Diazepam induces meiotic delay, aneuploidy and predivision of homologues and chromatids in mammalian oocytes

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The tranquilizer and anti-convulsant diazepam (DZ) is a suspected aneugen. In order to assess its aneugenic potential in mammalian oogenesis we exposed in vitro maturing mouse oocytes to the drug. Spindle formation and cell cycle progression, the behaviour of chromosomes and the distribution of mitochondria were characterized with respect to induction of numerical chromosomal aberrations. A concentration of 25 \( \mu \text{g/ml} \) DZ induced a pronounced delay in maturation and blocked a high percentage of oocytes in meiosis I. This arrest was partly reversible. Hyperploidy was slightly increased in oocytes matured in the presence of 5 \( \mu \text{g/ml} \) DZ and became significantly elevated in oocytes matured with 25 \( \mu \text{g/ml} \) DZ, relative to controls. Concomitantly, DZ induced spindle aberrations and displacement of chromosomes from the equator, but unlike in mitosis and in male meiosis most oocytes still possessed bipolar spindles. A significant fraction of meiotically delayed, metaphase I-blocked oocytes exposed to 25 \( \mu \text{g/ml} \) DZ contained univalents. Some DZ-treated oocytes progressing to meiosis II exhibited one or multiple single chromatids. Precocious chiasma resolution and equational segregation of chromatids from functional univalents in first anaphase (predisdivision) may be responsible for this condition, a mechanism also discussed in the aetiology of maternal age-related aneuploidy. DZ disturbed the spatio-temporal distribution of mitochondria during oocyte maturation, possibly by binding to peripheral-type benzodiazepine receptors on mitochondria, thus affecting the availability of ATP and calcium homeostasis. Blocks in maturation may also relate to binding of DZ to calmodulin. Data suggest that DZ exposes mammalian oocytes to predisdivision and aneuploidy. Thresholds, long lasting effects of DZ in vivo and sex-specific sensitivities in chemically induced aneuploidy of mammalian germ cells are critically evaluated.

Introduction

DZ belongs to the class of clinically effective benzodiazepines with hypnotic, anxiolytic, muscle relaxant and anti-convulsant activity. Pharmacological activity appears mainly related to high affinity binding of DZ to the central-type benzodiazepine receptors and modulators of \( \gamma \)-aminobutyric acid receptors of the nervous system (Costa and Guiotti, 1979) and the highly conserved peripheral-type benzodiazepine receptors on the outer membrane of mitochondria of steroidogenic cells and other cell types (Rose et al., 1992; Papadopoulos et al., 1997). At micromolar concentrations DZ exhibits anti-proliferative effects in tissue culture (Clarke and Ryan, 1980; Hsu et al., 1983; Wang et al., 1984; Lafi et al., 1987; Warr et al., 1993; Kunert-Radek et al., 1994; Camins et al., 1995), mainly by arresting cells in G2 phase or in mitosis (see for example Satya-Prakash et al., 1984; Natarajan et al., 1993; Warr et al., 1993). DZ was shown to inhibit centriolar (Andersson et al., 1981; Estervig and Wang, 1984) and spindle pole separation (Izzo et al., 1997). It severely disturbs bipolar spindle formation in mitotic and male meiotic cells (Hsu et al., 1983; Lafi et al., 1987; Callani et al., 1989; Gassner and Adler, 1995). The influence of DZ on spindle formation in mammalian oogenesis has not been characterized so far.

Several groups reported that DZ interferes with fidelity of chromosome segregation and increases aneuploidy (Hsu et al., 1983; Satya-Prakash et al., 1984; Lafi and Parry, 1988; Natarajan et al., 1993; Sbrana et al., 1993; Warr et al., 1993). DZ caused micronucleus formation when cells were exposed to the drug in vivo and in vitro (Lafi and Parry, 1988; Antoccia et al., 1991; Bonatti et al., 1992; Van Bao et al., 1992; Lynch and Parry, 1993). Structural chromosomal aberrations were observed by Lafi and Parry (1988), while most other studies failed to detect such an effect (Degraeve et al., 1985; Xu and Adler, 1990). Weak clastogenic activities might relate to impurities in the drug (reviewed by Giri and Banerjee, 1996). It has been suggested that the aneugenic activity of DZ contributes to genomic instability of cells and to the suspected tumour-promoting and carcinogenic activity of the drug (de la Iglesia et al., 1981; Diwan et al., 1986; Gibson et al., 1995). However, there may be species- and tissue-specific differences in pharmacology and sensitivity to DZ (Giri and Banerjee, 1996; Parry et al., 1996), since several studies failed to reveal any aneugenic properties of DZ, especially when the drug was administered in vivo (see for example Miller and Adler, 1992; Leopardi et al., 1993; Parry et al., 1996; Schmid et al., 1997) and when only the rises in hyperploid cell numbers were taken into account. Hypoploidy but not hyperploidy levels appear increased in most DZ-exposed cells (Lafi and Parry, 1988; Sbrana et al., 1993; Warr et al., 1993).

Chemically induced aneuploidy in germ cells can contribute to trisomy formation, reducing the developmental potential of the embryo and leading to physical and mental retardation of affected human individuals (reviewed by Eichenlaub-Ritter, 1994, 1996; Plachot, 1997). In fact, recent cytogenetic analysis of human sperm from DZ-poisoned males surviving suicide attempts in vivo (see for example Miller and Adler, 1992; Leopardi et al., 1993; Parry et al., 1996; Schmid et al., 1997) and when only the rises in hyperploid cell numbers were taken into account. Hypoploidy but not hyperploidy levels appear increased in most DZ-exposed cells (Lafi and Parry, 1988; Sbrana et al., 1993; Warr et al., 1993).

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Animals and oocyte culture

Oocytes were isolated from the ovaries of outbred 10-16-week-old virgin animals and cultured in M2 medium according to procedures previously described (for example, Eichenlaub-Ritter and Boll, 1989, Eichenlaub-Ritter and Betzendahl, 1995).

Treatment

Multipolar spindles, rises in hypoploidy levels and C-mitotic effects were reported in previous studies on mitotically dividing cells exposed to DZ at a concentration of 5 μg/ml DZ in M2 medium. However, DZ were reported to be toxic to oocytes and caused oocyte degeneration (Stenchever and Smith, 1981). To cover the potential aneugenic range which is still tolerated by oocytes and is in a physiologically and pharmacologically relevant range (Lukey et al., 1991) 5 and 25 μg/ml DZ were used in the present study. Oocytes were exposed for 8 or 16 h to 5 or 25 μg/ml DZ in M2 medium. Some oocytes were treated for only 3 h with 25 μg/ml DZ during 3-isobutyl-1-methylxanthine (IBMX; Sigma)-induced arrest of oocyte meiosis in the dictyate stage, followed by 16 h maturation and culture in drug-free medium. For this set of experiments controls were exposed to 100 mM IBMX alone (Eichenlaub-Ritter and Betzendahl, 1995) or to IBMX plus solvent 15% DMSO.

Each treatment was repeated several times with oocytes from two to three mice in each group. Oocytes within a group were isolated within 30 min in order to obtain good developmental synchrony. Maturation in vitro was considered normal when at least 65% of the oocytes in the control emitted a polar body. Data from repeated experiments were pooled for final analysis.

