Aneuploidy in mouse metaphase II oocytes exposed in vivo and in vitro in preantral follicle culture to nocodazole

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Aneuploidy tests are important in evaluating genetic hazards especially when chemical exposures are suspected to affect the fidelity of chromosome segregation in oocytes and embryos. In the current study, a newly established method, mouse preantral follicle culture, was employed to grow oocytes in vitro within follicles. The sensitivity of in vitro grown follicle enclosed oocytes was compared with oocytes maturing in vivo in the ovary. In both the cases, oocytes were exposed to the cytostatic chemical, nocodazole, from the time of hormonally stimulated resumption of meiosis. The in vivo study revealed a significant decrease in the number of ovulated mouse oocytes and an increase in meiosis I-arrested and hyperploid metaphase II oocytes at a single i.p. dose of 70 mg/kg body weight of nocodazole. A significant increase was also observed in the number of meiosis I-arrested and hyperploid mouse oocytes from preantral follicle culture, when they were cultured in the presence of ≥30 nM nocodazole during the final stages of maturation. This concentration is slightly lower than that previously shown to induce nondisjunction in denuded mouse oocytes or in cultured human lymphocytes. The higher sensitivity of the in vitro matured oocytes from preantral follicle culture than that of denuded oocytes may be related to a synergistic adverse influence of nocodazole on the oocyte, on somatic cell integrity and on cell-cell communication, which possibly also affects ovulation in vivo. When expressed in molarity relative to the mouse weight, the effective dose of the acute exposure in vivo is 3–4 orders of magnitude higher than the lowest effective concentration employed continuously in vitro. Reduced bioavailability of nocodazole to the target cells due to its poor water solubility may contribute to this difference. Preantral follicle culture can be helpful in analysing mechanisms in chemically induced aneuploidy in mammalian oogenesis, and in predicting the consequences of chemical exposures in vivo.

Introduction

Aneuploidy resulting from chromosome nondisjunction at female meiosis imposes a significant impact on human health (Hook, 1983; Parry et al., 1996; Aardema et al., 1998; Eichenlaub-Ritter, 1998, 2002, 2003). The incidence of aneuploidy has been estimated to reach at least 5% and possibly up to 20% in clinically recognized conceptions (Hassold and Hunt, 2001). Disturbances in chromosome segregation in oocytes and aneuploidy of the embryo are responsible for subfertility as they can cause implantation failure or congenital abnormalities leading to spontaneous abortion in humans. The adverse influence of chemicals on oogenesis and folliculogenesis is also likely to contribute to subfertility when they interfere with the differentiation and survival of the follicle or with ovulation. Thus, there is a need to study the consequences of chemical exposures during oogenesis in experimental animals as well as in suitable in vitro models to identify mechanisms of action, targets and thresholds for risk assessment.

Nocodazole, methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate, a synthetic anti-tumour drug interfering with microtubule assembly (De Brabander et al., 1976; Heath and Rethore, 1981), competes with colchicine for the same binding site on the Arg-390 of b-tubulin (Sackett and Varma, 1993) and directly affects microtubule polymerization and microtubule-dependent processes (Wilson et al., 1974; Heath and Heath, 1978; Heath and Rethore, 1981; Mejillano et al., 1996). Perturbation of the microtubule polymerization can activate the ‘spindle checkpoint’ (Nicklas, 1997; Gardner and Burke, 2000; Nasmyth, 2001; Musacchio and Hardwick, 2002; Shonn et al., 2003), leading to a persistent or transient cell cycle arrest. Anti-microtubule agents like nocodazole may also trigger apoptosis as a consequence of checkpoint activation (Decordier et al., 2002), which relates to the efficacy of these agents in cancer treatment (Sasaki et al., 2002; Masuda et al., 2003). However, nocodazole is also a potent mitotic aneugen which induces mitotic nondisjunction at low nanomolar concentrations, e.g. in cultured human lymphocytes (Elhaoui et al., 1995, 1997; Decordier et al., 2002; Kirsch-Volders et al., 2003).

We previously used denuded mouse oocytes maturing in in vitro experiments (further referred to as ‘denuded oocytes’) to analyse the activity of aneugens, including nocodazole, on spindle formation and chromosome segregation during mammalian oogenesis (Eichenlaub-Ritter and Boll, 1989; Shen et al., 2005). We showed that a 1 h exposure to nocodazole at micromolar concentrations during meiosis I resulted in the complete depolymerization of the oocyte spindle and meiotic block (Eichenlaub-Ritter and Boll, 1989). After the removal of nocodazole, recovery of oocytes and their progression to metaphase II hyperploidy was dramatically increased. Low concentrations of nocodazole, in the nanomolar range, do not completely depolymerize microtubules but may alter the morphology and stability of the spindle in mitotically dividing cells (Jordan et al., 1992; Vasquez et al., 1997). Similarly, when denuded mouse oocytes matured in the presence

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of 20–40 nM nocodazole, at low concentrations, which may also be relevant for the in vivo situation of potential exposures to the cytostatic chemical in humans, the pole-to-pole distance of the metaphase I and II spindle decreased significantly (Shen et al., 2005). However, only some of the denuded oocytes exposed to 30 and 40 nM nocodazole were meiotically blocked. Nondisjunction was significantly increased at a concentration of 40 nM nocodazole, suggesting the existence of a biological threshold for the aneugen (Shen et al., 2005). In the in vivo studies, a single dose of 35 mg/kg body weight (b.w.) of nocodazole administered to mice 1 h after mating resulted in a high incidence of chromosome malsegregation at meiosis II, detectable as chromosomal aberrations in one-cell embryos and as an increase in the embryonic death (Generoso et al., 1989). To date, there are no comparable experimental studies on the consequences of in vivo exposures to nocodazole for nondisjunction in oocytes before ovulation during meiosis I.

