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Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women

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To examine the effects of maternal ageing on the meiotic apparatus, we obtained oocytes from naturally cycling women in two age groups, including younger (aged 20–25 years) and older (aged 40–45 years) women. Using high-resolution confocal microscopy we obtained a detailed picture of the meiotic spindle and chromosome placement during various phases of meiosis. Our data revealed that the meiotic spindle in older women is frequently abnormal, both with regard to chromosome alignment and the microtubule matrix that comprise the meiotic spindle. The spindle in 79% of the oocytes from the older group exhibited abnormal tubulin placement and one or more chromosomes were displaced from the metaphase plate during the second meiotic division. In contrast, only 17% of the oocytes from the younger age group exhibited aneuploid conditions. The majority of eggs from this group possessed a well ordered, meiotic spindle containing chromosomes that were fully aligned within a distinct metaphase plate in the spindle. Chromosome management during meiosis is directed by microtubule assembly within the spindle. These data suggest that the regulatory mechanisms responsible for assembly of the meiotic spindle are significantly altered in older women, leading to the high prevalence of aneuploidy.

Key words: ageing/aneuploidy/human oocyte/meiosis

Introduction

The influence of maternal ageing on meiotic maturation in the human oocyte is poorly understood. Because of the limits in our ability to procure specimens for study, there are broad gaps in our understanding of the temporal and spatial aspects of chromosome segregation in this process. Oocyte maturation in individuals beyond prime reproductive years is often associated with an increased incidence of non-disjunction (e.g. Down's Syndrome) or aneuploidy, and a generalized increase in spontaneous abortion (Butcher and Fugo, 1967; Lanman, 1968; Hook, 1981; Angell *et al.*, 1994) Many women in the USA are currently attempting to reproduce when they are older and thus biologically less fertile (National Center for Health Statistics, 1988, 1989; Hollander and Breen, 1991). Because the uterine environment in many older women is fully competent to carry a pregnancy (Sauer *et al.*, 1990; Meldrum, 1993), it has been suspected that the reproductive failure they often experience can be attributed to the functional and structural qualities of the oocyte (Navot *et al.*, 1991).

Studies on cytoskeletal architecture of the human oocyte have focused primarily on the second metaphase (MII) of meiosis. At MII, the spindle is oriented perpendicularly to the cell surface like most mammalian oocytes (Szollosi, 1975; Egozcue, 1987) and appears to be bipolar with regard to its microtubule origins. The normal metaphase plate within the spindle is distinct, with the chromosomes in compact alignment within it (Pickering *et al.*, 1988). The meiotic spindle in the human oocyte is a highly sensitive and dynamic organelle, as exemplified by the disruption of its structure by short-term exposure to temperatures slightly below 37°C (Pickering *et al.*, 1990).

Much of the existing data on 'ageing' oocytes have been limited to animal studies focusing on postovulatory ageing (Longo, 1981; Gianfortoni and Gulyas, 1985; Eichenlaub-Ritter *et al.*, 1988b, Nogues *et al.*, 1988). While these studies are instructive regarding the biology of postovulatory oocytes, they contribute little to our understanding of oocyte function in the older individual. It is difficult to compare data regarding in-vitro ageing with true maternal ageing, particularly because many in-vitro studies examine the oocytes after prolonged culture periods. For example, Eichenlaub-Ritter *et al* (1988b) studied the meiotic spindle in oocytes from women up to 40 years of age, but examined the oocytes 48–72 h after retrieval from mature ovarian follicles. Most studies of the meiotic apparatus in the human have not documented the age of the individuals from whom the oocytes were obtained. Moreover, all specimens examined have been obtained from hypergonadotrophin-stimulated infertility patients undergoing in-vitro fertilization (IVF). The present study has circumvented these limitations by obtaining oocytes from normal, naturally cycling women from two discrete age groups. In this way we have begun to directly address the problems of meiotic malfunction during maternal ageing by examining the meiotic spindle and chromosome placement in oocytes obtained from natural cycles. This is the first study to examine spindle–chromosome relationships in oocytes from normal, naturally cycling women from younger and older age groups.

