Effects of low O$_2$ and ageing on spindles and chromosomes in mouse oocytes from pre-antral follicle culture

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To assess their quality, spindles were analysed in mouse oocytes from pre-antral follicle culture. High or low oxygen tension was present during the last 16 or 20 h post human chorionic gonadotrophin (HCG)/epidermal growth factor (EGF) addition. Most oocytes from pre-antral follicle culture possessed typical anastral spindles with flat poles resembling those of ovulated, in-vivo-matured oocytes of sexually mature mice, while denuded oocytes in-vitro matured to metaphase II (MII) formed significantly longer, slender spindles with pointed, narrow poles. Spindles in oocytes from follicle culture were only slightly shorter and less compact at the equator as compared with those of oocytes matured in vivo. Chromosomes were well aligned at the equator in MII oocytes obtained from follicle culture with high oxygen. Maturation rate was significantly reduced by lowering oxygen tension to 5% O$_2$. Prolonged culture and the presence of only 5% O$_2$ dramatically increased the percentage of MII oocytes with unaligned chromosomes. These observations indicate that sufficient oxygen supply and time of retrieval after initiation of resumption of maturation by HCG as well as the microenvironment and cell–cell interactions between oocytes and their somatic compartment are critical in affecting the oocyte’s capacity to mature to MII, to form a functional spindle, and to align chromosomes correctly.

Key words: ageing/oocyte/oxygen/pre-antral follicle culture/spindle

Introduction

To obtain oocytes with high meiotic and developmental competence (Cha and Chian, 1998; Moor et al., 1998; Picton et al., 1998; Bao et al., 2000) from cryopreserved, stored ovarian material of patients treated with aggressive chemotherapy (Hovatta et al., 1996; Gook et al., 1999; Newton et al., 1999) or from other infertile patients undergoing assisted reproduction treatment, appropriate in-vitro growth and maturation systems are needed (Nayudu, 1994; Bahadur and Steele, 1996; Park et al., 1997; Abir et al., 1998, 1999; Smitz and Cortvrindt, 1998, 1999; Blumenfeld et al., 1999; Newton et al., 1999). Patients of advanced age may have a realistic chance of conceiving a child when ovarian material retrieved from the ovary at an early age is stored and healthy, immature oocytes can be later matured in vitro for IVF.

Mouse models have been established to study factors essential for oocyte development during in-vitro folliculogenesis (reviewed by Hartshorne, 1997; Van den Hurk et al., 1997). By designing the appropriate conditions for follicle growth and differentiation, a large percentage of oocytes can be matured to metaphase II (MII) after reinitiation of meiosis by human chorionic gonadotrophin (HCG) and recombinant epidermal growth factor (rEGF) (Smitz et al., 1998). These oocytes are capable of normal embryonic development following in-vitro fertilization (Cortvrindt et al., 1996, 1998; Smitz et al., 1996). Using this culture system, 60% of grown oocytes fertilize, and 60% of the fertilized oocytes develop in vitro to blastocysts. This final yield is lower than from in-vivo-grown follicles from age-matched animals (100% fertilized, 60% blastocyst formation) so that culture conditions should be further improved, although live offspring were obtained after in-vitro culture of follicles from prepubertal mice. The formation of a functional spindle, completion of first meiotic division and high fidelity of chromosome segregation are essential features of a healthy oocyte (Eichenlaub-Ritter et al., 1986, 1988a,b, 1995; Battaglia et al., 1996; Eichenlaub-Ritter, 1998; Van Blerkom and Henry, 1998; Volarevic et al., 1998). Spindle morphology and chromosome alignment can be used as one indicator for the oocyte’s capacity to form a chromosomally balanced embryo. Therefore, anti-tubulin immunofluorescence has now been employed to study spindle formation and chromosome behaviour in oocytes grown and matured in pre-antral follicle culture under different conditions. Maturation to MII appears to be slower in in-vitro follicle culture as compared with in-vivo maturation (Cortvrindt et al., 1996). Prolongation of culture past 16 h post HCG addition
may further increase the yield of oocytes in MII. However, ageing of oocytes at MII may cause concomitantly a deterioration of the spindle and displacement of chromosomes from the spindle equator (Eichenlaub-Ritter et al., 1986, 1988b). To determine the optimal time for harvest of oocytes with normal spindles after reinitiation of meiosis by HCG/EGF for in-vitro fertilization, spindles and chromosome alignment were compared between mouse oocytes retrieved from culture 16 or 20 h post HCG and rEGF addition.

Under conditions set by a previously developed pre-antral follicle culture (Cortvrindt et al., 1996), follicles deteriorated rapidly, and few were able to emit a polar body (PB) and mature to MII when oxygen tension was reduced to 5% throughout the 12-day culture period (Smitz et al., 1996). Denuded oocytes isolated in HEPES-buffered, ungassed media in an atmosphere of ~20% O2 in air from antral follicles will mature to MII when placed into pre-equilibrated bicarbonate-buffered medium and cultured with 5% O2 and 5% CO2 to MII. Since oocytes cease to be directly coupled to their follicle cells by gap junctions during the final stages after initiation of resumption of maturation, the oxygen tension was lowered to 5% from the time of HCG/rEGF addition in one experimental group. It was expected that the oocytes might be protected against reactive oxygen species (ROS) generation once they resumed maturation under such conditions. The rate of meiosis reinitiation and progression to MII was then compared with the rate of maturation in the 20% oxygen group.

