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Differential chromosome behaviour in mammalian oocytes exposed to the tranquilizer diazepam *in vitro*

Fengyun Sun, Hang Yin[1] and Ursula Eichenlaub-Ritter[2]

Institute of Gentechnology/Microbiology, Faculty of Biology, University of Bielefeld, 33501 Bielefeld, Germany and [1]McGill University, Montreal, Canada

There are several reports demonstrating that aneugens may preferentially affect segregation of particular chromosomes in somatic cells. Much less is known on specific susceptibility of individual chromosomes to non-disjunction in mammalian meiosis in response to chemical exposures. To explore possible chromosome-specific behaviour and susceptibility to errors in chromosome segregation in mammalian oogenesis we employed spindle immunofluoresecence in combination with FISH with chromosome-specific probes to analyse congression of chromosomes X, 8 and 16 in diazepam (DZ)-treated, meiotically delayed meiosis I oocytes of the mouse. Concomitantly, we assessed the susceptibility of homologues to precociously segregate prior to anaphase I during DZ-induced meiotic arrest. About 50% of all oocytes exposed to 25 μg/ml DZ became meiotically delayed. Chromosomes failed to congress at the spindle equator in one-third of these meiosis I oocytes. The X chromosome was significantly more often located away from the spindle equator as compared with the expected random behaviour. Concomitantly, DZ exposure induced untimely segregation of homologous chromosomes of the gonosome and the autosomes in meiosis I. This occurred with similar frequencies. The observations confirm that DZ perturbs cell cycle progression, interferes with chromosome alignment, causes predivision and thus may predispose mammalian oocytes to errors in chromosome segregation. For the first time, chromosome-specific behaviour is reported in female meiosis in response to exposure to an aneugenic chemical.

Introduction

Fidelity of chromosome segregation may be related to chromosome-specific features such as chromosome size (Hieter *et al*., 1985), presence of heterochromatin (Fauth *et al*., 2000), size of centromere (Ford and Lester, 1982), chromatin condensation (e.g. decondensation of heterochromatin; Vig and Willcourt, 1998), size of kinetochore (e.g. mean surface area; Cherry *et al*., 1989), kinetochore constitution (e.g. centromere proteins; Nath *et al*., 1995), telomere length (e.g. differential rate of telomere shortening; Surralles *et al*., 1999) and replication timing of α-satellite sequence or the whole chromosome. In consequence, chemical exposure is likely to preferentially interfere with the behaviour and segregation of individual chromosomes, depending on the cellular target and mode of chemical action. Examples of differential sensitivity of individual chromosomes to non-disjunction or lagging in response to selected aneugenic chemicals have been reported for mitotically dividing somatic cells (see for example Xi *et al*., 1997; Bourner *et al*., 1998; Aly *et al*., 1999; Migliore *et al*., 1999), supporting the notion that mechanisms leading to aneuploidy may be either chromosome dependent or compound and dose related (Migliore *et al*., 1999).

Unlike mitosis, which may give rise to sporadic, local or mosaic aneuploidy in the individual, germline abnormalities will give rise to constitutional aneuploidy throughout the body. Numerical chromosomal aberrations cause a reduction in the developmental potential of the embryo and pose a high risk for spontaneous abortion, as well as physical and mental retardation of the affected individual in the human (for reviews see Eichenlaub-Ritter, 1996, 1998, 2000). Trisomy based on errors in chromosome segregation in oogenesis preferentially involves chromosomes with a particular recombinational history and is differentially correlated with maternal age (Jacobs and Hassold, 1995; Eichenlaub-Ritter, 1998). Chromosome-specific probes for the most common trisomies are usually employed in human preimplantation diagnosis with fluorescence *in situ* hybridization (FISH) to assess the chromosomal constitution of oocytes and embryos (Gianaroli *et al*., 1999; Verlinsky *et al*., 1999), but it is not clear whether they are the most sensitive and best markers for aneuploidy in preimplantation embryos (Bahce *et al*., 1999; Mahmood *et al*., 2000). The mechanisms responsible for specific susceptibilities of individual chromosomes to errors in segregation during oogenesis are still unknown (Warburton and Kinney, 1996; Lamb *et al*., 1997; Eichenlaub-Ritter, 1998). Even less information exists on differential susceptibility of individual chromosomes to chemically induced aneuploidy in female meiosis, which might contribute to high levels of numerical chromosomal aberrations in human embryos. The role of genetic components in susceptibility to aneugenic chemicals in meiosis is clearly an area where additional research is needed (Preston, 1996).

Diazepam (DZ) [7-chor-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine-2(3)*H*-on], a tranquilizer (valium), anticonvulsant, muscle relaxant and psychostimulant (Byck, 1975), has been shown to interfere with chromosome segregation in a number of cell types and cell lines when present in the culture medium (see for example Natarajan, 1993; Warr *et al*., 1993; Izzo *et al*., 1998). There has been some debate about the ability of DZ to increase aneuploidy rate *in vivo* (Miller and Adler, 1992; Leopardi *et al*., 1993; Marchetti *et al*., 1994; Parry *et al*., 1996). However, a recent study on the chromosomal constitution of sperm in DZ-poisoned patients surviving suicide or patients and experimental animals exposed constitutionally to the drug provided compelling evidence that DZ is in fact a meiotic aneugen (Schmid *et al*., 1999; Baumgartner *et al*., 2001). Previous work from our group (Yin *et al*., 1998a) has provided evidence that a high concentration of DZ (25 μg/ml medium) also affected meiotic maturation and fidelity of chromosome segregation in mammalian oocytes maturing

[2]To whom correspondence should be addressed.

in vitro. Thus, DZ interfered with the association of mitochondria with the spindle apparatus during meiotic prophase and induced a pronounced delay in oocyte maturation. After 16 h maturation, when the majority of untreated control oocytes had emitted a polar body and reached metaphase II of meiosis, a high percentage of the DZ-exposed mouse oocytes were still in meiosis I (Yin *et al*., 1998a).

DZ appears to affect the integrity of the centrosomes and centriolar separation (Andersson *et al*., 1981; Gassner and Adler, 1996; Izzo *et al*., 1998), thus causing monopolar or multipolar spindle formation in somatic and male meiotic cells (Gassner and Adler, 1996; Izzo *et al*., 1998). In contrast, most of the DZ-exposed oocytes had a typical barrel-shaped bipolar and anastral spindle with flat poles, although spindles were sometimes asymmetric (Yin *et al*., 1998a). In those oocytes maturing to metaphase II in the presence of DZ hyperploidy increased significantly (Yin *et al*., 1998a). The presence of univalent chromosomes in spread, meiotically delayed meiosis I oocytes and of single and multiple chromatids in metaphase II spread cells suggested that DZ induced predivision of homologues and chromatids when oocytes became exposed to the tranquilizer throughout maturation *in vitro* (Yin *et al*., 1998a).