Analysis of cell cycle progression and chromosomal constitution

Cell cycle progression was analyzed by determining numbers of oocytes with intact germinal vesicles (GV), germinal vesicle breakdown (GVBD) or polar bodies (PB) or oocytes which became parthenogenetically activated pronuclei after defined times of maturation. Cytogenetic analysis and C-banding was performed as previously described (Eichenlaub-Ritter and Betzendahl, 1995, Soewarto et al., 1995). The numbers of oocytes with GV and those with bivalents in meiosis I were scored in relation to those with metaphase II chromosomes or pronuclei to determine the extent of drug-induced meiotic arrest. Most of the oocytes in this study were spread according to our standard method. For this oocytes were first hypotonically treated, then singly transferred to a Petri dish with fixative and finally individually spread on slides (Eichenlaub-Ritter and Betzendahl, 1995, Soewarto et al., 1995). In this way maximal numbers of oocytes were obtained for chromosomal analysis. Oocytes in those experiments were also spread in a slightly different, less gentle way. They were directly placed on a slide in hypotonic solution and then spread by addition of a drop of fixative. This yielded very clear chromosome preparations with little cytoplasm, but often disrupted and spread oocytes too vigorously, so that groups of chromosomes were lost from the slide. Although only those oocytes possessing 17 or more metaphase II chromosomes were included in the analysis, hypoploidy rates in those sets of experiments employing the latter technique were elevated in comparison with the gentle technique (Eichenlaub-Ritter and Betzendahl, 1995). There were still differences in hypoploidy levels between DZ-exposed and control oocytes, although they did not reach statistical significance due to the small number of oocytes. Implying that these differences between groups still reflect inherent differences in response to chemical exposure. Taken together, hypoploidy rates differed significantly between controls and DZ-exposed groups. Overall hypoploidy rate was not influenced by spreading technique.

Only oocytes with well spread metaphase II chromosomes were evaluated (see Figure 2). Oocytes with <20 to 17 metaphase II chromosomes were regarded as hypoploid. Hyperploids included all oocytes with ≥20 metaphase II chromosomes (including those with 20 chromosomes and one or more single chromatids). Oocytes with 19 metaphase II chromosomes plus two chromatids of equal size were regarded as euploid. In one case we found an oocyte with 19 metaphase II chromosomes and two additional chromosomes of unequal size. Although we were unable to unambiguously decide whether both represent additional chromatids or one chromatid and one metaphase II chromosome, the oocyte was clearly euploid and therefore included in the group of hyperploids and not among euploids with 20 metaphase II chromosomes. Diploid oocytes contained 40 metaphase II chromosomes.

Homologues in metaphase I oocytes were only scored as univalent when they were separated from each other by at least one chromosome length and were located individually among a metaphase I spread on a slide. Predistribution of chromatids was also only scored in cases where arms of chromatids were clearly visible and separated from each other by a distance exceeding their length. In some of the spread oocytes predistribution of chromatids at anaphase I was confirmed by analysis of the reciprocal chromosomal constitution of the polar body. Statistical evaluation was by the χ² test using Yates correction.

Analysis of spindle disturbances and morphidional distribution

Preparation of oocytes for immunofluorescence was according to previously published methods (Eichenlaub-Ritter and Betzendahl, 1995; Soewarto et al., 1995). In short, the zona was removed by pronase and the oocytes extracted previously described (for example, Eichenlaub-Ritter and Boll, 1989, Eichenlaub-Ritter and Betzendahl, 1995).

Material and methods

Chemicals

Diazepam (DZ; CAS 439-14-5; a gift from Roche) was provided by I.-D. Adler (GF, Oberschleißheim, Germany). It was dissolved in 100% dimethylsulfoxide (DMSO, Sigma, Deisenhofen, Germany) and diluted to concentrations of 5 or 25 μg/ml in M2 medium shortly before use. The final concentration of solvent in M2 medium was 0.5%.

Animals and oocyte culture

Oocytes were isolated from the ovaries of outbred 10-16-week-old virgin MF1 mice at the day of diestrus of the normal hormonally unstimulated cycle (Eichenlaub-Ritter and Betzendahl, 1995). Animal breeding and isolation and culture of oocytes in M2 medium was performed according to procedures

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in warm extraction buffer containing non-ionic detergent and glycerol. They were attached to slides, fixed in ice-cold methanol and processed for immunofluorescence with a monoclonal anti-tubulin antibody (Eichenlaub-Ritter and Betzendahl, 1995; Soewarto et al., 1995). For analysis of mitochon-
drial behaviour live oocytes were incubated for 30 min in M2 medium containing 300 ng/ml Mitotracker™ CM™TMRos-H2 (M-7511; Molecular Probes, Eugene, OR). The non-fluorescent compound becomes converted to a fluorescent one after oxidation in the mitochondria, where it accumulates and reacts with accessible thiol groups on peptides and proteins. Distribution of mitochondria was examined in living oocytes under a fluorescent microscope. Chromosomes were vital stained with 500 ng/ml Hoechst 33342 (Sigma, Deisenhofen, Germany) for 30 min.

For confocal microscopy extracted, fixed and immunofluorescently labelled spindles of oocytes were examined with a Zeiss laser microscope equipped with a UV laser and one for longer wavelengths (Eichenlaub-Ritter and Betzendahl, 1995; Gassner and Adler, 1995). Images of optical sections through oocytes were later combined to yield a three-dimensional image of the spindle and to determine the relative positioning of individual chromosomes (Eichenlaub-Ritter and Betzendahl, 1995).

Results

Cell cycle kinetics and rate of maturation of mouse oocytes exposed to DMSO or DZ

Many classical aneugens which interfere with microtubule polymerization but also those influencing spindle formation indirectly affect cell cycle progression. A mitotic checkpoint sensing spindle integrity (Elledge, 1996; Nicklas, 1997) can induce a block in M phase or mitotic or meiotic delay (Eichenlaub-Ritter et al., 1996; Straight, 1997). To check for such an activity of DZ in female meiosis we first analysed meiotic maturation in DZ-exposed oocytes. With low concent-