The relative sensitivity of different cell types to aneugens can be influenced by differential susceptibility to mitotic/meiotic slippage (Dai et al., 2004), by differential response to the induction of apoptosis (e.g. in mitotic and meiotic cells have to be considered in aneuploidy research (Hunt and Hassold, 2002). Differential susceptibility to aneugens in chronic versus acute exposures and in species-specific responses suggest a need to be considered in aneuploidy research (Eichenlaub-Ritter et al., 2002; Witt et al., 2003). However, we still do not know much about the differential and synergistic effects of chemicals on germ cells and their somatic compartments, respectively, which contribute to the differences in the biological threshold concentrations in vivo and in vitro and are therefore of importance in hazard and risk assessments.

To study these chemical effects on oogenesis, we employed a new in vitro model of oocyte maturation, preantral follicle culture, in which oocytes from prepubertal mice are cultured together with the supporting somatic cell compartment (Cortvrindt and Smitz, 2002; Sun et al., 2004). Initially, follicles with two layers of granulosa (follicle) cells (preantral) and some theca cells were mechanically isolated from the ovary of 12–14-day-old mice and cultured in the presence of recombinant gonadotrophins for 12 days (Cortvrindt and Smitz, 2002; Sun et al., 2004). Under the culture conditions, granulosa and theca cells proliferate, the follicle cells differentiate into mural and cumulus granulosa cell layers, and an antral cavity is formed in the majority of the follicles. Oocytes grown in vitro reach meiotic competence. After adding recombinant human chorionic gonadotrophin (hCG) and recombinant epidermal growth factor (rEGF) on day 12 of the culture, cumulus follicle cells mucify, in vitro, and the metaphase II oocyte is ovulated in vitro after a final period of culture for 16 h (Figure 1). Under these conditions, normal metaphase II spindles are formed in the oocytes (Hu et al., 2001), and the chromosomes segregate with high fidelity, a prerequisite for the in vitro model for aneugen tests (Sun et al., 2004). We used this in vitro culture system to expose mouse oocytes within follicles to low concentrations of nocodazole during the last stages of maturation in culture. Comparing the data with recent observations on nocodazole-exposed, in vitro maturing follicle-cell denuded oocytes, it is possible to determine whether nocodazole influences the functionality of the somatic compartment and indirectly affects the survival and maturation of the oocyte during the last stages of oogenesis in vitro. Apart from chromosomal constitution, we analysed mucification of cumulus/in vitro ovulation, meiotic progression, spindle formation and chromosomal behaviour. In parallel, we tested the aneugenic effect of nocodazole on the first meiotic division of mouse oocytes in vivo by injecting mice with the cytostatic chemical at the time of hormonally stimulated resumption of oocyte maturation in vivo (Mailhes, 1995). The study showed that nocodazole induced nondisjunction in vivo and in vitro, and suggests that the influence of nocodazole on the somatic compartment is likely to contribute to adverse effects during ovulation and the fidelity of meiotic chromosome segregation.

Materials and methods

Chemicals

Nocodazole (CAS no. 31430-18-9) was purchased from Sigma (Germany). For in vitro studies, the stock solution was prepared at a concentration of 10 μM in dimethyl sulfoxide (DMSO). The stock solution was diluted to suitable concentrations just before use. The final concentration of DMSO in the medium was 0.1%. For in vivo studies, nocodazole was dissolved in DMSO at a concentration adjusted to inject the mice with 5 ml/kg b.w., the highest advisable dose of DMSO for animal treatment.

In vitro experiments

Preantral follicle culture. Female mice (12–14 days old) were F1 hybrids (C57Bl/6J × CBA/Ca) from mice originally obtained from Harlan Winkelmann (Borchern, Germany). They were reared and maintained in the departmental animal facilities, housed in a temperature- (23–25°C) and light-controlled room (12L:12D; 7 a.m.–7 p.m.), and fed with food and water ad libitum.

In the present study, 2316 preantral follicles from a total of 40 prepubertal mice were obtained for culture. The isolation medium was composed of L15 Leibovitz-glutamax (GIBCO, Invitrogen, Germany), 10% foetal calf serum (GIBCO, Invitrogen, Germany) and antibiotics (Sigma, Germany). Mechanical dissection of the ovary and isolation of the early preantral follicles was done as previously described. The current study was different from the previous cytogenetic study (Sun et al., 2004) in that 96 well culture plates (Corning, USA), instead of dishes with individual microdroplets of culture medium covered with a thick oil layer, were used as the culture vessel, and the medium was refreshed on every fourth and not on every second day of culture (Cortvrindt and Smitz, 2002). Each follicle was contained within 50 μl of medium, covered by 30 μl of mineral oil. Microwell culture prevents the cross talk among the cultured follicles through the oil overlay by the lipophilic steroids, and reduces the amount of dilution of lipophilic components into the oil phase. Thus, some nocodazole may segregate by diffusion into the oil phase during the last 16 h of culture, but this should not cause an extensive dilution of nocodazole in the medium due to the restricted surface area and the short exposure period. The culture medium consisted of a minimal essential medium with glutamax (GIBCO, Invitrogen, Germany), and was supplemented with 10 μl/ml recombinant luteinizing hormone (rLH, Serono, Germany, kindly donated), 100 μl/ml recombinant follicular stimulating hormone (rFSH, Serono, Germany, kindly donated), foetal calf serum (GIBCO, Invitrogen, Germany) and insulin-transferrin-selenium mixture (Sigma, Germany). All follicles were placed in an incubator at 37°C with 5% CO2 in air and 100% humidity. Follicles with two layers of granulosa cells (100–130 μm diameter) were initially cultured for 12 days with refreshments of 20 μl of medium on days 4, 8 and 12. The spent medium was pooled for each plate and kept at −80°C for later measurement of steroid production. The pattern of follicle development and oocyte growth in the plate culture was similar to that reported for the microdroplet culture on dishes (Sun et al., 2004). Steroid production in pooled, spent medium before nocodazole exposure was in the normal range (data not shown). The survival of follicles on day 12 of the culture was over 90%. At the end of the 12 day culture period, plates were randomly divided into control, solvent control and nocodazole-treated groups. Nocodazole was added to the last refreshment of medium on day 12 (Figure 1). The follicle-like structures cultured in wells were simultaneously stimulated for in vitro ovulation by rEGF (Promega, Germany) and rHCG (Serono, Germany, kindly donated) at a final concentration of 1.5 IU/ml rHCG and 5 ng/ml rEGF in the medium. Nocodazole was added at the same time as the in vitro ovulatory stimulus, at the appropriate concentrations, to have 20, 30 or 40 nM nocodazole present during the 16 h of culture before oocyte harvest. In response to the in vitro ovulatory stimulus the cumulus granulosa cells surrounding the oocyte mucify, which can
be assessed using a dissection microscope prior to oocyte harvest. A schematic diagram of the experimental protocol of the in vitro and in vivo exposure is provided in Figure 1.