The potential of DZ to affect chromosome congression, as indicated by our previous work, raises an obvious question as to whether DZ randomly interferes with the process of chromosome alignment. This question is critical when considering surveillance of environmental exposures (Warburton and Kinney, 1996) and the expression of checkpoint controls in mitosis and meiosis. We noticed that chromosomes were often located away from the spindle equator in DZ-treated oocytes. Spindle formation and chromosome alignment are dynamic events and the long meiotic prometaphase I in oocytes is characterized by polymerization of multiple microtubule asters. Interactions between chromosomes/kinetochores and multiple organizing centres participate in bipolar spindle formation. Oscillation of bivalents between poles of the bipolar spindle are typical for prometaphase I stages. However, from ~8–9 h maturation onwards metaphase I and II oocytes usually have well-aligned chromosomes assembled at the equatorial plane of the spindle. Displacement of chromosomes from the equator of mitotic and male meiotic cells may trigger reactions downstream of which activation of the anaphase-promoting complex (APC) and anaphase progression are inhibited in mitotically dividing cells (for reviews see Waters *et al*., 1999; Gardner and Burke, 2000). In contrast, displaced chromosomes in the oocytes of trichlorfon-exposed mouse oocytes did not result in maturation arrest (Yin *et al*., 1998b). Therefore, checkpoints sensing unaligned chromosomes may be rather permissive in mammalian oocytes. Failure to align properly at the equator during meiotic metaphase I may therefore present a particular risk factor for non-disjunction and aneuploidy.

In view of the most commonly used chromosomal probes in preimplantation diagnosis in the human, it appears essential to see whether information derived from studying one aneuploidy e.g. by FISH with specific probes, can be extrapolated to the rest (Warburton and Kinney, 1996). Therefore it appeared important to determine whether individual chromosomes in oocytes differed in sensitivity of their response to a chemical exposure. Unlike maternal age-related aneuploidy in human oocytes, which may affect over 30% of all oocytes (Griffin, 1996; Verlinsky *et al*., 1999; Gianaroli *et al*., 1999; Munne *et al*., 2000), the relative rate of chemically induced errors in chromosme segregation in oocytes of experimental animals is usually low (e.g. 5.5% in mouse oocytes matured in the presence of 25 μg/ml DZ; Yin *et al*., 1998a). To detect a preferential non-disjunction event for any of the 20 chromosomes in the mouse would require an analysis of hundreds or thousands of oocytes. We therefore employed spindle immunofluoresecence in combination with FISH with chromosome-specific probes instead of conventional cytogenetic analysis. Instead of conventional cytogenetic analysis in the current study to detect chromosome-specific behaviour in mouse oocytes maturing *in vitro* in the presence of the tranquilizer DZ. The high susceptibility of the X chromosome to malsegregation suggests that this chromosome represents a convenient marker for studies on aneuploidy induced by environmental agents. However, since it has been suggested that aneugenic chemicals may differentially affect specific chromosomes in relation to their specific mechanisms of action (Xi *et al*., 1997), other chromosomes should be simultaneously analysed to increase the sensitivity and reliability of the analysis. We therefore selected a probe specific for chromosome 16, which contains regions of homology/synteny to human chromosome 21 (see for example Reeves *et al*., 1998), besides those for the X chromosome in our study. Furthermore, the behaviour of chromosome 8 was assessed with a pancentromeric rather than a chromosome painting probe. We analysed the frequency of displacement of individual chromosomes/chromatids from the spindle equator in DZ-delayed meiosis I oocytes. Furthermore, we also explored possible chromosome-specific susceptibility of two automosomes, chromosomes 8 and 16, and the X gonosome to segregate precociously prior to anaphase I in response to DZ.

Materials and methods

Chemicals

DZ (CAS 439-14-5; a gift from Roche) was from the same source as used in a previous study of chemically induced aneuploidy in mouse oogenesis and spermatogenesis (Yin *et al*., 1998a; Baumgartner *et al*., 2001). It was disolved in 100% dimethylsulphoxide (DMSO) (Sigma, Deisenhofen, Germany) and diluted to a final concentration of 25 μg/ml in M2 medium shortly before use. The final concentration of the solvent in M2 medium was 0.5%. Previous work has confirmed that solvent alone has no influence on maturation or aneuploidy in mouse oocytes (Yin *et al*., 1998a).

Animals and oocyte cultures

For the study MF1 outbred mice were used since they possess a large follicle pool. Animals were obtained from Harlan and housed in our animal facilities under a 12 h light/dark cycle with food and water *ad libitum*. Oocytes were isolated from the ovaries of 10–16-week-old virgin MF1 mice on the day of diestrous of the natural, hormonally unstimulated cycle (Eichenlaub-Ritter and Betzendahl, 1995). Animal breeding, isolation and culture of oocytes in M2 medium were all carried out according to previously described procedures (Eichenlaub-Ritter and Boll, 1989; Eichenlaub-Ritter and Betzendahl, 1995).

Treatment with DZ

In each experiment oocytes from large antral follicles with an intact nucleus (germinal vesicle, GV) were isolated from ovaries of mice within 30 min for individual groups so as to obtain good developmental synchrony. Oocytes of the controls and the DZ group were then cultured in M2 medium in parallel for 16 h. In the DZ group 25 μg/ml DZ in M2 medium was present throughout the culture period. Experiments were repeated three times. Maturation *in vitro* was considered normal when at least 65% of oocytes in the control extruded a first polar body within 16 h of culture (Eichenlaub-Ritter and Betzendahl, 1995; Figure 1).

Analysis of cell cycle progression

Cell cycle progression was analysed under a stereo microscope by determing numbers of meiotically incompetent or G_2 arrested oocytes with an intact nucleus (GV), those which had resumed maturation and resolved their nucleus (germinal vesicle breakdown; GVBD) and those that had progressed to second meiosis after undergoing cytokinesis and extrusion of the first polar body

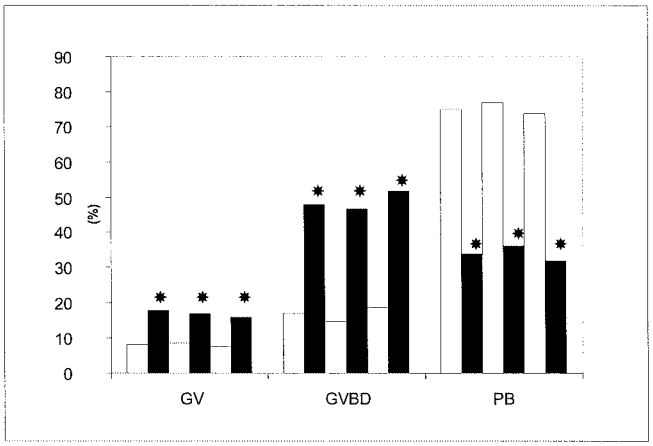

Fig. 1. Cell cycle progression in oocytes exposed to 25 µg/ml DZ after 16 h maturation in M2 medium. GV, dictyate stage arrested oocytes with intact germinal vesicle; GVBD, oocytes resuming maturation and undergoing germinal vesicle breakdown; PB, oocytes maturing to meiosis II and emitting a polar body. Open bars represent data for controls, black bars for DZ-exposed groups in three separate experiments (left to right). Numbers of oocytes in each of the three experiments for the control and DZ-exposed groups were 397, 278, 257 and 490, 594, 496, respectively. *Significant difference between control and DZ-exposed group, $P < 0.01$.

after 16 h culture (Figure 1). Furthermore, oocytes which had become parthenogenetically activated, containing one or several pronuclei, were recorded.