Materials and methods

Our experiments were designed to examine the meiotic spindle assembly of oocytes during natural menstrual cycles from younger (aged 20–25 years, group A) and older (aged 40–45 years, group B) women With approval from our Human Subjects Review Board, we

enlisted volunteers with a normal medical history and no history of infertility who exhibited normal menstrual cycle length (25–35 days) and possessed mid-luteal endocrine profiles consistent with normal ovulation (prolactin <20 ng/ml, progesterone >3 ng/ml, testosterone <60 ng/ml). Subjects were monitored by daily venous blood analysis for oestradiol, progesterone, luteinizing hormone and follicle stimulating hormone from cycle day 1 until ovarian follicle aspiration. Transvaginal ultrasound was used to follow the progress of the dominant ovarian follicle, beginning in the mid-follicular phase When the dominant follicle reached a mean diameter of 15–16 mm and/or the serum oestradiol concentration was >150 pg/ml, then 10 000 IU human chorionic gonadotrophin (HCG) were administered within 20 h. Follicular contents were collected 32 h after the administration of HCG using transvaginal ultrasound-guided aspiration.

Follicular contents were collected in 37°C Dulbecco's phosphate-buffered saline (Irvine Scientific, Irvine, CA, USA). The follicular aspirates were maintained at 37°C throughout the follicle aspiration procedure. When an oocyte–cumulus complex was identified, it was immediately transferred into culture medium in 4 ml culture tubes and placed in a humidified 5% CO_2 incubator. Culture medium consisted of bicarbonate-buffered human tubal fluid (HTF; Irvine Scientific) containing 1% bovine serum albumin (BSA; Sigma Chemical Co , St Louis, MO, USA) Within 15 min of follicle aspiration, the oocyte–cumulus complexes were placed in culture dishes containing 1 ml culture medium and returned to the incubator ready for immunocytochemistry.

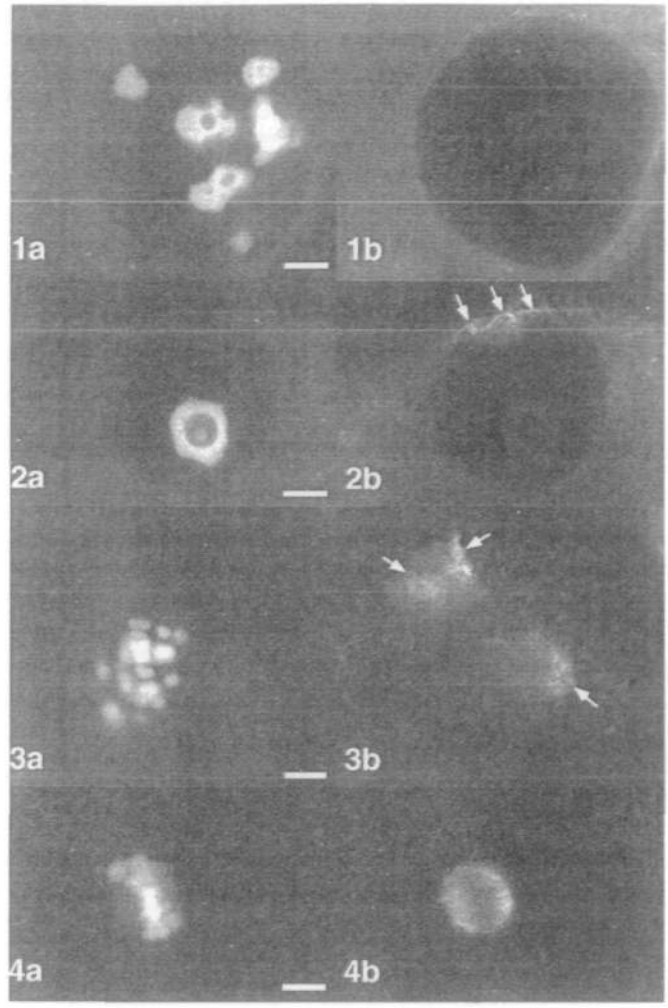
At selected time intervals the oocytes were prepared for immunocytochemistry, first by treatment of the oocyte–cumulus complex with 0 1 mg/ml hyaluronidase (Sigma Chemical Co.) in modified HTF containing 50 mM HEPES buffer and 1% BSA Hyaluronidase treatment lasted no more than 1 min, during which time the oocytes were removed from the cumulus matrix by gentle pipetting in a small-bore pipette. The oocytes were washed three times in the modified HTF buffer. The temperature was maintained at 37°C throughout the stripping procedure