Analysis of meiotic progression. Although maturation status can usually be assessed by the presence of a germinal vesicle (GV), the resolution of the nucleus (germinal vesicle breakdown, GVBD) or the presence of a polar body (PB), sometimes PBs cannot unambiguously be identified in all oocytes. Therefore, denuded oocytes were transferred individually into a Petri dish for vital staining of chromosomes with Hoechst 33342 for 20-30 min (Sigma, Germany). Nuclear staining was assessed with a Zeiss (Axiovert 10) inverted microscope, equipped with a mercury lamp and a heated stage. On the basis of the fluorescence patterns of the chromatin, the oocytes were then assigned to GV oocytes, which were maturation incompetent or meiotically blocked and therefore still arrested in the dictyate stage at the end of the culture period. For oocytes resuming maturation, meiotic progression was determined by analysing the number of GVBD oocytes and PB oocytes. GVBD oocytes had resolved their nuclear membrane and characteristically possessed one or two sets of condensed chromosomes but had not emitted a PB. Oocytes with PB had two spatially separated sets of chromosomes in the ooplasm and in the PB compartiment, and visibly had undergone cytokinesis. Oocytes were pooled according to their meiotic stages, and oocytes resuming maturation (GVBD and PB oocytes) were spread separately for chromosome analysis.

Chromosomal analysis. The spreading procedure was performed according to a modified Tarkowski (1966) method (Sun et al., 2004). C-banding was performed as previously described (Eichenlaub-Ritter and Betzendahl, 1995).

Cytogenetic analysis was performed with a phase contrast microscope (Zeiss) at 1000 × final magnification. First, nuclear maturation was determined. GVBD oocytes either contained bivalents, and accordingly reached meiosis I during maturation, or contained two sets of dyads (metaphase II chromosomes). PB oocytes contained metaphase II chromosomes in the haploid range with or without separated chromatids (predivision). The PB oocytes, in which chromosomal number could unambiguously be determined, were analysed further for ploidy (e.g. euploid oocytes had 20 metaphase II chromosomes, Figure 2A). Oocytes with PB with >20 metaphase II chromosomes, or exceeding the

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**Fig. 1.** Experimental design of in vitro and in vivo tests. (A) Preantral follicles were isolated from 12-14-day-old prepubertal mice and cultured for 12 days in the presence of rLH and rFSH with the medium refreshed on days 4, 8 and 12. At day 12 rHCG and rEGF were added to the culture medium and oocytes matured in vitro within the follicles for a further 16 h (control). In the other experimental groups, solvent or nocodazole was added when the culture medium was refreshed for the last time. In vitro ovulation/mucification, spindle formation, chromosome congression and chromosomal constitution were then analysed. (A') Representative features of follicles and oocytes grown and matured in preantral follicle culture: (a) Preantral follicles with a small GV oocyte obtained from a 12-day-old prepubertal mouse with two to three layers of granulosal cells and some theca cells (arrows) attached to the surrounding basal membrane; (b) attachment of follicle, outgrowth of theca cells (arrow), and initiation of proliferation of thca and granulosa cells surrounding the growing GV-stage oocyte on day 4 of the culture; (c) increased numbers of somatic cells containing a growing oocyte on day 8 of the culture; (d) follicle with antral-like cavity (translucent area, arrow) separating differentiated outer mural granulosa cells (arrowhead) from inner cumulus granulosal cells surrounding the in vitro grown GV-stage oocyte on day 12 of the culture; (e) in vitro ovulation on day 13 with mature oocyte (arrow, out of focus) surrounded by mucified, highly expanded cumulus cells (larger magnification shown in e') with cumulus mass containing a mature PB-stage oocyte (inset) 16 h after maturation in vitro, following hormonal stimulation on day 12; (f) oocyte-cumulus complex that failed to mucify with cumulus firmly attached to the oocyte, which may contain a GV-stage oocyte, which were maturation incompetent or meiotically blocked and therefore still arrested at meiosis I on day 13 of the culture. (B) Folliculogenesis was primed by i.p. injection of PMSG in prepubertal 28-day-old mice, followed 48 h later by stimulation with HCG and in vivo maturation for 17 h after an i.p. injection of DMSO as solvent control or nocodazole. The number of ovulated oocytes per female and the chromosomal constitution of these in vivo matured oocytes were analysed.
Fig. 2. (A–F) Typical patterns of chromosome constitution of oocytes with or without nocodazole treatment. (A) Euploid oocyte with 20 metaphase II chromosomes in control. (B) Hyperploid oocyte with 25 metaphase II chromosomes and one chromatid (arrow) from the 30 nM nocodazole group. (C) Hyperploid oocyte with one extra chromatid (arrow) from the 30 nM nocodazole group. (D) Diploid oocyte with 40 metaphase II chromosomes, which matured in the presence of 40 nM nocodazole. (E) Hyperploid oocyte with 22 metaphase II chromosomes (upper part) and corresponding hypoploid PB with 18 metaphase II chromosomes (lower part) from the 30 nM nocodazole group. (F) Hypoploid oocyte with 19 metaphase II chromosomes (upper part) and hypoploid polar body with 21 metaphase II chromosomes (lower part) from the 20 nM nocodazole group. Scale bars in A–F; 5 μm. (G–I) Spindle morphology and chromosome behaviour in oocytes matured in the presence or absence of nocodazole. (G) Control oocyte with normal metaphase II spindle and aligned chromosomes (G'). (H) Oocyte with aberrant spindle with unaligned chromosomes (H') from the 30 nM nocodazole group. (I) Presumably polyploid GVBD oocyte with two spindles and two sets of chromosomes (I') from the 30 nM nocodazole group. Scale bars in G–H, 10 μm.
euploid chromosome constitution with their overall content of dyads plus bivalents or chromatids (Figure 2B and C), were recorded as hyperploid. The number of oocytes with metaphase II chromosomes in the diploid range was recorded over the total number of oocytes with or without PB (GVBD) with countable metaphase II chromosomes (from 17 to 40) to assess the incidence of polyploidy. Premature separation of chromatids (predivision) prior to anaphase II was also analysed (Figure 2B and C), irrespective of ploidy (including oocytes with euploid, hyperploid or hypoploidy constitution), in the oocytes with PB. Cases in which premature separation of chromatids was observed, but ploidy could not be unequivocally determined (one oocyte from the control, and the solvent control, and two oocytes from the 20 nM nocodazole group with two sets of chromatids), were also recorded.