In vivo analysis of spindle formation

Oocytes of controls and the DZ group were viewed with a Nikon Photomicroscope equipped with a heated stage, polarizing optics and a computer-based image processing facility (Pol-Scope/Spindle View TM; Liu *et al*., 2000), kindly provided by MTG (Altdorf, Germany).

Analysis of chromosome behaviour: indirect anti-tublin immunofluorescence

Only those DZ-exposed oocytes arrested or delayed in meiosis I which had resumed maturation and undergone GVBD but failed to emit a polar body were used for the indirect anti-tubulin immunofluorescence and FISH study. Preparation of oocytes for immunofluorescence followed methods described elsewhere (Eichenlaub-Ritter and Betzendahl, 1995). Briefly, the zona pellucida was removed from oocytes by pronase digestion and gentle pipetting and the oocytes were then extracted in a 37°C microtubule-stabilizing buffer containing non-ionic detergent and glycerol. Extracted oocytes were attached to poly-L-lysine-coated slides and fixed briefly in –20°C methanol. To visualize the meiotic spindle, the slides were processed for immunofluorescence by exposure to a monoclonal anti-α-tubulin antibody (Sigma) for 45–60 min at 37°C, followed by several washes in phosphate-buffered saline (PBS) and reaction with a secondary, FITC-conjugated antibody (Sigma) for 60 min at 37°C (Eichenlaub-Ritter and Betzendahl, 1995; Soewarto *et al*., 1995). Chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI) (10 µg/ml PBS) and spindles examined prior to further processing for FISH.

Spindle formation was imaged using an Axiophot microscope (Carl Zeiss, Jena, Germany) equipped with filters for FITC and DAPI fluorescence. The number of oocytes with a bipolar or aberrant spindle, the number of unaligned chromosomes in bipolar meiosis I spindles and the number of oocytes with one or several unaligned chromosomes were recorded. Oocytes which were still arrested in the circular bivalent stage without bipolar spindles and those in early prometaphase I, where no equatorial plate with already aligned chromosomes was obvious, were excluded from the analysis. Only those oocytes were evaluated in which a large number of chromosomes had congressed at the spindle equator, defining an equatorial plane, and in which one or several displaced chromosomes were spatially separated from those aligned in the equatorial plane of the spindle. Each oocyte with an abnormal spindle and/or abnormal localization of chromosomes was recorded with a CCD camera (SensiCam; PCO CCD Imaging, Kelheim, Germany). Those oocytes with bipolar spindles possessing unaligned chromosomes were immediately processed for FISH. Since few oocytes of the control were arrested at meiosis I and of these only a low percentage exhibited chromosome displacement, they were not included in FISH analysis since low numbers of cells will preclude that the relative behaviour of one versus the other two chromosomes may be evaluated statistically.

Analysis of chromosome behavior: FISH with chromosome-specific probes

After removal of coverslips, slides with spindles and unaligned chromosomes were fixed in 1% formaldehyde containing 50 mM $MgCl_2$ for 5 min at room temperature. After washing in PBS (pH 7.0) preparations were digested with prewarmed 100 mg/ml RNase A in 2× SSC at 37°C for 15 min, followed by immersion in PBS and dehydration in an ethanol series (70, 90 and 100%) and air drying. Slides were then briefly digested with 0.1 mg/ml pepsin in 10 mM HCl (7 min, 37°C) and washed again in PBS. Finally, preparations were dehydrated and air dried again as above.

Whole chromosome-specific painting probes for the X chromosome (AGS, Heidelberg, Germany) and chromosome 16 (Cambio, Cambrige, UK), as well as a pancentromeric chromosome 8-specific probe (Boyle and Ward, 1992), were used in the study. Chromosome 8 (clones 84a and 85e; Boyle and Ward, 1992) was transformed in *Escherichiacoli* XL1-blue. DNA was isolated with a Qiagen Plasmid Maxi Kit (Qiagen, Chatsworth, MD) and labelled with biotin-dUTP (Boehringer, Mannheim, Germany) using the Gibco Nick Translation System (Boehringer). The labelled probe was mixed with Master Mix 2.1 (55% formamide, 10% dextran in 1× SSC).

Standard methods of hybridization were employed with some modifications according to the manufacturer's protocol or previous reports (Sun *et al*., 2000). In all, 10 µl of either probe specific for chromosome X, chromosome 16 or chromosome 8 was used for each slide. Probes were denatured at 70°C for 5 min, 65°C for 10 min and 75°C for 5 min, respectively, and were then incubated at 37°C for 30 min, 37°C for 70 min or on ice. The target DNA was denatured with 13 µl of denaturation mixture (50% formamide, 10% dextran in 2× SSC) for each slide at 70°C for 5 min on a hot block. Denaturation was stopped with 100% ice-cold methanol. Slides were dried on a hot block at 37°C for 3 min prior to application of probes. Finally, denatured probe was added, slides were covered and sealed and hybridization was carried out in a moist chamber at 37°C (chromosome X), 42°C (chromosome 16) or 37°C (chromosome 8) overnight.

Post-hybridization washing was the same for all probes and consisted of two steps: 15 min in prewarmed 50% formamide (2× SSC, pH 7.0) and 10 min in two changes of prewarmed 0.1× SSC at 45°C. After washing, the preparations were blocked in 0.5% blocking reagent (Boehringer) in 4× SSC at 37°C for 30 min. The biotinylated probes were detected with Cy3-conjugated avidin (Biotrend, Köln, Germany) and the signals amplified once by means of biotin-conjugated anti-avidin IgG from rabbit (Biotrend). Chromosomes were once more stained with DAPI (10 µg/ml PBS). After addition of an antifade solution of 1,4-diazobicyclo-octane (DABCO) in PBS (2 mg/ml PBS containing 20% glycerol; Sigma) slides were covered, sealed and stored at 4°C in the dark.

Analysis of chromosome behaviour: microscopic analysis and statistics

Microscopic analysis was performed using an Axiophot microscope (Carl Zeiss, Jena, Germany) equipped with filters for Cy3 and DAPI fluorescence.