Fig. 1. Cell cycle progression in oocytes exposed continuously or transiently to DZ during or prior to resumption of maturation in vitro. (A) Maturation for 8 h in the presence or absence of 25 μg/ml DZ. A lower percentage of DZ-exposed oocytes (horizontal lines) (n = 214) as compared with untreated controls (con, 8 h M2, vertical lines) (n = 497) spontaneously resume maturation and undergo GVBD during 8 h culture. (B) Number of oocytes reaching meiosis II and emitting a polar body (PB) during 16 h culture in the presence of DZ. Low concentrations of DZ (16 h 5 μg/ml DZ, open columns) (n = 408) do not influence maturation and polar body formation (PB) in comparison with controls (con, 16 h M2, solid columns) (n = 1065) or solvent (16 h DMSO, chequered columns) (n = 290). However, numbers of oocytes in GVBD are higher and those with PB are reduced by high concentrations of DZ (16 h 25 μg/ml DZ, hatched columns) (n = 1595). (C) Exposure to 25 μg/ml DZ during the first or second 8 h maturation and culture for a total of 16 h. The percentage of oocytes exposed for the second (8 h M2/8 h DZ, bold vertical lines) (n = 232) or first (8 h DZ/8 h M2, bold horizontal lines) (n = 81) 8 h of culture to 25 μg/ml DZ which emitted a polar body is lower than in controls (solid columns in B) but greater than in the group of oocytes cultured continuously in DZ (hatched columns in B). Numbers of oocytes in GVBD or with PB are similar in both groups, irrespective of early or late transient exposure to DZ. (D) Exposure to DZ prior to resumption of maturation during a 3 h arrest in the dictyate stage by 1BMX, followed by 16 h maturation without DZ. The number of oocytes reaching second meiosis with PB is only marginally lower in the group of oocytes exposed transiently to 25 μg/ml DZ for 3 h prior to resumption of maturation (3 h 1BMX & DZ, 16 h M2, hatched columns) (n = 504) as compared with controls (3 h 1BMX, 16 h M2, solid columns) (n = 199). Solvent alone slightly increases numbers of oocytes with PB (3 h 1BMX & DZ, 16 h M2, open columns) (n = 246). (E) Recovery of oocytes from DZ-induced meiotic arrest/delay during 3 h in N2 medium. (Left) The number of control oocytes (con, 16 h M2, solid columns) increases only marginally during additional 3 h culture (16 h M2, horizontal lines) (n = 116). (Middle) The number of oocytes exposed to solvent (dotted columns) does not change during additional culture for 3 h in the absence of DMSO (16 h DMSO, 3 h M2) (n = 49). (Right) In contrast, the percentage of oocytes with GV decreases and that with polar bodies (PB) increases in oocytes treated with DZ for 16 h (16 h 25 μg/ml DZ, open columns) during a 3 h recovery from DZ (16 h 25 μg/ml DZ, 3 h M2, boldly hatched columns) (n = 149).
trations of DZ (5 μg/ml) or solvent alone oocytes appeared to develop normally. However, the number of oocytes with GVBD was reduced after 8 h maturation in vitro in the presence of 25 μg/ml DZ in comparison with controls (Figure 1A). At this time of culture in vitro nearly all oocytes of the control had resolved their nuclear membrane and initiated spindle formation (see Figure 4a), while a considerable number of DZ-exposed oocytes were still in the GV stage.

A concentration-dependent delay or arrest of meiotic maturation by DZ also became evident in oocytes matured for 16 h in vitro in the presence of the drug (Figure 1B). The number of oocytes from the group exposed to solvent alone (5% DMSO) reaching metaphase II and emitting a first polar body was slightly but not significantly reduced relative to the untreated controls. A dose of 5 μg/ml DZ did not block meiotic progression but rather more oocytes as compared with solvent alone and controls emitted a polar body (Figure 1B). The number of oocytes in metaphase II with a polar body was dramatically reduced by >50% relative to solvent alone and controls and that of oocytes in the dictyate stage (GV) increased by nearly 10% when oocytes matured in the presence of 25 μg/ml DZ.

Unlike observations with other drugs (e.g. chloral hydrate; Eichenlaub-Ritter and Betzendahl, 1995), inhibition of maturation by high doses of DZ was reversible, since some oocytes of the group arrested in GV were capable of progressing to meiosis I and some with GVBD matured to meiosis II (e.g. they emitted a polar body) when oocytes recovered for 3 h in drug-free medium following a 16 h culture period in the presence of 25 μg/ml DZ (Figure 1E). In contrast, GV- and meiosis I-arrested oocytes in the control and solvent groups represented meiotically incompetent oocytes and were unable to develop to meiosis II when the period of culture in vitro was prolonged by 3 h. They remained arrested in the GV stage or meiosis I (GVBD) (Figure 1E, left and middle). Oocytes appeared sensitive to DZ at all stages of maturation (Figure 1C). The percentage of oocytes with a polar body in groups exposed to DZ during the first or second 8 h maturation was reduced relative to controls (solid columns in Figure 1B), but higher compared with the group exposed to DZ throughout maturation (hatched columns in Figure 1B). Treatment with DZ during the first as well as the last 8 h of culture resulted in a similar decrease in numbers of cells with a polar body relative to untreated controls (compare Figure 1C with B).

Exposure to DZ during a 3 h arrest in the dictyate stage prior to resumption of maturation on addition of IBMX followed by culture in drug-free medium for 16 h marginally reduced the numbers of oocytes emitting a first polar body relative to the IBMX group, while solvent alone appeared to enhance the rate of maturation slightly (Figure 1D).

From these observations it can be concluded that: (i) DZ affects cell cycle kinetics when it is present at higher concentrations (25 μg/ml) throughout maturation or during the first or second 8 h of culture; (ii) the higher but not the lower concentration of DZ reversibly arrests mammalian oogenesis; (iii) maturation to metaphase II of oocytes is not inhibited by drug exposure prior to resumption of maturation.

### Cytogenetic analysis of DZ-exposed oocytes: meiosis I versus meiosis II

Cytogenetic analysis confirmed the observation of a meiotic delay caused by DZ. As expected, the percentage of oocytes with bivalent chromosomes in meiosis I was significantly increased in groups exposed to 25 μg/ml DZ for 16 h, while that with metaphase II chromosomes declined (Table I) relative to the control. In contrast, the presence of 5 μg/ml DZ may even stimulate meiotic progression of incompetent oocytes to meiosis II, since significantly more oocytes in the DZ group had metaphase II chromosomes and fewer remained in meiosis I as compared with the control and solvent groups. Transient arrest of oocytes in the dictyate stage by IBMX alone or IBMX plus solvent had no pronounced effect on maturation kinetics. The percentage of oocytes maturing to metaphase II after recovery from IBMX and DZ was nearly identical to that of oocytes matured without DZ and only reduced relative to solvent (Table II). Neither the rise in the number of oocytes from the DZ group with metaphase I chromosomes nor the decrease in such with metaphase II chromosomes was significantly different from the controls. The overall rate of oocytes with metaphase II chromosomes was also comparable with the untreated controls which had not been blocked in the dictyate stage by IBMX (Table I).

Cytogenetic analysis confirmed that some oocytes developed to meiosis II and recovered from drug treatment when they matured for an additional 3 h in drug-free medium after culture for 16 h in 25 μg/ml DZ (compare Table I with III). However, the absolute percentage of oocytes maturing to meiosis II was still lower than in the solvent (16 h in DMSO plus 3 h in M2 medium without solvent) or control group (19 h culture; Table III). The percentage of metaphase II oocytes was not very different between controls and solvent-exposed oocytes, regardless of whether they matured for 16 h (72.5 and 73.7% respectively) or 19 h in vitro (72.0 and 74.5% respectively; see Tables I and III).