**Immunofluorescence.** Oocytes were processed for spindle immunofluorescence as described previously (Eichenlaub-Ritter and Betzendaal, 1995; Hu et al., 2001; Sun et al., 2004). In short, the zona pellucida was removed by brief immersion in papain (Boehringer Mannheim, Germany) treatment. Oocytes were extracted in a microtubule-stabilizing solution at 37°C. After attachment to the slides, fixation in methanol at −20°C and rehydration in phosphate-buffered saline, the spindle was labelled by a mouse monoclonal anti-o-tubulin antibody (Sigma) and an anti-mouse FITC-conjugated secondary antibody (Sigma). Chromosomes were stained by 4,6-diamidino-2-phenylindole (DAP, Sigma) or propidium iodide (Sigma). Spindle images were recorded with a Zeiss Axiopt fluorescence microscope equipped with sensitive charge coupled device (CCD) camera (SensiCam, PCO CCD imaging, Kelheim, Germany).

**In vivo experiments**

These experiments were approved by the internal ethical committee of ENEA and officially authorized by the Italian Ministry of Health.

Three groups of 4-week-old female MF1 mice (Harlan, Italy) were used in this set of experiments. They were maintained in the institutional animal facilities, housed in a temperature-(21°C) and light-controlled room (12L:12D, 7 a.m.–7 p.m.), and fed with food (Teklad Global Diet 2018, Harlan, Italy) and water *ad libitum*.

These mice were superovulated by an i.p. injection of 7.5 IU of pregnant mare serum (PMSG, Crono-gest, Intervet, The Netherlands) to stimulate folliculogenesis. After 48 h, 5 IU of HCG (Chorulon, Intervet, The Netherlands) was injected to stimulate ovulation. At the time of HCG injection, one group was treated with 5 ml/kg b.w. of DMSO to serve as the solvent control. This was the highest advisable dose of DMSO for animal treatment. A second group was injected to stimulate ovulation. At the time of HCG injection, one group was treated with 5 ml/kg b.w. of nocodazole in DMSO and a third group 70 mg/kg b.w. of nocodazole in DMSO (Figure 1).

**In vivo ovulated oocytes** were collected 17 h after the treatment from the ampulla of the oviducts.

**Chromosomal analysis.** After the removal of cumulus cells with a brief digestion with hyaluronidase, GVBD and PB oocytes were spread on a slide according to the mass harvest method of Mailhes and Yuan (1987). Chromosomes were stained by C-banding (Salamanca and Armendares, 1974) and analysed under a phase contrast microscope (Zeiss, Germany) at 1000× final magnification according to the criteria similar to those adopted for in vitro experiments. The frequencies of oocytes containing a full set of bivalents (metaphase I-arrested oocytes), of oocytes containing a diploid or nearly diploid number of dyads (polyploid oocytes) and of oocytes displaying premature separation of chromatids or premature anaphase II were measured from all analysable oocytes, including GVBD and PB oocytes. The number of metaphase II oocytes with PBs, with >20 chromosomes (actual observed range: 21–25) was also recorded over the total number of metaphase II oocytes in which chromosomal number could unequivocally be determined to evaluate the induction of nondisjunction.

**Statistical analysis**

Data on cytoplasmic progression, nuclear maturation and chromosomal constitution after in vitro and in vivo experiments were compared between each treated group and the appropriate control or solvent control by the χ²-test with Yates correction. The χ²-test with Yates correction was also used to statistically evaluate the percentage of follicles which did not show mucification by cumulus cells. Trend analysis compared the increase in the percentage of follicles failing to mucify with increasing nocodazole doses. Student’s t-test was used to compare differences between the mean number of oocytes ovulated in the control and the nocodazole-treated mice.

**Results**

**Development of early preantral follicles in vitro**

In the current study, 96 well plates were used for preantral follicle culture. When the medium was refreshed for the last time, including the addition of a combination of rHCG and rEGF, nocodazole was also added to give a final concentration of 20, 30 or 40 nM in medium. DMSO was used as a solvent (0.1% in solvent control). There was no visible influence of nocodazole on follicle or oocyte survival at 16 h after exposure. We also analysed progesterone in pooled, spent culture medium before the addition of nocodazole and at the end of oocyte maturation, but we did not find any pronounced effect (data not shown). However, the percentage of follicles with mucification, expansion and detachment of cumulus around oocytes (Figure 1f), was reduced in the treated groups. There was a slight increase in the percentage of follicles without mucification (Table I) in the 30 nM nocodazole group. The numbers of follicles failing to mucify increased further with maturation in the presence of 40 nM nocodazole. Although differences between the treated groups and the control did not reach statistical significance, a statistically significant (P < 0.05) trend for an increase in the percentage of follicles without mucification with increasing nocodazole concentration was detected.