Efficiency of labelling was high for each of the probes. Over 90% of oocytes selected for processing for FISH contained a specific signal(s). According to the number and orientation of signals in each oocyte, behaviour of chromosomes was assessed. The labelled chromosomes were thus aligned properly at the spindle equator or displaced from it. Separation status was assessed on the basis of the number of signals. With only one signal homologues were recorded as paired in a bivalent, as expected for meiosis I. When two spatially separated signals were seen for the painting probes homologues had precociously separated into their two univalents (predivision). The pancentromeric probe for chromosome 8 was always associated with two distinct spots, even in intact bivalents. These were usually facing opposite poles and located apart from each other on a straight line parallel to the long axis of the spindle. Only when these signals were displaced from each other in an oblique fashion and for more than the chromosome length or clearly associated with distinct chromosomes in the spindle halves was chromosome 8 considered to have separated precociously. FISH-labelled bivalents or univalent chromosomes were either located at the equator of the spindle, together with the majority of the other chromosomes (aligned), or located in one or other spindle half or entirely outside the spindle (unaligned). Typical examples of chromosome behaviour are shown in Figures 3 and 4. Each oocyte with displaced and/or predivided chromosome X, 16 or 8 was recorded.

The absolute number of displaced chromosomes was analysed in all labelled oocytes of each group and compared between groups and between groups and expected rates of displacement calculated on the basis of random behaviour. The incidence of displacement according to random behaviour was calculated by dividing the number of all displaced chromosomes by 20 (assuming that each of the 19 autosomes and the X chromosome bivalent had the same probability of failing to congress) or by dividing the total number of displaced chromosomes by 40 (assuming that some or all of the bivalents had precociously separated into their two individual homologues). Statistical

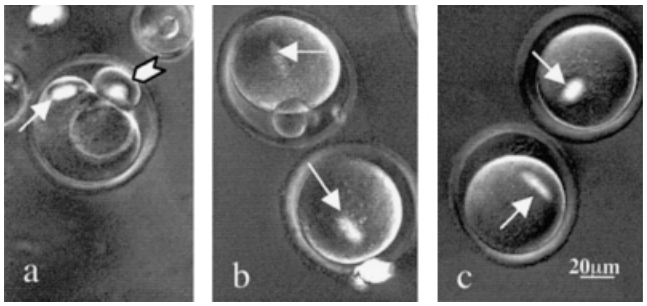

Fig. 2. Spindles in living oocytes observed with digital PolScope optics. (**a**) Birefringent spindle apparatus in control oocyte (arrow) and its first polar body (arrowhead) after *in vitro* maturation for 16 h. (**b**) Two oocytes of the DZ-exposed group with polar bodies and birefringent, bipolar spindles (arrows) in the oocyte cytoplasm. (**c**) Two meiosis I arrested or delayed oocytes of the DZ-group with bipolar, barrel-shaped and birefringent spindle apparatuses (arrows).

comparison between groups and expected and observed incidence of displacement was done using the χ^2 test with Fisher correction.

Results

Cell cycle progression of mouse oocytes in the control and experimental groups

The female mouse reaches sexual maturity at an age of 5–6 weeks. At this time each ovary contains ~10^4 oocytes in different stages of maturity. Meiotically competent oocytes isolated from large antral follicles spontaneously resume meiotic maturation *in vitro* under defined culture conditions when they are separated mechanically from the surrounding follicle and cumulus cells (Erickson and Sorensen, 1974). Accordingly, 75.0, 76.7 and 73.8% of all dictyate stage oocytes with an intact germinal vesicle obtained from the ovaries of sexually mature MF1 mice and cultured in M2 medium for 16 h emitted a polar body (Figure 1). In the presence of DZ (25 µg/ml) maturation rates were much lower, with 34.1 ± 2.05% of all oocytes reaching meiosis II. Compared with the corresponding control values, the maturation rates of DZ-exposed oocytes in three independent experiments were significantly decreased ($P < 0.01$) by a factor of 2.2, 2.1 and 2.3, respectively. Correspondingly, the number of oocytes with GVBD increased significantly ($P < 0.01$), ~3-fold compared with the controls (Figure 1). The rates of oocytes with GV consistently doubled and were significantly increased ($P < 0.01$) in the three individual experiments. There was no significant difference in maturation rate within controls or the DZ group between experiments. In support of previous observations the data suggest that 25 µg/ml DZ is a potent inhibitor of normal meiotic maturation and that DZ exposure throughout *in vitro* maturation causes cell cycle delay or arrest at the dictyate stage as well as at meiosis I.

Spindle formation and chromosome alignment in DZ-exposed oocytes

While DZ may induce monopolar or multipolar spindle formation in somatic and male meiotic cells, previous studies (Yin *et al*., 1998a) suggested that DZ interferes with meiotic progression but does not affect formation of a bipolar spindle in mammalian oocytes. In fact, when DZ-exposed, cultured oocytes in meiosis I (GVBD) or meiosis II (with polar body) were non-invasively viewed with a PolScope microscope in the present study, birefringent, bipolar structures were observed in all oocytes where a spindle was detected (Figure 2).

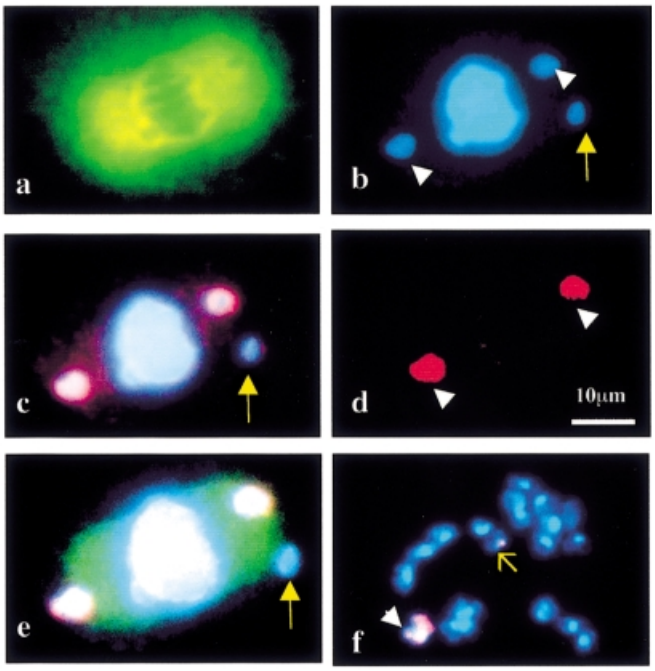

Fig. 3. Analysis of chromosome behaviour by combined FISH with an X chromosome-specific painting probe and anti-tubulin immunofluorescence to follow spindle formation and chromosome behaviour in mouse oocytes exposed to 25 µg/ml DZ during 16 h of *in vitro* maturation. (**a**) Bipolar, anastral metaphase I spindle in a DZ-arrested mouse oocyte with typical barrel-shaped, flat poles and a central plane with reduced fluorescence corresponding to the equatorial plane occupied by chromosomes. (**b**) Aligned chromosomes are in the equatorial plane. Three unaligned chromosomes, two located in each spindle half (white arrowheads) and one located beside the spindle apparatus (yellow arrow), can be recognized. (**c** and **d**) Two chromosomes which failed to congress at the equator (white arrowheads) are recognized by the X chromosome-specific painting probe, indicating predivision of the homologues. (**e**) Superimposition of the three labels suggesting that the two X homologues failed to congress at the equator but are still attached to spindle fibres, while the third unaligned chromosome (yellow arrow) is displaced without spindle attachment. (**f**) The X chromosome probe recognizing chromatin of one metaphase II chromosome (white arrowhead) and chromatin at the centromere of an additional chromosome (yellow arrow) in a spread metaphase II oocyte. Blue, DAPI stained chromosomes; green, FITC stained spindle microtubules; red, Cy3-labelled X chromosome.