Following cumulus removal, the oocytes were fixed at room temperature for 45 min in 0 1 M phosphate buffer containing 2% EM-grade paraformaldehyde and 0.01% Triton X-100. They were subsequently washed for at least 60 min in phosphate buffer containing 2% BSA. After washing, polyclonal antibodies to α-tubulin (Sigma Chemical Co.) were applied, followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma Chemical Co.). One of the final washing steps included 0.4 μg/ml propidium iodide to localize chromatin. The fixation and staining procedures were carried out at room temperature with the eggs free in suspension in small, humidified chambers to eliminate any possible adverse effects of adhering oocytes to coverslips. After staining, the oocytes were mounted in medium consisting of 50 mM triethylenediamine (Dabco, Kodak Chemical Co., Rochester, NY, USA) in glycerol/PBS, pH 8.6, to prevent rapid photobleaching.

Confocal fluorescence microscopy was used to obtain the FITC localization patterns using a Nikon Labphot microscope coupled to a Bio-Rad confocal laser Propidium iodide fluorescence was obtained simultaneously, and optical sections were collected and reproduced on a SPARC workstation Paired images were digitally reproduced to examine the co-localization of tubulin and chromatin.

Results

As the published reports have focused on examining human oocytes during the second metaphase of meiosis (MII), we selected this stage of maturation as the primary basis for comparison between oocytes from our two age groups. However, because there were no data regarding the timing of meiotic maturation in natural cycles, we needed to determine an optimal culture length in which oocytes could consistently be expected to reach MII. To accomplish this, the first nine oocytes retrieved from group A were prepared for immunocytochemistry either immediately after retrieval, when they were in prophase I of meiosis, or at intervals until they reached MII. Figures 1–4 are paired micrographs of the



Figures 1–4 Images are paired micrographs of single oocytes including propidium iodide (**a**) and fluorescein isothiocyanate (**b**) images. **Figure 1.** An oocyte prepared 33 h post-human chorionic gonadotrophin (HCG). The chromosomes in the germinal vesicle are clearly visible and randomly scattered throughout the prophase nucleus (**a**). No organized tubulin structures are visible (**b**). **Figure 2.** confocal images of an oocyte 35 h post-HCG. Few chromosomes from the germinal vesicle are visible in this plane of focus (**a**). However, the nucleation of microtubules can be seen between the germinal vesicle and plasma membrane (**b**, arrows). **Figure 3.** Paired confocal images of an oocyte 38 h post-HCG The chromosomes are closely associated with each other (**a**) and three distinct microtubule nucleation sites are present (**b**, arrows). A fourth microtubule nucleation site was visible below the focal plane of this micrograph **Figure 4.** Confocal micrographs of an oocyte prepared 43 h post-aspiration. This oocyte progressed to metaphase II and the chromosomes are arranged in a distinct metaphase plate (**a**) The microtubule matrix of the spindle is distinct and highly organized (**b**). Bar = 5 μm.