**Cytoplasmic maturation of nocodazole-exposed oocytes from follicle culture**

Less than 10% of the oocytes in any group failed to resume maturation (Table II, left panel), and most of the oocytes (>90%), including both controls and treated groups, were competent to resume meiotic maturation to either resolve the nucleus (GVBD) or also emit a PB (Figure 1). Vital staining with Hoechst 33342 revealed that solvent alone had no effect on meiotic progression of oocytes compared with the control group. However, the proportion of oocytes with PB decreased from >70% in the control and solvent control to 67.8, 61.7 and 40.9% in the 20, 30 and 40 nM nocodazole groups, respectively. Concomitantly, the percentage of oocytes at GVBD stage increased. Decrease in PB oocytes was significant at 30 and 40 nM nocodazole (Table II; P < 0.005 and 0.001, respectively).

**Nuclear maturation of oocytes from follicle culture treated with nocodazole**

Cytogenetic analysis of oocytes with GVBD and PB showed that nuclear maturation of oocytes in the solvent control was comparable to that in the untreated control group. In contrast, in the treated groups, the percentage of oocytes arrested at meiosis I stage with bivalent chromosomes increased, from between 16 and 20% in the controls to >40% at the highest concentration of nocodazole. Correspondingly, the overall percentage of oocytes with metaphase II chromosomes decreased.

**Table I.** Mucification of cumulus cell-enclosed oocyte complexes 16 h after hormonal stimulation and the treatment of antral follicles with 20, 30 or 40 nM nocodazole (noc).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of antral follicles</th>
<th>Failure in mucification in vitro ovulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>185</td>
<td>12 (6.5)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>106</td>
<td>6 (5.7)</td>
</tr>
<tr>
<td>20 nM noc</td>
<td>125</td>
<td>10 (8.0)</td>
</tr>
<tr>
<td>30 nM noc</td>
<td>131</td>
<td>12 (9.2)</td>
</tr>
<tr>
<td>40 nM noc</td>
<td>117</td>
<td>16 (13.7)</td>
</tr>
</tbody>
</table>
Table II. Meiotic and nuclear maturation, and chromosomal aberrations of in vitro grown mouse oocytes from preantral follicle culture matured within follicles in the presence of 20–40 nM nocodazole (noc) for the last 16 h following the in vitro ovulatory stimulus.

<table>
<thead>
<tr>
<th>Total number of oocytes</th>
<th>Maturation arrest</th>
<th>Meiotic development</th>
<th>Nuclear maturation</th>
<th>Chromosomal aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arrested with GV (%)</td>
<td>With GVBD (%)</td>
<td>With PB (%)</td>
<td>No. of GV BD and PB oocytes</td>
</tr>
<tr>
<td>Control</td>
<td>187</td>
<td>14 (7.5)</td>
<td>173</td>
<td>40 (23.1)</td>
</tr>
<tr>
<td>20 nM noc</td>
<td>220</td>
<td>10 (4.5)</td>
<td>210</td>
<td>49 (23.3)</td>
</tr>
<tr>
<td>30 nM noc</td>
<td>314</td>
<td>25 (8.0)</td>
<td>289</td>
<td>93 (32.2)</td>
</tr>
<tr>
<td>40 nM noc</td>
<td>244</td>
<td>22 (9.0)</td>
<td>222</td>
<td>85 (38.3)</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>7 (6.0)</td>
<td>110</td>
<td>65 (59.1)</td>
</tr>
</tbody>
</table>

GV, germinal vesicle; GVBD, germinal vesicle breakdown; PB, polar body. MII with chrom., metaphase II oocytes with chromatids/precocious anaphase II.

\( \chi^2 \): significant difference to control: \( P < 0.005; \) \( ^*P < 0.001 \).

\( ^a \)Including PB oocytes with two sets of chromatids in haploid range.

Unlike in denuded untreated or solvent-exposed oocytes analysed in previous studies, which tend to have only a few oocytes progressing through first anaphase in the absence of cytokinesis (e.g. 0.4% in control and 0.9% in DMSO/solvent control; Shen et al., 2005), a consistently higher percentage of polyploid, ‘diploid’ meiosis II oocytes with two sets of metaphase II chromosomes was previously observed in oocytes from preantral follicle culture (e.g. 5.7% and 6.8% of diploids in the untreated control in two sets of experiments; Sun et al., 2004). Currently, the number of diploids was also higher compared with the standard values in in vitro matured denuded oocytes, and varied between replicate experiments, with inconsistent and insignificant differences between the control and the solvent control. However, the percentage of such polyploid oocytes increased in this study in a dose-related fashion from a total of 7.2 and 1.9% in the control and control and the solvent control, respectively, to 29% in the group treated with the highest concentration of nocodazole (\( P < 0.005 \)) (Figure 2D).

Chromosome nondisjunction and prediision in nocodazole-exposed oocytes from follicle culture

No hyperploids were found in the control and the solvent control. The percentage of hyperploid oocytes (Figure 2E) increased to 3.9, 12.2 and 22.7% in the mouse oocytes retrieved from follicles cultured in the presence of 20, 30 and 40 nM nocodazole, respectively (Table II, right panel). The increase compared with controls reached significance in the 30 and 40 nM nocodazole groups (\( P < 0.005 \) and 0.001, respectively). Precocious separation of chromatids was comparatively rare among oocytes, which had resumed maturation (Table II). From the oocytes with prediision, one oocyte with complete chromatid separation and two sets of chromatids were found in each of the control and solvent groups. Two oocytes in the 20 nM nocodazole group contained a single chromatid and two had all chromatids separated into two sets. In the 30 nM nocodazole group, two oocytes had premature centromere separation of all chromatids and one oocyte possessed a single chromatid (Figure 2C). In the 40 nM nocodazole group, one oocyte exhibited premature centromere separation of all chromatids and one contained a single chromatid.