Processing for indirect immunofluorescence showed that some oocytes (5.5%) were still in the circular bivalent stage or very early prometaphase I. Chromosomes were unordered and no bipolar spindle was visible in these oocytes. However, most oocytes that progressed to meiosis I in the presence of DZ had bipolar prometaphase I or metaphase I spindles (Figures 3 and 4). Nearly 100% all of the few oocytes arrested at meiosis I in controls had normal bipolar spindles and their chromosomes congressed to the equator (Table I). In contrast, about one-third of all DZ-exposed oocytes had aberrant spindles. Still, 69.2% of the 569 DZ-exposed metaphase I oocytes in the DZ group had a bipolar, normal spindle apparatus. Unaligned chromosomes were present in 31.5% of the miosis I arrested oocytes with bipolar spindles (Table I). Several prometaphase I spindles possessed prominent asters at the side of the spindle in addition to displaced chromosomes (data not shown), in accordance with previous reports (Soewarto *et al*., 1995; Yin *et al*., 1998a). Failure to congress to the equator was only rarely found in meiotically incompetent metaphase I oocytes of the controls which failed to mature to metaphase II (Table I). Only those oocytes of the DZ group containing unaligned chromosomes were further processed for

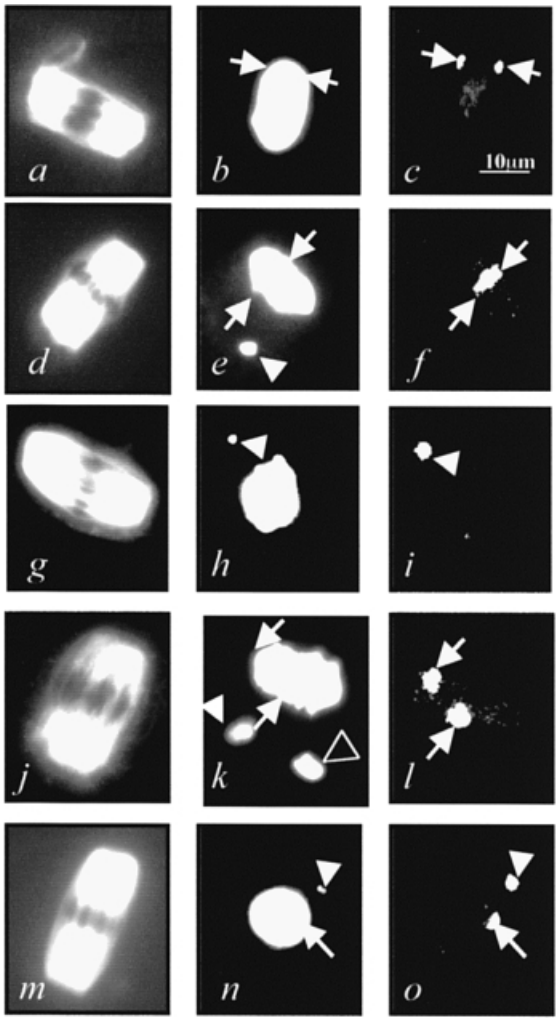

Fig. 4. Spindles and chromosome behaviour in DZ-exposed meiosis I oocytes as recognized by a chromosome 16-specific painting probe and a pancentromeric probe for chromosome 8 of the mouse. Bipolar, barrel-shaped meiosis I spindle (**a**) with well-aligned chromosomes (**b**) and orientation of the centromeres of the two chromosome 8 homologues to opposite spindle poles (arrows in b and **c**). Bipolar meiosis I spindle (**d**) with one unaligned chromosome (arrowhead in **e**) and well-aligned chromosomes including the bivalent of chromosome 16 (arrows in e and **f**). Normal metaphase I spindle with one displaced chromosome (arrowhead in **h** and **i**) reacting with the chromosome 16-specific probe in FISH. Bipolar metaphase I spindle of a DZ-exposed oocyte with separated, aligned homologues of chromosome 16 (arrows in **k** and **l**) and two displaced chromosomes, one within one spindle half (solid arrowhead in k) and another chromosome outside the spindle (open arrowhead in k). Bipolar metaphase I spindle (**m**) with one displaced chromosome (arrowhead in **n** and **o**), recognized by the chromosome 8-specific pancentromeric probe (o), in addition to an aligned chromosome (arrow in n and o), suggesting predivision and failure of congression of one homologue. (a), (d), (g), (j) and (m), spindle immunofluoresecence; (b), (e), (h) and (k), DAPI stained chromosomes in respective oocytes; (c) and (o), FISH with pancentromeric chromosome 8-specific probe; (f), (i) and (l), FISH with chromosome 16-specific painting probe. Bar in (c) for (a)–(o) 10 µm.

Table I. Analysis of spindle formation and chromosome alignment in meiosis I oocytes after 16 h maturation in the presence of 25 µg/ml DZ

	No. of oocytes	Spindle		Chromosomes	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	126	122 (96.8)	4 (3.2)	118 (93.7)	8 (6.3)
DZ group	569	394 (69.2)	175 (30.8)	390 (68.5)	179 (31.5)

FISH with chromosome-specific probes. To conclude, DZ significantly affected congression of chromosomes at meiosis I, although bipolar spindle formation was observed in most meiosis I arrested oocytes.

Chromosome behaviour in DZ-exposed, meiotically delayed, in vitro maturing mouse oocytes

Analysis of oocytes processed for spindle immunofluoresecence, in which chromosomes were stained with DAPI, confirmed that DZ severely interfered with chromosome congression in first meiosis (Yin *et al*., 1998a; Tables I and II). Between one (Figure 4d–f, g–i and m–o) and five chromosomes were found away from the spindle equator in individual oocytes. In most oocytes the chromosomes recognized by chromosome-specific probes in FISH were well aligned at the equator. In these cases two distinct signals facing opposite spindle poles were seen, as shown for the pancentromeric chromosome 8-specific probe (Figure 4c). Using the chromosome 16-specific painting probe, the whole stretched bivalent was observed at the centre of the spindle with its long axis perpendicular to the spindle equator (Figure 4f). The chromosome X painting probe reacted weakly with centromeres of one additional chromosome (probably chromosome 3, according to information from the manufacturer), but the signal for the X chromosome could clearly be distinguished from the spotted appearance of the centromere staining of the other unknown chromosome (Figure 3f). All of the three chromosomes recognized by FISH failed to congress to the equator in some of the oocytes arrested in meiosis I. Displacement was seen for the whole bivalent (see Figure 4i) or for one (Figure 4o) or both (Figure 3d and e) of the two precociously segregated homologues.