### Aneuploidy in DZ-exposed oocytes

The observations of a meiotic delay by 25 but not 5 μg/ml DZ implies that the drug influenced mammalian oogenesis

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**Table I.** Chromosomes in oocytes treated with DZ for 16 h. Number (percentage) of oocytes in dictyate stage with GV, in meiosis I with bivalents (MI chr), in meiosis II with metaphase II chromosomes (MII chr) or activated (act) in the absence of drug, in solvent (0.5% DMSO), or 5 μg/ml or 25 μg/ml DZ.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Number of oocytes</th>
<th>Number (%) of oocytes with GV</th>
<th>MI chr</th>
<th>MII chr</th>
<th>act</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>397</td>
<td>24(6.0)</td>
<td>83(20.9)</td>
<td>288(72.5)</td>
<td>2(0.5)</td>
</tr>
<tr>
<td>DMSO</td>
<td>171</td>
<td>106(6.8)</td>
<td>35(14.6)</td>
<td>126(73.7)</td>
<td>0(0)</td>
</tr>
<tr>
<td>DZ (5 μg/ml)</td>
<td>312</td>
<td>165(5.1)</td>
<td>27(8.7)**</td>
<td>265(84.9)**</td>
<td>4(1.3)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>490</td>
<td>71(4.5)**</td>
<td>225(45.9)**</td>
<td>190(38.8)**</td>
<td>4(0.8)</td>
</tr>
</tbody>
</table>

Significant differences from "control, "DMSO; "P<0.05, **P<0.01

**Table II.** Chromosomes in oocytes arrested for 3 h in dictyate stage with IBMX with or without 25 μg/ml DZ or solvent and matured for additional 16 h.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Number of oocytes</th>
<th>Number (%) of oocytes with GV</th>
<th>MI chr</th>
<th>MII chr</th>
<th>act</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h arrest in dictyate stage by IBMX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>127</td>
<td>18(14.2)</td>
<td>18(14.2)</td>
<td>90(70.9)</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>DMSO</td>
<td>219</td>
<td>13(5.9)</td>
<td>17(7.8)</td>
<td>187(85.4)</td>
<td>2(0.9)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>428</td>
<td>43(10.0)</td>
<td>81(18.9)**</td>
<td>299(69.9)**</td>
<td>5(1.2)</td>
</tr>
</tbody>
</table>

Significant differences from "DMSO, "P<0.01
in vitro in a concentration-dependent way. Therefore, it was of interest to also analyse its aneugenic properties. The numbers of oocytes with a normal chromosomal constitution of 20 metaphase II chromosomes (Figure 2b) appeared unaffected by treatment of oocytes with solvent and was only marginally, but non-significantly, reduced by low concentrations of DZ (Table IV). In contrast, the percentage of euploid oocytes was significantly reduced when 25 μg/ml DZ was present in the medium throughout maturation. Although hyperploidy levels were more than twice as high in the group of oocytes matured in the presence of 5 μg/ml DZ as compared with controls and solvent alone (2.3% in the group exposed to 5 μg/ml DZ as compared with 0.4 and 0.9% in controls and solvent alone respectively), this rise in chromosomal aberrations did not reach significance. However, the rate of hyperploids (Figure 2d, f and g) with more than 20 metaphase II chromosomes was significantly ($P < 0.01$) increased in oocytes matured for 16 h in the presence of 25 μg/ml DZ relative to the control. The difference from solvent alone reached borderline significance ($P < 0.07$).

The numbers of oocytes included in studies on temporarily restricted exposure to DZ were low. Still, the presence of two oocytes with $>20$ metaphase II chromosomes in each of the groups exposed to 25 μg/ml DZ during the first or second 8 h of maturation (corresponding to 5.1 and 2.6% respectively), as well as three hyperploid oocytes (6.1%; Table IV) in a small group of oocytes cultured for 16 h in DZ followed by 3 h recovery, suggests that oocytes are sensitive to DZ-induced aneuploidy throughout maturation (Table IV). The frequency of hyperhaploidy in oocytes treated for 16 h with DZ (5.5%) was similar to the hyperploidy rate in oocytes treated in the same way but with 3 h recovery afterwards (6.1%). Since many meiosis I-arrested oocytes progress to metaphase II after removal of the drug, the similar overall rate of hyperploidy between these groups implies that it may be predominantly "normal" oocytes which recover from drug treatment and mature past anaphase I. However, considering the small numbers of aneuploids in the recovered group (three of 49; Table IV), the notion that oocytes resume meiosis by an error-free mechanism under these conditions needs to be substantiated by further analysis.

Hypoploidy rates were generally higher in DZ-exposed oocytes as compared with controls, although the absolute percentage of hypoploids varied among experiments (see Materials and methods). Combined, the data imply that high concentrations of DZ cause significant increases in hyperploidy and possibly also predispose oocytes to chromosome loss and hypoploidy, although rises in hypoploidy may mainly relate to the presence of solvent.

Numbers of diploids (Figure 2e) were slightly (but not significantly) increased in DZ-exposed oocytes relative to the controls (Table IV). Again, this effect may be related to the presence of solvent rather than drug.

**P<0.01.**

| Table III. Chromosomes in oocytes after prolonged culture in M2 (16 h and 3 h; control (19 h), or maturation for 16 h in 0.5% DMSO or 25 μg/ml DZ, followed by 3 h in M2) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Concentration (μg/ml) | Number of oocytes | Number (%) of oocytes with | | |
| | | GV | MII chr | MI chr | act |
| Control (19 h) | 93 | 13(14.0) | 12(12.9) | 67(72.0) | 1(1.1) |
| DMSO (16 h/3 h) | 47 | 11(23.4) | 1(2.1) | 35(74.5) | 0(0) |
| DZ (25 μg/ml) | 119 | 23(19.3) | 27(22.7)** | 69(58.0)*** | 0(0) |

Significant differences from *control or †DMSO (16 h/3 h); *P<0.05, †P<0.01.

**Predivision of chromosomes in DZ-exposed oocytes during first or second meiosis**

There are several mechanisms which may cause errors in chromosome segregation at meiosis. DZ may cause non-disjunction when homologues fail to separate during anaphase I. Alternatively, untimely resolution of chiasmata may result in formation of functional univalents prior to anaphase I (termed predivision; Angell et al., 1994). The latter have a high risk of random segregation to either pole or equational segregation of chromatids at first meiosis instead of meiosis II. To test for activity of DZ in inducing such aberrant chromosome behaviour we characterized the chromosomal constitution of meiotically incompetent oocytes and oocytes blocked in prophase I by drug treatment for the presence of univalents. The numbers of meiosis I-arrested oocytes in these groups were always low, since most oocytes in the control and solvent groups spontaneously progressed to meiosis II during 16 h in vitro culture. Only one of the oocytes of the DMSO group contained univalents after 16 h maturation (Table V). Others in the solvent and control groups had exclusively bivalents. In contrast, several of the oocytes blocked in meiosis I by DZ contained pairs of short or long homologues (Figure 2a), which presumably separated during the drug-induced meiotic arrest prior to anaphase I. Of oocytes treated with 25 μg/ml DZ 8.9% possessed homologues in addition to bivalents (Figure 2b). Asynchronous premature separation of some homologues and formation of functional univalents was also found in both groups of oocytes exposed to the drug only temporarily (25 μg/ml DZ), during the first or last 8 h of culture (Table V) or during a 3 h arrest in the dictyate stage (12 of 81 cells, corresponding to 14.8%; Table V). Of meiosis I-blocked oocytes exposed to DZ for 16 h followed by a 3 h recovery or continuously cultured for 19 h in 25 μg/ml DZ 9.6% contained univalents as well as bivalents. Upon prolonged culture predivision also appeared to take place in a few of the meiosis I-blocked maturation-incompetent oocytes of the controls.