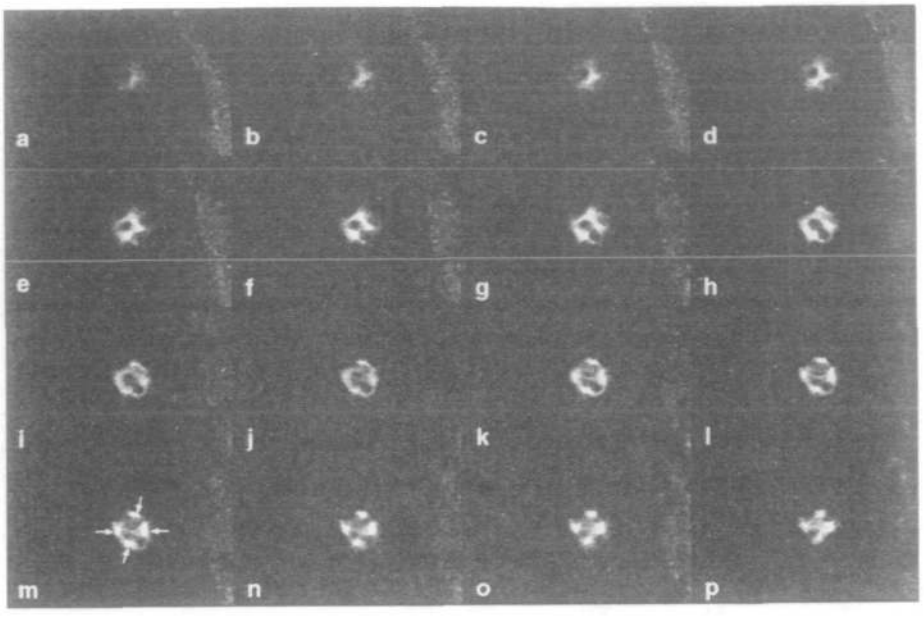


Figure 5. Human oocyte from group A stained for tubulin 40 h post-human chorionic gonadotrophin. This oocyte was prepared immediately after abstriction of the first polar body became evident. Optical sectioning was performed on the confocal microscope at 0.1 μm increments. Each image (**a–p**) represents a single focal level, beginning at the upper tangent of the spindle. Four discrete microtubule nucleation sites are visible within this spindle (**m**, arrows).

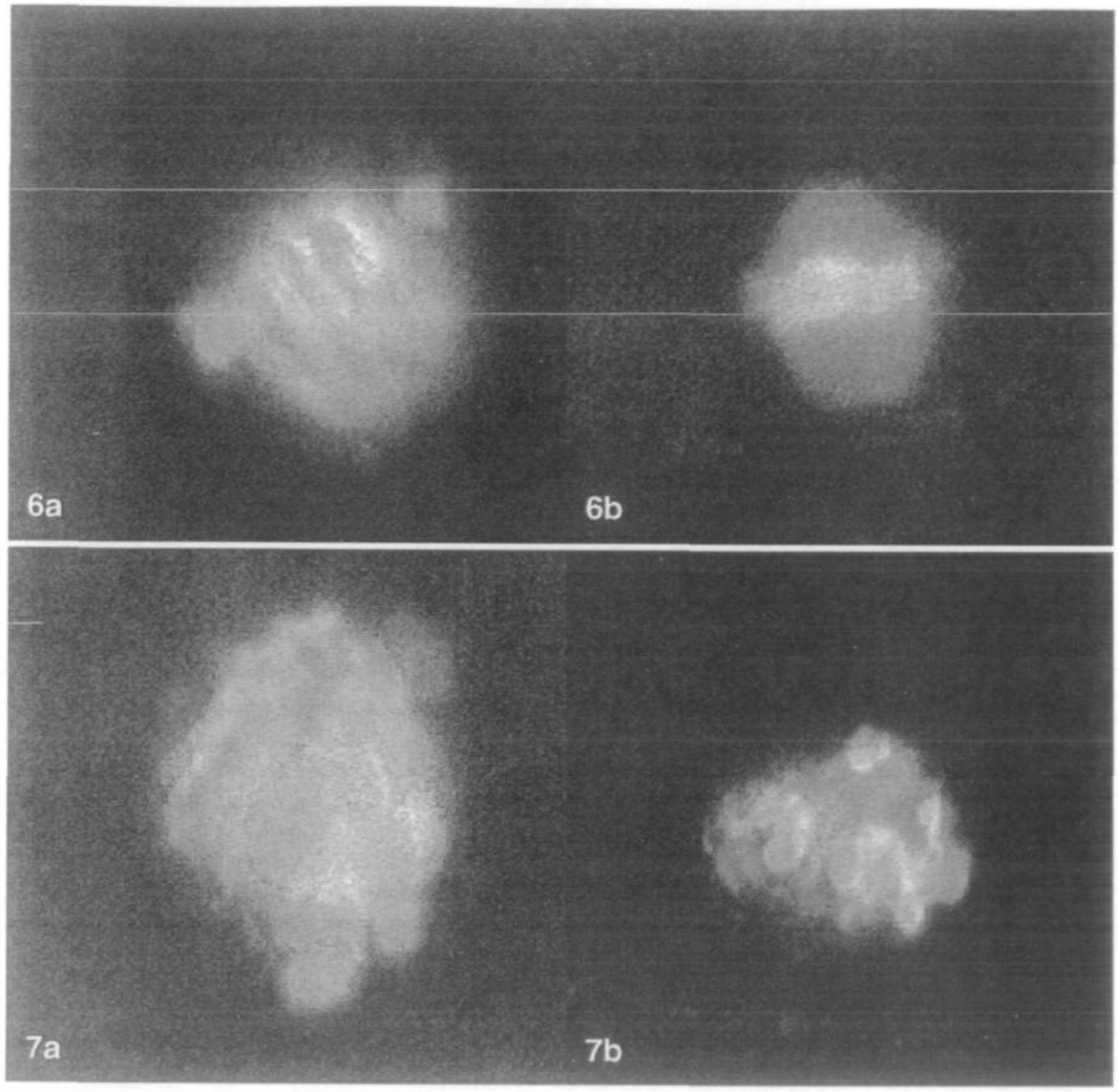
observations on the relationship between chromosomes and microtubule nucleation during meiosis. As expected, the condensed chromosomes were visible throughout germinal vesicle breakdown and became organized as the oocyte progressed to MII. Sites of microtubule nucleation were seen beginning at one pole of the prophase nucleus, which appeared to become more prominent as maturation progressed. Like animal models, it appeared that the first metaphase spindle was transient because we were unable to observe this stage. By the time the oocyte reached MII the spindle was distinct, with chromosomes aligned in the metaphase plate. These data were constrained by virtue of the number of oocytes observed and the fact that each oocyte was only observed at a single point in time. However, these observations and those made on oocytes from IVF patients (data not shown) were useful in determining that MII can consistently be expected within 44 h after HCG. However, it must be stressed that these data did not accurately establish the timing of the phases of meiosis in oocytes from naturally cycling women. Larger numbers of oocytes will be needed to accurately establish this timing. The time frames used for these observations were utilized to maximize our chances of securing oocytes at MII.