Displacement affected all three chromosomes for which chromosome-specific probes were available, but with different frequencies (Table II). The X chromosome was displaced in 11 of the 69 metaphase I oocytes with one or several unaligned chromosomes. In six oocytes of 52 with displacement, chromosome 16 was located away from the spindle equator and in three of 40 oocytes with unaligned chromosomes, signals specific for chromosome 8 were non-congressed and located away from the equator (Table II). This corresponds to 10, 4.8 and 3.5% of all unaligned chromosomes for chromosomes X, 16 and 8, respectively (Table II). χ^2 analysis indicated that the X chromosome was significantly more frequently involved in displacement than expected by mere chance ($P < 0.05$). The X chromosome would be expected to have failed to congress with random displacement in only ~6–3 (5.5–2.75) of all cases. Displacement of chromosomes 16 and 8 was within the expected range. In one of the cases of displacement of chromosome 8 one signal was detected away from the equator while the other was aligned (Figure 4o). This suggests that homologues precociously segregated in this oocyte, but the univalency did not consistently interfere with congression.

In total, in the 161 oocytes a total of 320 displaced chromosomes were found. One would expect therefore that each individual bivalent would be displaced in ~16 (320 divided by 20) or the homologues would fail to congress in only 8 (320 divided by 40) cases. However, we found 20 cases in which one of the labelled chromosomes was displaced (11 + 6 + 3 cases). This excess over expectation appears mainly due to the non-random behaviour of the X chromosome. Displacement of the X chromosome was three times as frequent as that of chromosome 8. There was no significant difference

Table II. Displacement of chromosomes in metaphase I blocked oocytes after 16 h culture in the presence of 25 μg/ml DZ

	Chromosome		
	X	16	8
Oocytes with one or several unaligned chromosomes	69	52	40
Total number of unaligned chromosomes	110	125	85
Expected frequency of displacement for each chromosome	110/20 or 40 5.5–2.75	125/20 or 40 6.25–3.13	85/20 or 40 4.25–2.13
Observed incidence of displacement	11[a]	6	3

[a]Significant difference from expectation, $P < 0.05$.

Table III. Predivision (univalents, two FISH signals) of chromosomes in meiosis I arrested oocytes after 16 h culture in the presence of 25 μg/ml DZ

Chromosome	No. of oocytes	Predivision (%)
X	161	3 (1.9)
16	171	4 (2.3)
8	135	4 (3.0)

between chromosome 16 and chromosome 8 with respect to their involvement in displacement among all unaligned chromosomes seen in each experiment.

Predivision of chromosomes induced by DZ at first meiosis

The observations by Yin *et al*. (1998a) on C-banded, spread chromosome preparations of DZ-treated meiosis I and II oocytes suggested that DZ induced predivision of chromosomes (precocious resolution of chiasmata and homologue separation as well as untimely segregation of chromatids of 'functional univalents' prior to anaphase II) during first and/or second meiosis. Therefore, we used FISH labelling in the present study to see whether one or several separated signals were present in DZ-exposed meiotically delayed oocytes. In fact, we found two signals and precocious separation for the X chromosome (Figure 3), as well as for chromosome 16 (Figure 4l) and chromosome 8 (Figure 4o). Since numbers of metaphase I arrested oocytes were low (Figure 1) and displacement is an infrequent event (Table I), the few oocytes available from the control were not included in the analyis of preferential behaviour by FISH.

The cases in which two signals for the X chromosome or chromosome 8 or 16 were found are listed in Table III. More than one signal was detected in 3, 4 and 4 DZ-arrested oocytes, respectively, with at least one displaced chromosome processed for indirect immunofluoresecence and FISH with chromosome X-, 16- or 8-specific probes. Two signals were found for aligned chromosomes located apart from each other in distinct regions of the equatorial plane (Figure 4 l). In one case, both separate red Cy3 signals for chromosome X were displaced from the equator (Figure 3a–e), indicating that untimely segregation into the two homologues was associated with failure to congress at the equator. In two other cases (one for chromosome 16 and one for chromosome 8), one univalent was apparently aligned whereas the other had not congressed at the equator (Figure 4o). Frequency of presegregation in this selected group of oocytes was between 1.9 and 3% for the three chromosomes (Table III). Thus, our observations clearly show that DZ can induce precocious division of bivalents during female meiosis I and that one consequence of precocious separation may be a failure to congress at the equator. However, predivision may not necessarily result in failure to congress.

There was no significant difference in the relative frequency of predivision between the groups with respect to the involvement of an individual chromosome. The overall incidence of predivision was high, with 2.4% of all FISH-labelled oocytes possessing more than one signal (11 of 467 oocytes). In spreads of untreated metaphase I oocytes we never found univalents and in only one oocyte of the solvent control was presegregation detected (Yin *et al*., 1998a), suggesting that all precocious disjunction events in the DZ group occurred in response to the tranquilizer. We never observed more than two areas of label (chromosome X or 16) or more than two spots (chromosome 8), indicating that precocious separation of homologues in bivalents may take place during meiotic arrest or delay, while predivision of chromatids of such homologues is not triggered by DZ prior to anaphase I or meiosis II.

Discussion

Preferential behaviour of chromosomes in response to chemical exposure

The aim of the present study was to evaluate the behaviour and susceptibility of individual chromosomes to errors in segregation. Our data provide evidence that the X chromosome remained displaced from the spindle equator significantly more often than expected during DZ-induced delayed metaphase I, unlike the other two autosomes, which had roughly the same random displacement rate as expected for any chromosome. Furthermore, our observations on DZ-exposed oocytes confirm earlier findings that DZ affects meiotic progression and spindle formation in mammalian oocytes (Yin *et al*., 1998a). Moreover, we show in this study that nearly one-third of all meiosis I delayed oocytes possess aberrant spindles, although bipolar spindle formation could still occur. In one-third of oocytes with a bipolar spindle failure of chromosome congression and/or detachment and displacement of chromosomes was observed.

Observations on somatic cells have repeatedly suggested that chemically induced aneuploidy affects chromosomes at mitosis differentially (Guttenbach and Schmid, 1994; Bourner *et al*., 1998; Xi *et al*., 1997; Aly *et al*., 1999). There are several reports which suggest that the sex chromosomes are susceptible to malsegregation under many conditions. For instance, non-disjunction of the X chromosome occurred more frequently as compared with chromosomes 1, 8, 11, 17 and 18 in binucleated human lymphocytes exposed to colchicine, vinblastine or carbendazim (Marshall *et al*., 1996). A similar preferential involvement of the X chromosome in errors in segregation has been observed in response to griseofulvin and

vanadate exposure (Migliore *et al*., 1999). It has been known for many years that the X chromosome is particularly susceptible to spontaneous loss in lymphocyte cultures from aged women, a phenomenon observed both for the inactive and the active X chromosome (Hando *et al*., 1994; Catalán *et al*., 1995, 2000; Surralles *et al*., 1996). Both preferential involvement of the X chromosome in human aneuploidy and the relationship between this type of chromosome aberration and age are well documented for somatic cells (Fitzgerald, 1975; Galloway and Buckton, 1978; Ford and Russell, 1985; Nowinski *et al*., 1990; Richard *et al*., 1993). Here we present evidence that the X chromosome also exhibits a unique susceptibility to displacement in response to a chemical in meiotically dividing mammalian oocytes.