When true or functional univalents separate precociously in an equational fashion during first anaphase or when the two sister chromatids of a metaphase II chromosome disjoin prior to anaphase II, this gives rise to one or multiple chromatids in metaphase II (Hunt et al., 1995) and, therefore, may predispose oocytes to an error in chromosome segregation at anaphase II. In view of the formation of univalents in first meiosis we therefore also determined the number of oocytes with chromatids in metaphase II, regardless of whether they were hypo- or hyperploid. In fact, disturbances in the synchrony of meiotic events as seen in first meiosis were also found in second meiosis. Several of the oocytes of the DZ-exposed groups progressing to metaphase II (Figure 2f and g), but none of those in the solvent group and only one of the controls cultured for 16 h in drug-free medium, contained one or multiple chromatids (Table VI). All of the oocytes treated for 16 h with 25 μg/ml DZ possessed only a single chromatid, indicating that they were derived by predivision. Accordingly, we observed a single extra chromatid in a DZ-treated oocyte...
and its partner in the polar body (Figure 2g). In contrast, chromatid-containing control oocytes cultured for 19 h had two chromatids of equal size (as in Figure 2c), suggesting that they were derived by separation of sister chromatids after meiosis I, e.g. during the extended meiotic arrest in metaphase II. Instead of by precocious segregation at anaphase I. Strikingly, a high percentage of those oocytes (5.6%) exposed to DZ during a 3 h interval prior to resumption of maturation contained chromatids. Only one of the 11 oocytes in this group exhibited two chromatids of about the same size. All the rest
Table IV. Cytogenetic analysis of aneuploidy in oocytes matured to meiosis II in the presence or absence of DZ or solvent

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Number of oocytes</th>
<th>Number (%) of oocytes with chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>= 20</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>16 h of maturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>227</td>
<td>192(84.6)</td>
</tr>
<tr>
<td>DMSO</td>
<td>116</td>
<td>89(76.7)</td>
</tr>
<tr>
<td>DZ (5 μg/ml)</td>
<td>173</td>
<td>124(71.1)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>256</td>
<td>169(66.0)<strong>,</strong></td>
</tr>
<tr>
<td>First (f) or second (s) 8 h of maturation in DZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DZ (5 μg/ml) f</td>
<td>39</td>
<td>28(71.8)</td>
</tr>
<tr>
<td>DZ (25 μg/ml) s</td>
<td>77</td>
<td>55(71.4)</td>
</tr>
<tr>
<td>3 h in diacyt stage by IBMX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48</td>
<td>44(91.7)</td>
</tr>
<tr>
<td>DMSO</td>
<td>120</td>
<td>96(80.0)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>196</td>
<td>155(79.1)</td>
</tr>
<tr>
<td>After prolonged culture in M2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (19 h)</td>
<td>59</td>
<td>53(89.8)</td>
</tr>
<tr>
<td>DMSO (16 h/3 h)</td>
<td>32</td>
<td>27(84.4)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>49</td>
<td>34(69.4)</td>
</tr>
</tbody>
</table>

Significant differences from *control or bDMSO (16 h/3 h); *P < 0.05, **P < 0.01.

Table V. Predvision (univalents) in meiosis I oocytes after culture without DZ or with DZ throughout maturation (16 h or 16 h plus 3 h recovery or 19 h), or during the first (f) or second (s) 8 h of culture, or during arrest in diacyt stage by IBMX

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Number of oocytes</th>
<th>Predvision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 h of maturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83</td>
<td>0(0)</td>
</tr>
<tr>
<td>DMSO</td>
<td>35</td>
<td>12(2.9)</td>
</tr>
<tr>
<td>DZ (5 μg/ml)</td>
<td>27</td>
<td>1(4.5)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>225</td>
<td>20(8.9)**</td>
</tr>
<tr>
<td>DZ (25 μg/ml) f</td>
<td>12</td>
<td>2(16.7)**</td>
</tr>
<tr>
<td>DZ (25 μg/ml) s</td>
<td>38</td>
<td>2(7.9)</td>
</tr>
<tr>
<td>3 h arrest in diacyt stage by IBMX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18</td>
<td>0(0)</td>
</tr>
<tr>
<td>DMSO</td>
<td>17</td>
<td>0(0)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>81</td>
<td>12(14.8)</td>
</tr>
<tr>
<td>Prolonged culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (19 h)</td>
<td>12</td>
<td>2(16.7)</td>
</tr>
<tr>
<td>DMSO (16 h/3 h &amp; 19 h)</td>
<td>11</td>
<td>0(0)</td>
</tr>
<tr>
<td>DZ (25 μg/ml)(16 h/3 h &amp; 19 h)</td>
<td>73</td>
<td>7(9.6)</td>
</tr>
</tbody>
</table>

Significant differences from *control (16); *P < 0.01.

diacytate stage by IBMX.

Significant differences between control and DZ-treated groups, corresponding to 1.7%; P < 0.001.) and for DZ-treated meiosis II oocytes to exhibit chromatids (25 of 825 oocytes for the DZ group, corresponding to 3%, versus only four of the 644 oocytes of the control and solvent groups, corresponding to 0.6%; P < 0.001).

Spindle formation and chromosome behaviour in DZ-exposed oocytes

Several ultrastructural studies have revealed that DZ may induce severe spindle aberrations, especially formation of monopolar or multipolar spindles in cells possessing centrioles (see for example Gassner and Adler, 1996). Oocytes do not possess centrioles (Szdllbsi et al., 1988; Messinger and Albertini, 1991; Battaglia contained either a single chromatid or two chromatids of different size, consistent with predvision of two homologues and equational division of univalents at anaphase I (Table VI).

Although absolute numbers of oocytes with predvision in meiosis I and II were low in each experiment, there was a clear and significant tendency for DZ-exposed meiosis I oocytes to possess univalents (44 of 456 oocytes of the entire group of oocytes exposed to DZ, corresponding to 9.6%, versus three of the 176 oocytes of the control and DMSO groups, corresponding to 1.7%; P < 0.01) and for DZ-treated meiosis II oocytes to exhibit chromatids (25 of 825 oocytes for the DZ group, corresponding to 3%, versus only four of the 644 oocytes of the control and solvent groups, corresponding to 0.6%; P < 0.001).

**Diazepam induces meiotic delay, aneuploidy and predvision**

**Table VI. Predvision (chromatids) in meiosis II oocytes after culture without DZ or with DZ throughout maturation (16 h or 16 h plus 3 h recovery or 19 h), or during the first (f) or second (s) 8 h of culture, or during arrest in diacyt stage by IBMX**

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Number of oocytes</th>
<th>Predvision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 h of maturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>227</td>
<td>1(0.4)**</td>
</tr>
<tr>
<td>DMSO</td>
<td>116</td>
<td>0(0)</td>
</tr>
<tr>
<td>DZ (5 μg/ml)</td>
<td>173</td>
<td>2(0.1)**</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>256</td>
<td>5(2.0)</td>
</tr>
<tr>
<td>DZ (25 μg/ml) f</td>
<td>39</td>
<td>0(0)</td>
</tr>
<tr>
<td>DZ (25 μg/ml) s</td>
<td>77</td>
<td>2(2.6)**</td>
</tr>
<tr>
<td>3 h arrest in diacyt stage by IBMX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48</td>
<td>0(0)</td>
</tr>
<tr>
<td>DMSO</td>
<td>120</td>
<td>1(0.8)**</td>
</tr>
<tr>
<td>DZ (25 μg/ml)</td>
<td>196</td>
<td>11(5.6)**</td>
</tr>
</tbody>
</table>

Significant differences from control (16); *P < 0.05, **P < 0.01. **P < 0.001.