With these data we also noticed that the early MII spindle appeared to be more complex than simply a bipolar arrangement. Therefore we subjected some oocytes to optical sectioning through the spindle (Figure 5). With these observations it appeared that the early metaphase spindle was tetrapolar because four distinct microtubule nucleation centres were visible. With these data we postulate that the human oocyte recruits four microtubule organizing centres (MTOC) which eventually merge to form a bipolar spindle in the mature metaphase stage. However, the techniques employed for these experiments may not adequately preserve the delicate structure of centrosomal domains, so an accurate assessment of this hypothesis awaits further work.

The majority (26/35) of oocytes obtained from our donors were allowed to remain in culture for 12–16 h (44–48 h post-HCG) after follicle aspiration until MII was reached. In all specimens, the second polar body was visible and no oocytes were observed to suffer from prophase arrest. In the majority of the specimens from group A the chromosomes were evenly aligned within the metaphase plate and the MII spindle was uniform in shape (Figures 6 and 8). In contrast, this type of normal MII spindle morphology was seen in very few of the oocytes from group B. The majority of oocytes in group B were either abnormally shaped or disordered with regard to microtubule placement (Figures 7 and 9). Moreover, the chromosomes were not tightly aligned as in the younger group, with one or more chromosomes being considerably displaced from the metaphase plate. Using an arbitrary measure of 2–3 μm chromosome displacement from the plane of the metaphase plate, we determined that 83% of the oocytes from group A exhibited normal MII spindle assembly. In contrast, 79% of oocytes from group B were grossly abnormal with regard to chromosome placement in the metaphase plate (Table I).

Discussion

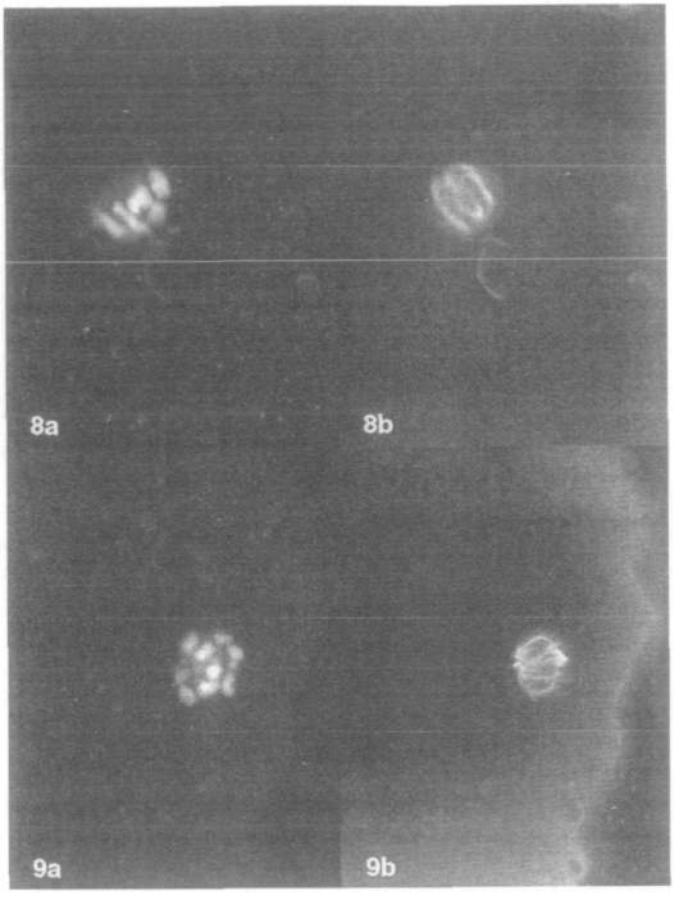
These data demonstrate the direct relationship between maternal age and meiotic spindle status in human oocytes. By obtaining oocytes from normal, naturally cycling women, we have been able to demonstrate that chromosome management during oocyte maturation appears to be linked directly to a spindle assembly process which is dysfunctional in older individuals. Because our comparisons between oocytes from young and older individuals were confined to the MII phase of meiosis, we cannot accurately extrapolate what may be occurring during the first meiotic division or other phases of meiosis. However, spindle assembly for the second meiotic



