</td>
<td>449</td>
<td>17</td>
<td>5</td>
<td>2</td>
<td>62</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(46.1)</td>
<td>(3.8)</td>
<td></td>
<td>(1.1)</td>
<td>(0.4)</td>
<td>(0.7)</td>
<td></td>
<td></td>
<td></td>
<td>(58)</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>741/29</td>
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<td>0</td>
<td>392</td>
<td>36*</td>
<td>21**</td>
<td>1</td>
<td>15</td>
<td>377</td>
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</tr>
<tr>
<td></td>
<td>(25.6)</td>
<td>(9.2)</td>
<td></td>
<td>(5.4)</td>
<td>(0.3)</td>
<td>(3.8)</td>
<td></td>
<td></td>
<td></td>
<td>(38)</td>
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</table>

PCS = premature chromatin separation; PA = premature anaphase; SC = single chromatid.

*P < 0.05 versus control.

**P < 0.01 versus control.
and in-vitro experimental protocols were replicated until an adequate number of oocytes were available for cytogenetic analysis.

**Cytogenetic analysis and statistical analysis of data**

Similar to cytogenetic techniques for other cell types, the number of oocytes placed onto slides was less than the number actually collected due to cell lysis during hypotonic treatment and fixation. Chromosomes were C-banded (Salamanca and Armendares, 1974) to enable an objective distinction between complete dyads and those separated into two chromatids or premature centromere separation (PCS). Due to the tortuous morphology of mouse metaphase II (MII) oocyte chromosomes, sister chromatids separated at the centromere could resemble two dyads if C-banding is not used (Figure 1A). This situation could then result in the erroneous recording of an extra chromosome. The frequencies of hypoploidy \( n = 10–19.5 \), haploidy \( n = 20 \), hyperploidy \( n = 20.5–29.5 \), and diploidy \( n = 30–40 \) were calculated relative to the total number of MII oocytes analysed. The range in chromosome numbers for each classification encompasses our historical values. Likewise, the proportions of oocytes with PCS, premature anaphase (PA) (Figure 1B), and single unpaired chromatids (SC) (Figure 1C) were calculated relative to the number of MII oocytes analysed. PCS refers to MII oocytes with one to all

![Figure 1](image-url)

**Figure 1.** Cytogenetic analysis of mouse oocytes following nicotine treatment. Cytogenetic analysis was performed as described above; chromosome configurations are shown: (A) metaphase II (MII) oocyte, premature centromere separation (PCS) \( n = 20 \); (B) MII oocyte, premature anaphase (PA) \( n = 20 \); (C) MII oocyte, single chromatid (SC) \( n = 19.5 \); (D) MI oocyte; (E) MII oocyte, diploid; and (F) MII oocyte, hyperploid, \( n = 21 \).
Effect of nicotine on oocyte maturation