Fig. 2. Chromosomal constitution of oocytes cultured in DZ-containing medium. (a) Pair of univalents (arrowheads) in a metaphase I arrested oocyte matured for 16 h in the presence of 25 μg/ml DZ. (b) Control oocyte with 20 metaphase II chromosomes after 16 h culture. (c) Aged control oocyte cultured for 16 h without DZ with 19 metaphase II chromosomes and two chromatids (arrowheads). (d) Hyperploid oocyte with 21 metaphase II chromosomes after culture for 16 h in the presence of 25 μg/ml DZ. (e) "Diploid" oocyte with 40 metaphase II chromosomes after 3 h arrest in the diacyt stage in the presence of IBMX and 25 μg/ml DZ. Centromere separation of some chromosomes (arrowheads) appears to have been caused by spreading, since chromatids are still oriented towards each other and only separated by a small distance. (f) Hyperploid oocyte which underwent anaphase I during a 3 h recovery from treatment with 25 μg/ml DZ (16 h) with an extra single chromatid (arrowhead), apparently derived by predvision. (g) 22 metaphase II chromosomes and a single chromatid (arrowhead) in an oocyte and (g') 17 metaphase II chromosomes plus a single chromatid in its respective PB after culture for 16 h in the presence of 25 μg/ml DZ. Bar 5 μm.
condensed chromosomes (Figure 4c and c’)- In comparison, control oocytes possessed a much more compact microtubule with a highly aberrant cytoskeleton. For instance, whirls the side of the spindle (Figure 4e) in addition to displaced possessed prometaphase I spindles with prominent asters at 4a and a’). In contrast, many cells matured in the presence of 25 μg/ml DZ for 8 h were still at early prophase I stage at this time. Many DZ-exposed oocytes still possessed a prometaphase I or metaphase I spindle after 16 h maturation (Figures 3c and 4e and e’), when controls had nearly all developed to metaphase II (Figures 3d and d’). Some of the DZ-exposed, meiosis I-arrested oocytes had normal spindles but displaced chromosomes (Figure 3c), while others possessed prometaphase I spindles with prominent asters at the side of the spindle (Figure 4e) in addition to displaced scattered chromosomes (Figure 4e’). Some DZ-treated oocytes were, even after 16 h culture, at an earlier stage of meiosis with a highly aberrant cytoskeleton. For instance, whirls of unpolarized microtubules were associated with scattered condensed chromosomes (Figure 4c and c’). In comparison, control oocytes possessed a much more compact microtubule configuration at an earlier time of maturation (4 h after the start of culture), when they were still in the process of spindle assembly (Figure 4b and b’). Infrequently, tripolar spindles with unordered bivalents and, only once, a monopolar spindle with scattered unattached bivalents were observed in the group of oocytes exposed to 25 μg/ml DZ (Figure 4h and h’). Unlike male meiosis, most of the spindles of DZ-exposed oocytes possessed two poles, in spite of other aberrations, like an asymmetric shape, fusiform poles or an unusual association with laterally displaced asters. Oocytes in anaphase I and telophase I were also sometimes detected in the DZ-exposed experimental group (Figure 4f and f’), whereas most oocytes of the control and solvent groups were in metaphase I with well-aligned chromosomes (Figure 4d). There was no evidence for lagging of chromosomes in the few DZ-exposed oocytes fixed in anaphase I.

As seen in DZ-blocked meiosis I stages, most oocytes escaping arrest in the first division and progressing to metaphase II during 16 h culture also possessed a bipolar spindle, in spite of the presence of 25 μg/ml DZ during maturation (Figure 3d). However, unlike the controls, with their well-aligned chromosomes and symmetrically shaped spindle, many of the DZ-exposed oocytes exhibited spindle abnormalities. For instance, some spindles had fusiform poles but well-aligned chromosomes (Figure 3d). Others had scattered chromosomes and an oblique orientation of the spindle equator relative to the long axis of the spindle (Figure 4g and g’). Two DZ-exposed oocytes contained two spindles in addition to displaced chromosomes (Figure 4i and i’).

Quantitative analysis confirmed that DZ severely affects spindle formation and interferes with chromosome alignment in first as well as second meiosis (Table VII). The percentage of oocytes with aberrant spindles or unaligned chromosomes was very low in the control and only marginally higher in the group of oocytes exposed to solvent. Nearly one third of all oocytes matured in the presence of 25 μg/ml DZ had an aberrant spindle or one or more unaligned chromosomes (the latter feature was only evaluated in cells with a roughly bipolar spindle apparatus or when chromosomes were unattached to the spindle, as in Figure 4h’). Oocytes with spindle aberrations or chromosomal displacement in the control and solvent groups nearly all comprised cells in meiosis II. In contrast, in the DZ-exposed group these aberrations were seen with about equal frequencies in first and second meiosis.

Disturbances in the association of mitochondria with the spindle

Since DZ can bind to receptors on mitochondria, inhibit porphyrin and heme transport (Taketani et al., 1995) and affect steroidogenesis (Amsterdam and Suh, 1991; Papadopoulos et al., 1997), possibly by interfering with acyl-CoA-mediated reactions (Knudsen et al., 1993), it may influence the function of mitochondria in oocytes. Therefore, the distribution and activity of mitochondria was characterized by Mitotracker™ CMTMros-H2 staining in oocytes exposed to high concentrations of DZ. In fact, labelling showed that the DZ-induced delay in cell cycle progression was accompanied by a disturbance in the association of mitochondria with the periphery of the spindle (Figure 5b and b’). While mitochondria formed a typical broad belt around the prometaphase I spindle of controls (Van Blerkom, 1991; Calarco, 1995) after 8 h culture (Figure 5a and a’), there was only a small band of mitochondria found in association with chromosomes in the circular bivalent stage.
Diazepam induces meiotic delay, aneuploidy and predivision

Fig. 4. Characteristic disturbances in the spindle and chromosome alignment of DZ-exposed oocytes. (a) Typical barrel-shaped anastral spindle and well-aligned bivalents (a') in control oocyte at metaphase I. Untreated oocyte initiating spindle formation shortly after GVBD with microtubular asters (b) in the vicinity of condensed bivalents (b'). Prophase I-arrested DZ-exposed oocyte with unordered, unfocused and extended whorl of microtubules (c) with peripherally located, scattered condensed bivalents (c'). Typical metaphase II spindle (d) and well-aligned chromosomes (d') of control oocyte matured for 16 h. First meiotic spindle (e) with unaligned chromosomes and peripherally located bivalents (arrowhead in e') of DZ-exposed oocyte. Telophase I spindle (f) and chromosomes (f') after 16 h culture in DZ. Asymmetric metaphase II spindle with fusiform poles (g) and unaligned chromosomes (g') in DZ-exposed oocyte. Monopolar spindle (h) and unordered chromosomes (h'), some of which appear unattached in an oocyte blocked in meiosis I after DZ. An oocyte which emitted a PB but had two aberrant microtubule assemblies, an unfocused whorl of microtubules and a bipolar metaphase II spindle (i) with unaligned chromosome (arrowhead in i') or clusters of chromosomes in the centre of microtubular assemblies. (a-i) Anti-tubulin immunofluorescence; (a'-i') DAPI-stained chromosomes. Bar 5 μm.