No hyperploids were found in the control and the solvent control. The percentage of hyperploid oocytes (Figure 2E) increased to 3.9, 12.2 and 22.7% in the mouse oocytes retrieved from follicles cultured in the presence of 20, 30 and 40 nM nocodazole, respectively (Table II, right panel). The increase compared with controls reached significance in the 30 and 40 nM nocodazole groups (\( P < 0.005 \) and 0.001, respectively). Precocious separation of chromatids was comparatively rare among oocytes, which had resumed maturation (Table II). From the oocytes with prediision, one oocyte with complete chromatid separation and two sets of chromatids were found in each of the control and solvent groups. Two oocytes in the 20 nM nocodazole group contained a single chromatid and two had all chromatids separated into two sets. In the 30 nM nocodazole group, two oocytes had premature centromere separation of all chromatids and one oocyte possessed a single chromatid (Figure 2C). In the 40 nM nocodazole group, one oocyte exhibited premature centromere separation of all chromatids and one contained a single chromatid.

Table III. Spindle morphology and chromosome behaviour in metaphase II oocytes from control, solvent control and nocodazole (noc)-exposed follicles.

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<thead>
<tr>
<th>Number of oocytes/Total aberrant meiosis II oocytes (%)/Oocytes with aberrant spindle (%)/Oocytes with displaced or dispersed chromosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Solvent control</td>
</tr>
<tr>
<td>20 nM noc</td>
</tr>
<tr>
<td>30 nM noc</td>
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</tbody>
</table>

\( \chi^2 \)-test: significant difference to control: \( P < 0.001 \).

Spindle morphology and chromosome congrression in nocodazole-exposed metaphase II oocytes from follicle culture

The spindle apparatus plays a key role in the fidelity of chromosome segregation in both mitosis and meiosis. It is considered as a major target for disturbances by aneugens. In the current study, the influence of nocodazole on the morphology of metaphase II spindles and on chromosome alignment was therefore analysed in controls and the 20 and 30 nM nocodazole groups (Table III). In the control, most oocytes possessed normal spindles (Figure 2G and G’). The solvent alone did not increase the percentage of metaphase II oocytes with aberrant spindles. However, nocodazole induced an increase in the number of aberrant oocytes at 20 and 30 nM concentration (Table III), and the percentage of oocytes with aberrant spindles (Figure 2H) and/or unaligned, dispersed or displaced chromosomes (Figure 2H’) was also significantly increased in both the dose groups. Nearly 50% of all meiosis II oocytes with PB in the 30 nM nocodazole group were abnormal indicating a high susceptibility to second meiotic errors (Table III). In addition, some highly aberrant GVBD oocytes with two spindles and two sets of chromosomes were also observed (Figure 2I and I’) in the treated groups.

Ovulation, maturation and nondisjunction of oocytes exposed to nocodazole in vivo

Nocodazole interfered with the process of ovulation significantly reducing the mean number of oocytes ovulated and
Table IV. Results of the experiments testing nocodazole (noc) effects on mouse oocytes after in vivo exposure

<table>
<thead>
<tr>
<th>Mean number of oocytes/ female</th>
<th>Nuclear maturation</th>
<th>Chromosomal aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GVBD and PB oocytes</td>
<td>MII oocytes with bivalents (%)</td>
</tr>
<tr>
<td>5 ml/kg DMSO</td>
<td>33.6 ± 1.6</td>
<td>441(0)</td>
</tr>
<tr>
<td>35 mg/kg noc</td>
<td>33.8 ± 1.5</td>
<td>329 1.0 (0.3)</td>
</tr>
<tr>
<td>70 mg/kg noc</td>
<td>27.7 ± 3.0*</td>
<td>264 18.6 (8.4)</td>
</tr>
</tbody>
</table>

MII, meiosis II; GVBD, germinal vesicle breakdown; PB, polar body; Predivision, meiosis II oocytes with premature segregation of individual chromatids (single chromatids) or premature anaphase II.

*Significantly lower than the corresponding solvent control value: Student’s t-test P < 0.05.

**Significantly higher than the corresponding solvent control value: χ²-test P < 0.001.

*Excluding oocytes in which chromatids could not be individually recognized on all chromosomes.

harvested per female at the highest dose tested (Table IV). Nocodazole (35 mg/kg b.w.) had no apparent effect on ovulation, nuclear maturation and aneuploidy induction. A dose of 70 mg/kg b.w. of nocodazole induced a significant increase in the percentage of meiosis I-arrested oocytes compared with the solvent control (P < 0.001). Notably, at the same dose, nocodazole significantly increased the frequency of hyperploid oocytes compared with the solvent control (P < 0.001) from 0 to 7.3%. The frequency of oocytes with premature centromere separation of all (premetaphase II) or nearly all dyads (predivision) decreased dose-dependently in nocodazole-treated mice, although differences between the treated groups and the control did not reach statistical significance. If the decrease in oocytes with chromatids and premature anaphase II was actually nocodazole-related, it could be a secondary consequence of a transient arrest at the metaphase I stage, thus shortening the time spent by the oocytes at metaphase II, when, in the absence of fertilization, degeneration may start with relaxation of chromatid cohesion at the centromeres.