While it is the centromeres of sister chromatids which separate at mitotic anaphase, it is chiasma resolution which triggers anaphase at first meiosis. Therefore, it is not astonishing that positioning and number of chiasmata greatly influence and determine the specific risk of individual chromosomes for errors in segregation at male and female meiosis (for references see Eichenlaub-Ritter, 2000; Hunt, 2000). Achiasmatic chromosomes have a particularly high risk for non-disjunction at all maternal ages in mammalian oogenesis (for references see Eichenlaub-Ritter, 2000). The X chromosome is only infrequently achiasmatic and behaves generally like an autosome in female meiosis (Handel and Hunt, 1992). However, X-linked DNA polymorphism studies in 47, XXY and 47, XXX patients resulting from meiotic aneuploidy have demonstrated that the additional X chromosome originated from the oocyte in ~50% of cases, compatible with an error at maternal meiosis I (Jacobs *et al*., 1988; Hassold *et al*., 1990; May *et al*., 1990; Macdonald *et al*., 1994). In the mouse the X chromosome has a fairly long recombination map and on average contains one or two chiasmata (Hultén *et al*., 1995). Therefore, we did not initially expect to find any specific behaviour of this chromosome in mouse oocytes but rather were interested in the behaviour of mouse chromosome 16, with syntenic regions to human chromosome 21. Unexpectedly, the X chromosome was often non-randomly displaced, suggesting that attachment to spindle fibres was particularly susceptible to the activity of DZ in this chromosome. Displacement was not causally related to the absence of chiasmata, since only a minority of oocytes with a displaced X chromosome exhibited two signals after FISH, indicating that homologues were achiasmatic or disjoined precociously.

Recently, Hunt and co-workers reported a meiotic drive and preferential retention of the single X chromosome in the oocyte of X0 female mice during first meiosis (LeMaire-Adkins and Hunt, 2000). They suggested that the presence of an anastral spindle in oocytes and the particular mechanisms governing spindle formation and chromosome segregation in unequal division during oogenesis may contribute to the peculiar behaviour of the X and other chromosomes. They argued that failure of one or more univalents or bivalents to migrate into the polar body might, in consequence, be responsible for an excess of the trisomic rather than the monosomic condition in the embryo. Displacement of the X chromosome, suggesting a failure of proper spindle attachment as well as precocious separation, may also predispose the DZ-exposed oocytes to non-disjunction of the affected chromosomes and, as in human oogenesis, to hyperploidy at metaphase II. Together the data on mitotically dividing somatic cells as well as those on meiotic oocytes, including those of the present study, suggest that chromosomes differ in their susceptibility to spontaneous, chemically induced and age-related non-disjunction. Although we have no conclusive evidence for a non-random excess of X chromosomes in DZ-exposed, aneuploid oocytes, the results obtained in this study show for the first time that chromosomes differ in their response to chemical exposure in mammalian oogenesis.

The significance of displacement or failure of congression with respect to cell cycle and aneuploidy in oocytes

One-third of all oocytes delayed in metaphase I in response to DZ exhibited chromosome displacement. We previously speculated that disturbances in mitochondrial distribution, a possibly insufficient energy supply and reduced activity of motor proteins might be responsible for the meiotic delay upon DZ treatment (Yin *et al*., 1998a). In untreated mammalian oocytes anaphase I is usually only executed after each bivalent has established stable bipolar attachment with microtuble fibres, at the very end of meiotic prophase I, shortly before anaphase transition (Brunet *et al*., 1999). In accordance, when the spindle is depolymerized by antimitotic agents like nocodazole, there is meiotic arrest (see for example Eichenlaub-Ritter and Boll, 1989). Spindle formation is aberrant in Mlh1-deficient mice due to the absence of recombination and bivalent chromosomes. Oocytes which possess nearly exclusively univalents do not enter first anaphase (Woods *et al*., 1999). However, some oocytes of Mlh1 knockout mice escape the block in spite of a lack of chromosome congression, provided they have a critical number of bivalents. This indicates that congression failure is not a strong factor in triggering the spindle checkpoint in mammalian oogenesis. Oocytes from X0 mice, which frequently show unordered chromosomes at meiosis I, also do not block or delay maturation prior to anaphase I (LeMaire-Adkins *et al*., 1997). Since DZ interferes with the alignment of chromosomes this may be one aetiological factor in the generation of aneuploidy.

A failure to properly align can be related to depletion of or a significant reduction in availability of high energy sources needed for the activity of microtubule motor proteins involved in congression of chromosomes at the metaphase plate (e.g. CENP E; Yen *et al*., 1991), tethering the kinetochore to spindle microtubules (Lombillo *et al*., 1995; Wood *et al*., 1997) and triggering the spindle checkpoint in response to the status of chromosome capture and/or alignment (Chan *et al*., 1998, 1999; Yao *et al*., 2000; Yucel *et al*., 2000). If DZ interferes with motor protein activity this might explain why DZ induces not only spindle aberrations (Izzo *et al*., 1998) but also chromosome lagging and kinetochore-positive micronucleulei in human fibroblasts (Natarajan, 1993) and in the Cl1 cell line (Natarajan, 1993; Izzo *et al*., 1998) and significant increases in the frequency of micronucleated polychromatic erythrocytes (Leal Garza *et al*., 1998). CENP-E is also present at the kinetochore in mammalian oocytes and may participate in congression at first meiosis (Lee *et al*., 2000). Depriving ooplasm of certain motor proteins of the dynein and kinesin family produces a distinct phenotype characterized by congression failure, failure in bundling of microtubules at the poles or disordered spindle fibres (Walczak *et al*., 1998). If DZ interferes with the activity of motor proteins by disturbing the energy supply, this would be expected to result in disturbances in maturation and chromosome alignment similar to those seen in this and a previous study.

When meiotic arrest is overcome, displacement of a bivalent or a homologue is likely to lead to non-disjunction: chromosomes would migrate to the same daughter cell or would be randomly segregated, resulting in aneuploidy. In fact, a significant increase in hyperploidy has been reported in mouse oocytes matured *in vitro* in the presence of 25 μg/ml DZ (Yin *et al*., 1998a). Congression failure, unaligned chromosomes and a permissive checkpoint were also discussed in maternal age-related risks for aneuploidy in oocytes (Eichenlaub-Ritter, 2000; Hunt, 2000; Steuerwald *et al*., 2001). We found that failure in congression did not cause a major meiotic delay in first meiosis in mouse oocytes exposed to the pesticide trichlorfon (Yin *et al*., 1998b). In conclusion, displacement of chromosomes from the spindle equator, in particular when they are associated with a failure of chromosomes to attach to spindle fibres, as seen with combined FISH and tubulin immunofluorescence in the present study, are likely to predispose to errors in segregation in a chromosome-specific fashion. Further studies with other chemicals now need to be performed to assess whether the X chromosome may be a particularly sensitive marker for aneugenic activity in mouse oogenesis or whether the response seen here is mostly due to a drug-specific response.