Figures 6 and 7. Composite micrographs of confocal images of the meiotic spindles in oocytes obtained from naturally cycling women in groups A and B (aged 20–25 and 40–45 years respectively). Each oocyte was stained for α-tubulin and chromatin 48 h post-human chorionic gonadotrophin and examined using confocal fluorescence microscopy. Chromatin is represented in red (propidium iodide) and microtubules in green (fluorescein isothiocyanate). **Figure 6.** (**a**) An oocyte obtained from group A (age = 22 years) at metaphase II (MII) exhibiting a uniform matrix of microtubules in the spindle and a distinct, uniform chromosome distribution within the metaphase plate. This spindle was slightly tilted, giving the appearance that the metaphase plate was closer to one of the poles. (**b**) Another oocyte from group A (age = 24 years) exhibiting similar characteristics to (**a**). **Figure 7.** (**a**) Confocal micrographs of the MII spindle in an oocyte obtained from group B (age = 40 years) that was stained for α-tubulin and chromatin 48 h after human chorionic gonadotrophin administration. Note that the microtubules in are in disarray compared with the oocytes in Figure 6. A distinct metaphase plate of chromosomes is not visible because most chromosomes were not well aligned. This spindle was slightly tilted, making it appear slightly larger than its actual size because of the broad spindle poles (**b**) Micrograph of another oocyte from group B (age = 42 years) in which a single chromosome (arrow) can be seen to be dislocated from the metaphase plate. Original magnification ×2090.

division is clearly altered in older individuals, and the variability in its structure is associated with chromosome misalignment in the metaphase plate.

It is interesting to note that the low incidence of chromosome displacement (17%) in the younger group corresponds with the incidence of chromosome aneuploidy in karyotyped oocytes from naturally cycling women (Gras *et al.*, 1992). The high rate of abnormal spindles in the older group (79%) is reflective of the high rate of aneuploidy in older individuals (Angell *et al.*, 1994). It has been shown in the CBA mouse that the pole–pole distance in the meiotic spindle becomes shorter than normal during maternal ageing (Eichenlaub-Ritter *et al*, 1988a). Their spindles also appeared to suffer from considerable chromosome misalignment as age increased. The timing of critical meiotic events may be altered in these animals, leading to irregular spindle assembly and incomplete chromosome segregation (Brook *et al.*, 1984; Eichenlaub-Ritter and Boll, 1989). Meiotic spindle disorganization and non-disjunction have also been demonstrated in eggs from ageing *Xenopus* females (Mikamo, 1968). One may speculate that the causes of spindle disorder in oocytes from older individuals may be due to altered regulatory factors that produce abnormal spindle



Figures 8 and 9. Paired confocal images of oocytes, including propidium iodide staining of chromosomes (**a**) and fluorescein isothiocyanate labelling of microtubules (**b**). **Figure 8.** Micrographs of an oocyte from group A prepared for microscopy 45 h post-human chorionic gonadotrophin (HCG) administration The metaphase plates of chromosomes (**a**) are in alignment and the microtubule spindle (**b**) is uniform. **Figure 9.** Micrographs of an oocyte from group B prepared for microscopy 46 h post-HCG Numerous chromosomes can be observed to be misaligned from the metaphase plate (**a**), and the spindle (**b**) is shorter in both pole–pole distance and irregular microtubule placement compared with group A oocytes Original magnification ×570