Discussion

Influence of nocodazole on meiotic progression and oocyte nuclear maturation

Chemicals with aneugenic potential can interfere with oocyte maturation, both in vivo and in vitro, and can result in a meiotic delay or an arrest. Nocodazole was shown to induce a cell cycle arrest at high concentrations (Eichenlaub-Ritter and Boll, 1989; Everett and Searle, 1995; Brunet et al., 2003; Wassmann et al., 2003). Mitotic slippage in response to nocodazole exposure can cause polyploidy and susceptibility to apoptosis or secondary nondisjunction (Verdooldt et al., 1999). In the current study, a low dose of nocodazole, resembling pharmacological exposure levels, was used to analyse the aneugenic activity on oocytes from preantral follicle culture. Indeed, nocodazole influenced both oocyte cytoplasmic and nuclear maturation. There was a dose-related arrest at meiosis I, demonstrated by increased numbers of oocytes in GVBD and in oocytes containing bivalents. This was similar to the results of the in vivo assay which also had significant increases in the number of oocytes ovulated in meiosis I. Nocodazole also interfered with cytoplasmic maturation and caused ‘meiotic slippage’ in preantral follicle culture, as can be deduced from the increased polyploidy suggesting a synchrony in the formation of a first PB and anaphase I progression. There was a significant increase in the number of polyploid oocytes with two sets of metaphase II chromosomes in preantral follicle culture but not in the in vivo study with ovulated oocytes. Since the number of ovulated oocytes decreased in vivo, it is conceivable that oocytes with this type of disturbance were possibly retained within the ovary. Likewise, oocytes from follicle culture could be particularly susceptible to the uncoupling of cytokinesis from anaphase I, or the concentration of nocodazole at the target site achieved in vivo by a single i.p. injection was below the threshold for induction of this effect.

Susceptibility of spindle formation to nocodazole-induced disturbances

Microtubule depolymerizing agents can disrupt microtubule-dependent processes by altering microtubule dynamics at low concentrations (Jordan and Wilson, 1990; Jordan et al., 1992; Toso et al., 1993; Dhamodharan et al., 1995; Tanaka et al., 1995). This can suppress the dynamic instability by decreasing microtubule elongation and shortening velocities both in vivo and in vitro (Vasquez et al., 1997). Suppression of dynamic instability of kinetochore microtubules (Mitchison et al., 1986; Hamaguchi et al., 1987; Mitchison, 1989), especially of microtubules at centromeres (Andrews et al., 2004; Bringmann et al., 2004; Lan et al., 2004), or steric hindrance of kinetochore/microtubule attachment might lead to a disruption of the balance of forces in the spindle and result in the shortening of the spindle (Nicklas, 1988). Wendell et al. (1993) showed that kinetochore microtubule number was reduced by about 25% in cells arrested in mitosis by 2 nM vinblastine. Using polarizing microscopy, we found that 20 nM nocodazole caused a reduction in the overall spindle length, an indicator for reduction in the microtubular lattice of the spindle of denuded, nocodazole-exposed mouse oocytes (Shen et al., 2005). About 33 nM nocodazole was sufficient to induce monopolar spindles in somatic cells (Jordan et al., 1992). In accordance, the current study shows that a high number of oocytes treated with 20 nM nocodazole in preantral follicle culture had aberrant metaphase II spindles, although we did not find typical monopolar configurations. Since oocytes possess multiple microtubule organizing centres instead of two centriolar centrosomes (for review see Eichenlaub-Ritter et al., 2004), formation of a monopolar...
spindle may be a rather rare event in oogenesis. There was no increase in first meiotic nondisjunction at this low nocodazole concentration. However, the severe spindle aberrations at meiosis II and the failure to assemble chromosomes at the spindle equator at metaphase II in the group exposed to only 20 nM nocodazole can contribute to chemically induced second meiotic error, after fertilization of the oocyte. Indeed, numerical chromosome aberrations including hypodiploidy, hyperdiploidy, haploidy and triploidy have been reported in one-cell embryos when metaphase II oocytes of hormonally unstimulated mice were exposed to 35 mg/kg b.w. of nocodazole (Generoso et al., 1989). The same paper did not report cytogenetic observations after in vivo exposure of meiosis I oocytes, but a uterine content analysis did not suggest that nocodazole caused the loss of conceptuses after treatment at this stage. This observation agrees with our finding that 35 mg/kg b.w. of nocodazole also did not induce aneuploidy in metaphase II oocytes collected after gonadotrophin-induced ovulation. Currently, we cannot determine whether hormonal stimulation influenced the response in the current in vivo study, but altogether these observations suggest that metaphase II spindles might be especially sensitive to nocodazole-induced alterations. In addition, accumulation of damage during a chronic exposure spanning the whole period of meiotic resumption, as in the follicle culture, could contribute to second meiotic disturbances, while a transiently high concentration in earlier prometaphase I induced by the acute exposure at meiotic resumption by the single i.p. in vivo administration may have a less severe, reversible effect.

**Chromosomal aberrations in metaphase II oocytes**

Nocodazole increased the number of oocytes with hyperploidy dose-dependently in metaphase II oocytes after a single dose in vivo as well as after low dose-exposure in follicle culture during the last 16 h of oocyte maturation. An aneugenic activity of nocodazole has been observed in many cell types, including yeast Saccharomyces cerevisiae (Wood, 1982; Zimmermann et al., 1984; Mayer and Goin, 1987), human lymphocytes cultured in vitro (Correll and Ford, 1987; Sandhu et al., 1988; Elhajoui et al., 1995, 1997; Decordier et al., 2002), meiotically dividing crane-fly spermatocytes (Ladrach and LaFountain, 1986), PIK1 cells (Cimini et al., 2001) and human lung diploid fibroblasts (MRC-5 cells) (Cimini et al., 2002). Nocodazole induced a dramatic increase of aneuploidy in denuded, young and old metaphase II oocytes of CBA/Ca mice exposed transiently for only 1 or 2 h to micromolar concentrations (Eichenlaub-Ritter and Boll, 1989) and in mouse oocytes of (C3H/HeH × 101/H)F1 hybrids or homozygous Rb(16.17)Bnr mice (Evert and Searle, 1995). Moreover, it has been demonstrated that nocodazole led to remarkably high frequencies of embryonic lethality as well as varied numerical chromosome abnormalities with changes in ploidy when 35 mg/kg b.w. of nocodazole was administered once at the time of sperm entry (Generoso et al., 1989). A 16 h continuous exposure to relatively low concentrations of 40 nM nocodazole induced a significant rise in the hyperploidy frequency in follicle-cell denuded metaphase II oocytes of the MF1 mouse exposed throughout in vitro maturation (Shen et al., 2005). The present study not only fully confirmed the aneugenic potential of nocodazole at meiosis I but also provided evidence that continuous exposures of the oocyte within a follicle throughout nuclear maturation may be especially critical for a loss of fidelity of chromosome separation at meiosis II. In addition, increases in polyploidy were pronounced in follicle-enclosed oocytes (here 29% polyploids with 40 nM nocodazole) but not so much in isolated, denuded mouse oocytes (4.4% in the 40 nM nocodazole group; Shen et al., 2005).