Table II. Nicotine-induced cytogenetic abnormalities in mouse oocytes in vitro

<table>
<thead>
<tr>
<th>Nicotine (mg/kg)</th>
<th>No. oocytes collected</th>
<th>No. oocytes analysed</th>
<th>No. MI</th>
<th>No. diploid</th>
<th>No. MII</th>
<th>MII oocytes (%)</th>
<th>PCS</th>
<th>PA</th>
<th>SC</th>
<th>hypoploid</th>
<th>haploid</th>
<th>hyperploid</th>
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<td>6</td>
<td>187</td>
<td>37</td>
<td>(19.8)</td>
<td>0</td>
<td>7</td>
<td>17</td>
<td>169</td>
<td>1</td>
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<tr>
<td>(46.5)</td>
<td></td>
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<td>(14.6)</td>
<td>(2.7)</td>
<td></td>
<td>(3.7)</td>
<td>(9.1)</td>
<td>(90.4)</td>
<td>(0.5)</td>
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<tr>
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<td>189</td>
<td>28</td>
<td>3</td>
<td>158</td>
<td>34</td>
<td>(21.5)</td>
<td>0</td>
<td>4</td>
<td>12</td>
<td>145</td>
<td>1</td>
</tr>
<tr>
<td>(45.3)</td>
<td></td>
<td></td>
<td>(14.8)</td>
<td>(1.6)</td>
<td></td>
<td>(2.5)</td>
<td>(7.6)</td>
<td>(91.8)</td>
<td>(0.6)</td>
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</tr>
<tr>
<td>5.0</td>
<td>504/10</td>
<td>208</td>
<td>18</td>
<td>1</td>
<td>188</td>
<td>29</td>
<td>(15.4)</td>
<td>(0.5)</td>
<td>6</td>
<td>10</td>
<td>174</td>
<td>4</td>
</tr>
<tr>
<td>(50.4)</td>
<td></td>
<td></td>
<td>(8.7)</td>
<td>(0.5)</td>
<td></td>
<td>(3.2)</td>
<td>(5.3)</td>
<td>(92.6)</td>
<td>(2.1)</td>
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<tr>
<td>10.0</td>
<td>883/20</td>
<td>190</td>
<td>26</td>
<td>6</td>
<td>158</td>
<td>7</td>
<td>(4.4)</td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>142</td>
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</tr>
<tr>
<td>(44.2)</td>
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<td></td>
<td>(13.7)</td>
<td>(3.2)</td>
<td></td>
<td>(1.9)</td>
<td>(7.0)</td>
<td>(89.9)</td>
<td>(3.2)</td>
<td></td>
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</tbody>
</table>

PCS = premature chromatin separation; PA = premature anaphase; SC = single chromatid.

*P < 0.05 versus control.

**P < 0.01 versus control.

of their dyads separated at the centromere and an even number of chromatids; whereas, SC designates MII oocytes with an uneven number of chromatids and no PCS. Since MI (Figure 1D) and diploid (Figure 1E) oocytes are distinct categories and are disregarded when computing aneuploidy, their frequencies were calculated relative to the total number of oocytes analysed. Hyperploidy (Figure 1F) was used as the criterion for aneuploidy because an unknown proportion of hypoploidy can result from chromosome loss during slide preparation. The number and type of structural chromosome aberrations were also recorded when present. The criteria used for rejecting a cell from the analysis included: excessive chromosome clumping or overlapping, excessive chromosome spreading such that an artefactual loss of chromosomes could have resulted, and inadequate C-banding that precluded an objective count of centromeres. χ² and Fisher’s Exact Tests were used for statistical analyses of data. Fisher’s Exact Test was used when the events were rare (fewer than five cell frequencies).

Results

The in-vivo data show that the frequencies of PCS were significantly (P < 0.05) increased following 10.0 mg/kg nicotine and that the frequencies of PA were significantly (P < 0.01) elevated after 5.0 and 10.0 mg/kg nicotine (Table I). PCS and PA are indicators of spontaneous oocyte activation concomitant with a possible increase in the rate of OM. 10 mg/kg nicotine also resulted in a 50% reduction in the number of oocytes collected (ovulated). On the other hand, in vitro nicotine treatment did not perturb the rate of OM and failed to show any significant (P < 0.05) effects for the parameters measured (Table II). Although there was a trend toward higher frequencies of hyperploidy with increasing nicotine doses in vitro, these differences were not significant. These data suggest that nicotine enhanced the rate of OM in vivo and reduced the number of oocytes ovulated but did not affect the frequencies of aneuploidy.