of meiotically delayed DZ-exposed oocytes (Figure 5b and b'). In the latter small clusters of mitochondria were found in the more central cytoplasms and larger ones were randomly distributed all over the cell. Similarly, after 16 h culture mitochondria in meiosis I oocytes were still not associated with the periphery of the spindle, as is characteristic for controls (Calarco, 1995). Rather, they appeared more dispersed and unorderd, comparable with what was seen in oocytes examined after 8 h culture. Even those oocytes progressing to metaphase II under the influence of the drug often displayed scattered mitochondria (Figure 5d and d'). An unusually small band of these organelles was present in association with the spindle. In conclusion, DZ not only affected spindle formation and cell cycle progression, but also compromised mitochondrial distribution and possibly influenced their function.

Discussion

Mechanisms of the DZ-induced delay in GVBD and anaphase progression

The present study shows that high concentrations of DZ interfere with meiosis in mammalian oocytes. Firstly, the initial stage between isolation from the follicle, resumption of maturation and resolution of the nuclear membrane is slowed down significantly and, secondly, oocytes become reversibly arrested in meiosis I. Unlike typical microtubule-depolymeriz-

575
ing aneugen, DZ does not appear to exert its C-mitotic effects by primarily interfering with the microtubular cytoskeleton, since only extremely high toxic doses of DZ caused assembly of microtubules with aberrant morphology and had a moderate effect on tubulin polymerization in vitro (Wallin and Hartley-Asp, 1993). The drug has little influence on polymerization velocity and does not alter steady-state microtubule assembly at physiologically relevant concentrations (Brunner et al., 1991). Therefore, it is unlikely that the delay in maturation of oocytes due to DZ results primarily from an interference of the drug with the polymerization equilibrium. A DZ-induced delay in microtubule polymerization (Brunner et al., 1991) might influence spindle formation after resolution of the nuclear membrane, but this does not explain the delay in GVBD, since resolution of the nuclear membrane does not rely on the presence of microtubules. Recently it was shown that DZ may inhibit protein synthesis (Parry et al., 1996). Protein synthesis is not initially required for commitment to resume maturation in mouse oocytes as nuclear membrane breakdown and chromosome condensation occur in the presence of cycloheximide in murine oocytes (e.g. Soewarto et al., 1995). Therefore, we do not believe that the initial delay in resumption of meiosis was caused by interference by DZ with protein synthesis, although it may have influenced oogenesis at a later stage.

Van Blerkom (1991) and Calarco (1995) have shown that resumption of maturation is accompanied by characteristic movements and a redistribution of mitochondria in oocytes. A broad ring of mitochondria becomes associated with the nucleus prior to GVBD and encloses the spindle in meiosis I. DZ disturbs mitochondrial distribution and possibly also function, for instance due to binding to peripheral-type benzodiazepine receptors (Wang et al., 1984; Krueger and Papadopoulos, 1992). The natural ligands for these receptors

Table VII. Analysis of spindle formation and chromosome alignment after 16 h of culture in the absence (control) or presence of DZ, or after exposure to DZ for the second (s) 8 h of culture

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Number of oocytes</th>
<th>Spindle</th>
<th>Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>normal (%)</td>
<td>aberr (%)</td>
</tr>
<tr>
<td>Control 126</td>
<td>122 (96.8)</td>
<td>4 (3.2)</td>
<td>118 (93.7)</td>
</tr>
<tr>
<td>DMSO 44</td>
<td>41 (93.2)</td>
<td>3 (6.8)</td>
<td>40 (90.9)</td>
</tr>
<tr>
<td>DZ (25 μg/ml) 29</td>
<td>26 (89.7)</td>
<td>3 (10.3)</td>
<td>26 (89.7)</td>
</tr>
<tr>
<td>DZ(25 μg/ml) s 27</td>
<td>75 (66.4)</td>
<td>38 (33.6)*</td>
<td>78 (69.0)</td>
</tr>
<tr>
<td></td>
<td>24 (88.9)</td>
<td>3 (11.1)</td>
<td>22 (81.5)</td>
</tr>
</tbody>
</table>

Significant differences from *control or bDMSO: *P<0.01.
aberr: aberrant, asymmetric or unusual spindle; equa: chromosomes at spindle equator; unali: some chromosomes unaligned. not at equator.

Fig. 5. Vital staining of mitochondria (a-d) and chromosomes (a'-d') in control (a and c) or DZ-exposed (b and d) oocytes after 8 (a and b) or 16 (c and d) h maturation in vitro. Mitochondria of control oocytes (a) are clustered in a dense aggregate around the metaphase I spindle of control oocytes after 8 h culture, but form only a small ring close to the chromosomes of meiotically delayed or meiosis I-blocked DZ-exposed oocytes (b). Mitochondria form a broad, nearly symmetrical belt around the metaphase II spindle (large arrowheads) of control oocytes (c) and are also present in association with dispersed chromosomes (small arrowhead in c') of the polar body (small arrowheads) of control oocytes. Fewer and less well organized clusters of mitochondria (large arrowheads in d) are present in oocytes or their polar bodies (small arrowhead) which progressed to metaphase II in the presence of DZ and possess only unordered chromosomes (d') Bar 5 μm.
Sex-specific differences in spindles, checkpoint controls and the response of germ cells to DZ

Diazepam induces meiotic delay, aneuploidy and predivision in mouse sperm in response to DZ, at least not in vivo (Schmid et al., 1997). Diazepam binding inhibitor protein is expressed in spermatids and sperm, but when DZ interferes with its function, this is only important for events following meiosis (Kolmer et al., 1997). While spermatocytes exposed to DZ predominantly exhibit a monopolar spindle (Gassner and Adler, 1995), the majority of spindles in DZ-exposed oocytes appear to be bipolar, although they have other aberrant features. This sex-specific difference is most probably related to the fact that spermatocytes form astral spindles and possess a centrosome at each fusiform spindle pole. Therefore, when centrosomes fail to separate during prophase a monopolar spindle results. The presence of a monopolar spindle substantially slows down progression into anaphase in mitotic cells (Ito et al., 1994) and may constitute the major reason for meiotic arrest and diploidy in DZ-exposed spermatocytes (Gassner and Adler, 1995; Baumgartner et al., 1997).