**Cell–cell signalling and chemically induced aneuploidy in follicle-enclosed oocytes**

In the present study, aneuploidy rose significantly at 30 nM nocodazole in the follicle culture, while a significant increase in aneuploidy of nocodazole-exposed denuded oocytes occurred only at 40 nM nocodazole (Shen et al., 2005). This indicates that exposure of the somatic compartment with the oocyte may impair the health and function of the follicle directly and indirectly (e.g. by also disturbing granulosa and theca cell function). It may thus synergistically affect the quality of the enclosed oocyte. There are distinct differences in the regulation of meiotic resumption in the denuded oocyte and follicle-enclosed oocyte maturation. In the case of denuded oocytes, maturation is spontaneous because inhibiting signals from follicle cells and components of follicular fluid are removed by the isolation (Edwards, 1965). In contrast, follicle-enclosed oocyte maturation in vivo is accompanied by the generation of a positive stimulus of the membrana granulosa and the cumulus cell origin in the presence of functional heterologous gap junctional communication with the oocyte (e.g. Downs, 1995; Byscov et al., 1997; Albertini et al., 2001; Matzuk et al., 2002; Su et al., 2003; Ashkenazi et al., 2004). It is clear that oocytes are not passive but have an active role in follicular function (e.g. Matzuk et al., 2002; Su et al., 2003). They actually resume maturation before gap junctional communication ceases. One kind of cellular process that extends through the zona pellucida from the follicle cell to the oolemma, termed transzonal projection, contains microtubules (Albertini and Barrett, 2003). It is conceivable that nocodazole exerts some effect on the cross-talk between the compartments and may influence the maturation of the enclosed oocyte directly as well as indirectly through alteration of general follicle function. There was no evidence for disturbed hormonal homeostasis by nocodazole but a pronounced cell cycle arrest and disturbance in meiosis II spindle formation could partially result from disturbed cell-to-cell communication.

**Dose-response to nocodazole in vitro and in vivo, and in somatic cells**

For many years, it has been commonly accepted that risk assessments of genotoxic chemicals are based on linear models for extrapolating low dose effects from experimental data. The underlying assumption is the absence of a threshold for the induction of mutations (Speit et al., 2000). However, recent data indicate that biologically meaningful threshold effects exist for some types of mutagenic events, e.g. aneuploidy-inducing chemicals that interact with non-DNA targets (Kirsch-Volders et al., 2000; Elhajoui et al., 1995, 1997). Most studies on thresholds were performed with somatic cells in the past. Little is known about the relative risk of aneuploidy induction in somatic versus germ cells, or on gender differences in susceptibility. A recent study on aneuploidy induced by nocodazole in denuded mouse oocytes suggested that the biological threshold for induction of nondisjunction could be similar in female meiosis and cultured human lymphocytes (Eichenlaub-Ritter et al., 2002; Kirsch-Volders, et al., 2003). This was in agreement with the notion that the dose-response to aneugens is similar between somatic and meiotic cells.
(Parry et al., 2000), a concept that may have to be critically re-evaluated.

Preantral follicle culture has been previously used to demonstrate the sensitivity of follicle-enclosed oocytes to aneugens (Sun et al., 2004). In the current study, 20 nM nocodazole significantly increased the number of aberrant meiosis II oocytes, while only 9.1% of in vitro matured denuded mouse oocytes had unaligned chromosomes after maturation to metaphase II in presence of 20 nM nocodazole in the medium (Shen et al., 2005). As shown here, the percentage was about three times higher (28.4%) in mouse oocytes exposed to the same concentration during the last hours of maturation in preantral follicle culture. Similarly, only 21.7% of the denuded oocytes that matured in the 30 nM nocodazole group had displaced chromosomes (Shen et al., 2005), while > 40% of the in vitro grown and matured oocytes from preantral follicle culture showed this aberration after exposure to the same nocodazole dose during the last 16 h of culture. The actual effective dose to induce spindle aberrations in metaphase II oocytes of cultured follicles may be even lower, but we did not test doses in this range. Hyperploidy was significantly increased by 30 nM nocodazole in the present study. Since paracrine signalling at the resumption of meiosis involves gap junctional coupling and nocodazole has been reported to inhibit the formation/function of gap junctions, e.g. in Novikoff hepatoma cells (Johnson et al., 2002), disruption of these critical activities can indirectly affect the spindle function and the fidelity of chromosome segregation and contribute to the high susceptibility to meiotic errors at oogenesis in follicle culture.

The experiments involving follicle-enclosed oocytes unequivocally showed that nocodazole induced perturbations of nuclear maturation and aneuploidy also after in vivo exposure of the mouse. However, these effects were only observed at the highest tested dose (70 mg/kg b.w.). Doses of 35 and 70 mg/kg b.w., expressed in molarity relative to the mouse weight, correspond to 115 × 10^3 and 230 × 10^3 nM nocodazole, respectively. These acute doses are between 10^3 and 10^4 times higher than the continuous in vivo effective nocodazole concentrations. Biodistribution and bioavailability play an important role in in vivo studies. In our experiments, evidence was provided at autopsy that nocodazole bioavailability to oocytes was reduced by precipitates observed in the peritoneum after the administration of both tested doses. Nocodazole has poor solubility in water; thus it is conceivable that the DMSO solution by precipitates observed in the peritoneum after the administrations. 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F.Sun et al.


Aneuploidy in mouse metaphase II oocytes


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