Discussion

An obvious finding from this study was that the in-vivo and in-vitro results differed. This was not unexpected due to possible differences in pharmacokinetics, catabolism, and the influence of follicular cells (Mailhes, 1995; Eichenlaub-Ritter et al., 1996). In addition, there are numerous mechanisms whereby compounds may interact with both the biochemical pathways responsible for OM and the chromosome segregation organelles and it has not been established if these mechanisms are similar between treatment modes. Although there is a paucity of comparative in-vivo and in-vitro aneuploidy data, the present study found that nicotine enhanced the rate of OM and that the frequencies of PCS were significantly (P < 0.05) increased following 10.0 mg/kg nicotine. On the other hand, in vitro nicotine treatment did not perturb the rate of OM and failed to show any significant (P < 0.05) effects for the parameters measured (Table II). Although there was a trend toward higher frequencies of hyperploidy with increasing nicotine doses in vitro, these differences were not significant. These data suggest that nicotine enhanced the rate of OM in vivo and reduced the number of oocytes ovulated but did not affect the frequencies of aneuploidy.
PCS, and PA oocytes. Elevated frequencies of ovulated MI and diploid oocytes are considered indicators of a decrease in the rate of OM and are often found in populations of oocytes exposed to microtubule-damaging compounds (Mailhes and Marchetti, 1994). Conversely, higher levels of PCS and PA suggest spontaneous activation and an acceleration in OM (Mailhes et al., 1998).

Although nicotine did not significantly elevate the frequencies of MI and diploid mouse oocytes (Tables I and II), species may differ in their sensitivities to nicotine-induced OM delay. We found 8.7% MI oocytes after 16 h culture in 5 mmol/l nicotine; whereas at this same dose and culture time, 30% of Syrian hamster oocytes were blocked in MI (Racowsky et al., 1989). Also, higher levels of diploidy and similar frequencies of aneuploidy were found in oocytes from smokers than nonsmokers (Zenes et al., 1995). Differences in culture media, scoring criteria and sample size may also have contributed to these apparent differences.

Both PCS and PA have been proposed as indicators of spontaneous activation and a more rapid rate of OM (Mailhes et al., 1998). The frequencies of PCS and PA were significantly (P < 0.05) higher in the mice receiving 10.0 mg/kg nicotine in vivo, but were not elevated in vitro (Tables I and II). This finding appears consistent with the reported retardation in the rate of spontaneous mouse oocyte activation during in-vitro culture (Abbott et al., 1998) and the higher frequencies of oocytes in MII from women who smoke cigarettes (Zenes et al., 1997). Unpublished data from our laboratory also show that PCS levels are higher in mouse oocytes following in-vivo exposure to clomiphene citrate (London et al., 1999a) and tamoxifen (London et al., 1999b) than during in-vitro exposure.

PCS may predispose oocytes to aneuploidy if sister chromatids segregate randomly (Mailhes et al., 1998), and at least two mechanisms can lead to PCS in MII oocytes. One involves premature separation of homologues during meiosis I and the equational division of chromatids during anaphase I (Angell, 1991; Hunt et al., 1995; Soewarto et al., 1995). The second entails premature centromere separation of chromatids during meiosis II with the possibility of random segregation of sister chromatids during anaphase II (Rodman, 1971). Although important, the molecular mechanism(s) responsible for both PCS and PA have not been elucidated.

Nevertheless, at least two possibilities exist regarding nicotine-induced PCS. Both may elevate PCS levels by increasing the rate of OM which results in the unfertilized oocyte residing longer in MII. As oocytes age in MII, they exhibit increased sensitivities to both calcium-induced oocyte activation (Xu et al., 1997) and PCS (Mailhes et al., 1998). Nicotine may accelerate OM because it can elevate cystolic calcium concentrations (Qiu et al., 1998; Gueorguiev et al., 1999), which normally occur following fertilization or parthenogenesis (Lorca et al., 1994; Homa, 1995), and it enhances mitogen-activated protein kinase activity (Tang et al., 1998), which plays a major role during OM (Morin et al., 1994; Gotoh and Nishida, 1995; Moos et al., 1995).

Although the results of this study fail to support the hypothesis that temporal perturbations during OM predispose oocytes to aneuploidy, they do support the proposal that relatively high doses of nicotine in vivo increase the frequencies of PCS and PA and reduce the number of oocytes ovulated. The results also suggest that nicotine does not elevate aneuploidy levels in mouse oocytes.

References


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