There appear to be several differences in chromosome behaviour and epitopes expressed at kinetochores between astral and anastral spindles (see for example Khodjakov et al., 1996) and mitotic and male meiosis as compared with oogenesis (for discussions see Rieder et al., 1993; Duesbery et al., 1997). Multiple MTOCs are recruited by chromosomes after GVBD in oocytes and align at the flat poles of the anastral spindle (Eichenlaub-Ritter and Boll, 1989; Eichenlaub-Ritter and Betzendahl, 1995). Like mitotic cells, oocytes possess a metaphase checkpoint sensing the presence of a spindle (see for example Kubiak et al., 1993), for instance they become reversibly blocked in meiosis I with condensed chromosomes when the spindle is totally depolymerized by nocodazole (Eichenlaub-Ritter and Boll, 1989). However, cells generally do not sense the presence of a chromosome with only one kinetochore (Khodjakov et al., 1997) or an asymmetric or multipolar spindle as long as the kinetochores are under tension (Sluder et al., 1997). The cytoplasm of vertebrate oocytes is sufficient to support bipolar spindle formation in the absence of centrosomes (Gaglio et al., 1997; Heald et al., 1997). Therefore, mouse oocytes may still succeed in polymerizing an anastral bipolar spindle in spite of the presence of DZ and disturbed centrosomal maturation. This capacity to organize an asymmetric but bipolar spindle in oocytes in contrast to spermatocytes may be one reason for an inefficient meiotic checkpoint, by still providing the basis for bi-orientation of chromosomes in meiosis II of DZ-exposed oocytes.

There appear to be several differences in chromosome behaviour and epitopes expressed at kinetochores between astral and anastral spindles (see for example Khodjakov et al., 1996) and mitotic and male meiosis as compared with oogenesis (for discussions see Rieder et al., 1993; Duesbery et al., 1997). Multiple MTOCs are recruited by chromosomes after GVBD in oocytes and align at the flat poles of the anastral spindle (Eichenlaub-Ritter and Boll, 1989; Eichenlaub-Ritter and Betzendahl, 1995). Like mitotic cells, oocytes possess a metaphase checkpoint sensing the presence of a spindle (see for example Kubiak et al., 1993), for instance they become reversibly blocked in meiosis I with condensed chromosomes when the spindle is totally depolymerized by nocodazole (Eichenlaub-Ritter and Boll, 1989). However, cells generally do not sense the presence of a chromosome with only one kinetochore (Khodjakov et al., 1997) or an asymmetric or multipolar spindle as long as the kinetochores are under tension (Sluder et al., 1997). The cytoplasm of vertebrate oocytes is sufficient to support bipolar spindle formation in the absence of centrosomes (Gaglio et al., 1997; Heald et al., 1997). Therefore, mouse oocytes may still succeed in polymerizing an anastral bipolar spindle in spite of the presence of DZ and disturbed centrosomal maturation. This capacity to organize an asymmetric but bipolar spindle in oocytes in contrast to spermatocytes may be one reason for an inefficient meiotic checkpoint, by still providing the basis for bi-orientation of chromosomes in meiosis II of DZ-exposed oocytes.

DZ severely affects alignment of chromosomes at the spindle equator. The displacement of chromosomes may be related to the altered mitochondrial distribution, to spindle asymmetry and to impaired activity of microtubule motor proteins and kinases associated with kinetochores. Odd chromosome movements, malsegregation and persistent malorientation of chromosomes were also noticed in mitotic and male meiotic cells when they were exposed to inhibitors of protein kinases (Nicklas et al., 1993). We cannot a priori decide whether restrictions on calcium-calmodulin signalling are the primary cause of the meiotic block or whether displacement of chromosomes is also involved. Using immunofluorescence in combination with FISH and chromosome-specific probes we recently identified several DZ-exposed oocytes with unaligned chromosomes in meiosis I which contained two univalents of the X chromosome in addition to other displaced chromosomes of...
unknown identity (Yin et al., in preparation). The X chromosome possesses at least one chiasma in normal female meiosis (Hulten et al., 1995). Therefore, the two homologous X chromosomes most probably represent functional univalents which separated precociously prior to anaphase I, in spite of the presence of other chromosomes displaced from the spindle equator. This can be taken as tentative evidence that the metaphase checkpoint is permissive in mammalian oogenesis or that DZ disturbs it.

**Significance of predivision for genetic stability in mammalian oocytes**

Precise and controlled timing of events is essential for fidelity of chromosome segregation and genomic stability (Hartwell and Weinert, 1989). Predivision appears to be the primary cause for errors in chromosome segregation in oocytes of aged women (Angell, 1997) and of aged rodents (Sakurada et al., 1996; Eichenlaub-Ritter, unpublished). When functional univalents are formed during prophase I they are predisposed to either random segregation in meiosis I or precocious equational segregation of chromatids in first anaphase, with the risk of errors in the second meiotic disjunction (Hunt et al., 1995). Precocious separation of chromatids during metaphase II of oogenesis may contribute to aneuploidy (Dailey et al., 1996; Sakurada et al., 1996). DZ-exposed mouse oocytes resemble an ‘aged’ phenotype in several ways. As in aged human oocytes (Volareck et al., 1998), meiotic resumption is delayed, aberrant spindles are formed and displaced chromosomes are frequently observed. Moreover, many DZ-exposed oocytes contain single chromatids, most of which seem to be the result of equal separation of chromatids. Similarly to human oocytes with a reduced developmental potential and displaced chromosomes (Van Blerkom et al., 1995), the DZ-treated oocytes also exhibit disturbances in mitochondrial distribution. Reductions in ATP content due to impaired mitochondrial function have been implicated in compromised spindle function in aged human oocytes (Van Blerkom et al., 1995). Currently it is unknown whether environmental exposure contributes to mitochondrial dysfunction and to age effects (Eichenlaub-Ritter, 1996; Plachot, 1997). In fact, predivision in IBMX-arrested DZ-exposed oocytes implies that this type of drug exposure may have long lasting consequences, when damaged oocytes are not efficiently eliminated from the pool by follicular atresia and fertilized oocytes with precociously separated chromatids progress to anaphase II.

**DZ-induced aneuploidy: is there a threshold and a risk in vivo**

The concentration of protein in serum influences the free concentration of DZ in vivo, for instance during the female cycle (Routeledge et al., 1981), and may contribute to sex-specific differences. We exposed oocytes to DZ in medium containing serum albumin, providing conditions such that there were no cytotoxic effects and meiotic arrest was reversible. The concentration of DZ causing significant rises in hyperploidy in the oocytes in vitro is in the same range as that inducing numerical chromosomal aberrations and micronuclei in tissue cultures (e.g. 10–50 µg/ml; Natarajan et al., 1993; Wart et al., 1993). There is some circumstantial evidence for an increase in hypoploidy in lymphocytes of suicide cases in vivo after the first hour of poisoning with DZ (Van Bao et al., 1992). However, plasma levels of DZ in the human range between 20 and 150 ng/ml with chronic treatment and are therefore much lower than doses employed in the present in vitro study. Therefore, DZ may have only negligible or minimal aneugenic activity in oogenesis in vivo when it is present at physiologically relevant concentrations. In accordance with this, Marchetti et al. (1994) did not find aneuploidy in mouse oocytes exposed to DZ in vivo. However, the concentration-dependent reduction in numbers of ovulated oocytes implies that DZ may interfere with oogenesis and hormonal regulation and, hence, mitochon- 

**References**


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