Table I. Number of human oocytes in each age group exhibiting normal or displaced chromosomes

Group (age)	Chromosome displacement	
	<3 μm	≥3 μm
A (20–25 years)	10	2
B (40–45 years)	3	11

The measurements of chromosome displacement in the metaphase plate of the MII spindle were made from confocal images The 3 μm displacement of chromosomes was selected after carefully examining the images and represented a distinct visual departure from the plane of the metaphase plate The oocytes that were assessed to have displaced chromatin all exhibited one or more misaligned chromosomes

components and/or alter the timing of the phases of meiosis resulting in microtubule irregularities and unusual chromosome placement.

Eichenlaub-Ritter *et al.* (1988b) and Pickering *et al.* (1988) revealed that the spindles of human oocytes were sensitive to prolonged in-vitro culture and exhibited dramatically altered morphological character from the normal condition. While we did not expose our oocytes to prolonged culture, it is conceivable that oocytes from older individuals are sensitive to even the short-duration culture. Thus, our results would be reflective of altered spindle biochemistry in older individuals However, the characteristics we observed with these oocytes do not resemble the spindle morphology seen with prolonged culture, and we suspect other mechanisms are attendant rather than culture sensitivity.

The assembly mechanisms for the meiotic apparatus are unique compared with mitotic spindle formation. Unlike somatic cells, oocytes from most species do not possess centrioles which are thought to be intimately involved with microtubule nucleation in the mitotic spindle of somatic cells. In mouse (Mattson and Albertini, 1990; Messinger and Albertini, 1991) and rat oocytes (Albertini, 1987, 1992), numerous microtubule organizing centres, the centrosomes, can be found adjacent to the prophase nucleus, often called the germinal vesicle (GV). They appear to be recruited for spindle assembly as the transition from prophase to metaphase begins. These domains eventually give rise to an apparently bipolar spindle. There is compelling evidence that the appearance of centrosomal foci near the GV in the mouse oocyte is dependent upon the maturity and meiotic competence of the cell (Wickramasinghe *et al*, 1991). Our data suggest that the human oocyte may follow the same pattern of centrosome recruitment because numerous microtubule nucleation sites were visible adjacent to the GV (Figure 2). We are currently investigating the recruitment of centrosomal domains during meiotic maturation in the human oocyte and speculate that at least four such domains are required for normal spindle assembly (Battaglia *et al.*, 1996).

It has been speculated that oocytes from older women may be part of a distinct cohort of oocytes which are primarily recruited for ovulation later in life If these oocytes are intrinsically dysfunctional, it could explain the abnormal meiotic structure we observed to be so prevalent in older individuals. It has been demonstrated that oxidative stress, as related to mitochondrial function, may be integral to oocyte competency and embryo quality (Van Blerkom *et al.*, 1995) Tarin (1995) has also suggested that oxidative stress may be the basis for age-related aneuploidy in oocytes. Experiments are needed to examine the effect of reactive oxygen on spindle assembly and the biochemistry of chromosome segregation to determine if hypotheses like the free radical theory of ageing (Harman, 1956) may apply. Aside from spindle structure, the chromosomes themselves may play an integral role in the onset of aneuploidy By examining human lymphocytes, Ford and Lester (1982) have demonstrated that chromosome displacement at mitotic metaphase is affected directly by chromosome size. In an elegant study of oocytes from XO female mice, Hunt *et al.* (1995) have provided evidence that the kinetochore–chromosome complex has a profound influence on meiotic maturation and chromosome segregation Whether these relationships have a direct effect on assembly of the structure of the meiotic spindle remains to be determined.

It is conceivable that the cause of spindle disorder in oocytes from older individuals may be from exogenous cues that create a suboptimal environment for oocyte maturation. We have observed subtle differences in the endocrine environment of follicular fluid and blood serum during maternal ageing, some of which may influence oocyte maturation (Klein *et al.*, 1996a,b). Regardless of whether the 'abnormal signal' causing aneuploidy arrives from exogenous sources or is endogenous to the oocyte, subtle changes in the timing/biochemistry of spindle assembly are likely to be responsible for chromosome displacement and microtubule irregularities. Further examination of the details of all phases of meiosis will be necessary to delineate the causes of the maternal age-related phenomena we have observed.

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