In conclusion, we caution against performing FISH studies with only a selected number of chromosome-specific probes in the evaluation of the aneugenic potential of a chemical in mammalian oogenesis since this may lead to an underestimation of risk due to chromosome-specific behaviour and chromosome-specific susceptibility to non-disjunction and malsegregation. Conventional chromosome analysis (e.g. C-banding), comparative genomic hybridization (see for example Wells and Delhanty, 2000) or spectral karyotyping (see for example Marquez *et al*., 1998) may produce more reliable data, since errors in segregation of all chromosomes can be assessed. For retrospective studies, including analysis of spare human oocytes, and prospective studies, such as preimplantation diagnosis and chromosomal analysis of blastomeres, this may be of particular importance.

Relevance of observations for human exposure and threshold concentrations

Epidemiological studies in humans suggest that therapeutic doses of DZ pose a very low risk for loss of genomic integrity, e.g. they do not appear to influence cancer risk in man (see for example Rosenberg *et al*., 1995). The concentration of DZ inducing aneuploidy in mouse oocytes (25 μg/ml) and producing predivision exceeds plasma concentrations of DZ in the human by >1000-fold. In fact, exposure of female mice to DZ *in vivo* did not result in increases in aneuploidy in oocytes even at the highest tolerated dose when the chemical was administered once at hormonally induced resumption of maturation (Marchetti *et al*., 1994). A reduction in the number of oocytes released at the highest doses administered in the latter study may be related to the influence of DZ on steroidogenesis and endocrine disturbances. Low, chronic concentrations of DZ do not appear to influence steriod hormone levels in rodents and are therefore not expected to interfere with ovulation (Weizmann *et al*., 1997). ATP concentrations vary considerably in mammalian oocytes (Van Blerkom *et al*., 1995) and oocytes may tolerate some reduction in the availabilty of high energy products, e.g. in response to interference with mitochondria by DZ. Judging from the normal meiotic progression at 5 μg/ml DZ, this may be below the threshold concentration that interferes with cellular functions affecting the fidelity of chromosome segregation (Yin *et al*., 1998a). However, significant increases in disomy were detected in sperm of mice chronically exposed to DZ in an *in vivo* study employing FISH with chromosome-specific probes. Numbers of oocytes are currently too low to detect small increases in aneuploidy rate. Quantitative comparisons between disomy rates in mice and those in sperm of men chronically exposed to benzodiazepine indicate that the human may be at least 10 times more sensitive as compared with the rodent (Baumgartner *et al*., 2001). Although our *in vitro* and previous *in vivo* studies in rodents imply that the risks for induction of aneuploidy in oocytes of exposure to DZ at therapeutic doses may be low, further research is needed to define threshold mechanisms and the biological relevance of our observations (Aardema *et al*., 1998; Bentley *et al*., 2000; Kirkland and Müller, 2000). New studies should preferably be performed in *in vitro* models, in which large numbers of oocytes and follicles are available for dose–response studies and in which earlier, critical stages of folliculogenesis and oogenesis and not only the effects of exposure to DZ on naked oocytes resuming maturation can be examined, as during folliculogenesis and oogenesis modulation of steroidogenesis and follicle function might have indirect and adverse effects on oocyte quality and its capacity to mature to meiosis II and faithfully separate chromosomes.

Predivision as a marker for aneugenic activity

The permissive nature of the checkpoint sensing unaligned chromosomes in mammalian oocytes becomes obvious when considering the precocious separation of homologues in DZ-exposed oocytes as recognized by FISH. Two signals were found fairly frequently in DZ-exposed oocytes delayed in meiosis I and in oocytes with perfectly aligned chromosomes, as well as in those where one or several chromosomes were displaced from the equator. The conclusion from this is that chromosome displacement does not prevent chiasma resolution or anaphase I progression, at least in DZ-exposed oocytes. In contrast, chiasma resolution between homologues of one univalent does not necessarily result in instantaneous segregation of all other chromosomes. With respect to predivision, all tested chromosomes were equally susceptible to untimely resolution of chiasmata upon DZ exposure, probably because of the similar average chiasma frequencies of chromosomes X, 16 and 8 in the mouse (Hultén *et al*., 1995; Lawrie *et al*., 1995). We did not process control oocytes for FISH analysis since previous studies had shown that in none of the ~10% of controls oocytes which had apparently not reached full meiotic competence and were therefore arrested at meiosis I was a bivalent found. In only one case was predivision seen in a DMSO-exposed metaphase II oocyte (Yin *et al*., 1998a). Therefore, predivision appears to be causally related to the DZ exposure, rather than meiotic arrest *per se*. Premature homologue and chromatid segregation predispose oocytes to aneuploidy *in vivo* (see for example Mailhes *et al*., 1997, 1998). On the assumption that all chromosomes in DZ-exposed oocytes have the same chance of presegregating, up to 40% of all oocytes should possess univalents in delayed meiosis I (~2% for each chromosome). This estimated value is much higher than that reported from cytogenetic analysis of spread oocytes (8.9%) (Yin *et al*., 1998a). The discrepancy can be related to differences in the selected cell population used for analysis. While all oocytes at meiosis I, including those

without bipolar spindles and those with unaligned and aligned chromosomes, were previously cytogenetically analysed, here only those oocytes with bipolar spindles and displaced chromosomes were evaluated for predivision. Therefore, precocious resolution of chiasmata may occur more frequently in oocytes with displaced chromosomes as compared with oocytes with aligned chromosomes.

Univalents have a high risk for random segregation and non-disjunction or, instead, premature separation of chromatids at anaphase I. Both mechanisms contribute to aneuploidy in human oocytes, although predivision may be more frequent in the mouse as compared with the human (Angell *et al*., 1993; Marquez *et al*., 1998; Eichenlaub-Ritter, 2000; Hunt, 2000; LeMaire-Adkins and Hunt, 2000). Precocious separation of homologues and segregation of chromatids at anaphase I instead of anaphase II also pose a risk for secondary meiotic errors. Thus, single chromatids cannot properly orient towards opposite poles at metaphase II and may segregate randomly at the completion of meiosis II. In accordance with the present data, we frequently found single or multiple chromatids in DZ-exposed metaphase II oocytes (Yin *et al*., 1998a). Therefore, the study confirms once more that high concentrations of DZ have an aneugenic potential in oogenesis which may predispose to errors in chromosome segregation, besides severely interfering with meiotic progression and spindle formation. We never found more than two signals for any FISH probe. Therefore, segregation of chromatids during meiotic arrest in first prometaphase does not appear to occur in mammalian oocytes prior to the initiation of anaphase I.

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