To assess the influence of sexual maturity of the animal and the presence or absence of follicle cells during maturation in vitro on spindle morphology and chromosome behaviour, spindles of oocytes from follicle culture were qualitatively and timely compared with those grown and matured in vivo or with those in denuded, in-vitro-matured oocytes obtained from antral follicles of prepubertal as well as sexually mature, adult female mice.

Materials and methods

**Animals/oocytes**

All mice used were F1 C57BL/6J×CBA/Ca hybrids, and were housed according to national standards. The experimental set-up is schematically in Figure 1. Oocytes in the in-vivo (MII oocytes) and in-vitro groups [in-vitro-grown (IVG) and in-vitro-matured (IVM) oocytes] were retrieved at the period of reinitiation of meiosis when they had reached MII stage.

IVG oocytes were obtained by isolation of pre-antral follicles from 12- to 14-day-old mice, after a culture period of 12 days, after which nuclear maturation was induced by HCG/EGF (Cortvrindt et al., 1996). This ovulatory stimulus caused muci-fication of the cumulus–oocyte complex (COC) and progression of meiosis in the follicle-enclosed oocyte. The IVG experiments were performed three times. To ensure identical conditions, follicles obtained from several mice were initially allocated randomly to plates and cultured in parallel. On addition of HCG/rEGF, plates were divided for further in-vitro maturation. One group of follicles was cultured as before, while the plates with follicles from the other group was placed for the last 16 or 20 h into an atmosphere containing only 5% O2 (while oocytes were still included in their follicle). The analysis at 16 h was selected since a timed analysis of meiosis progression had shown that maturation in follicle culture appears to be slower in vitro as compared with in-vivo conditions, and a relatively large number of oocytes had emitted a PB by this time. Initial analysis of spindles indicated that some oocytes were still in the process of division (anaphase or telophase); thus, a prolongation of culture time appeared justified.

IVM oocytes were obtained from 24-day-old (prepubertal) or adult (3-month-old) hybrid females primed with 5 IU pregnant mare's serum (PMS; Intergonan, Intervet, Tönishofen, Germany). At 48 h after PMS administration, oocytes were released from large antral follicles and mechanically denuded prior to in-vitro maturation for 15 h in M16 medium (Sigma, Deisenhofen, Germany) in 5% CO2 in air (Figure 1). This group was included as a reference group since the oocytes matured without follicle cells. In our experience oocytes of this strain reach MII at about 12 h past isolation, and do not

![Figure 1](image-url)
exhibit any sign of ageing, such as detachment of the spindle from the cortex or deterioration of spindles when processed for fluorescence at 15 h of culture.

The in-vivo controls (MII oocytes, Figure 1) consisted of ovulated oocytes isolated from the ampulla either of 26-day-old or sexually mature, young adult (3-month-old; adult) hybrid mice after priming with 5 IU PMS and administration of 5 IU HCG (Predalon®, Organon, Oberschleißheim, Germany) 48 h later and oocyte retrieval 14 h later. A time of 14 h corresponds to the optimal time when oocytes of this strain are suitable for in-vitro fertilization. The analysis on IVM and MII oocytes was carried out on oocytes obtained from at least five independent experiments performed independently in two laboratories.

**Conditions of pre-antral follicle culture and media**

Pre-antral (100–130 µm) follicles obtained from 12- to 14-day-old hybrid mice were isolated in L15 medium + 10% fetal calf serum (FCS; Gibco, Eggenstein, Germany) + penicillin/streptomycin and cultured individually in 20 µl droplets (1 follicle per droplet) of α-minimal essential medium (α-MEM with glutamax; Gibco) with 5% FCS, and with 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS; Sigma). In addition, 100 µl/ml recombinant FSH (rFSH; kindly donated by Ares Serono, Geneva, Switzerland), and 10 µIU/ml of recombinant LH (LH; Ares Serono) was present in culture medium, according to previously established conditions (Cortvrindt et al., 1996, 1998). On each second day, 10 µl of culture medium from each drop was removed and replaced by freshly prepared pre-equilibrated medium (Cortvrindt et al., 1996). On day 12 of culture, half of the volume of the culture droplet was removed and replaced by the same culture medium in which HCG/EGF was added. This caused mucleation of the follicle-enclosed cumulus and progression of meiosis in its oocyte. The COC were collected at the preset time point (16 or 20 h), and the cumulus cells mechanically removed by pipetting the COC gently in and out through a pre-pulled glass pipette.

**Indirect anti-tubulin immunofluorescence (spindle and chromosome staining)**

The preparation of oocytes for indirect immunofluorescence was carried out (using the same conditions and protocols) independently by two laboratories and different investigators. Results were matching, and there was no significant inter-laboratory or inter-operator variation. IVM and in-vivo-matured MII oocytes from young and adult females were processed repeatedly, and in parallel. For practical reasons, oocytes from follicle culture were processed for indirect immunofluorescence separately from IVM or in-vivo-matured MII oocytes using identical protocols for extraction and fixation. The different groups (low and high oxygen tension) were processed in parallel, and those matured for 16 h and 20 h on the same day. There was no significant inter-experiment variation.

Processing of oocytes for anti-tubulin immunofluorescence was carried out as described previously (Eichenlaub-Ritter and Boll, 1989; Eichenlaub-Ritter and Betzendahl, 1995). All procedures were carried out on a heated stage. In short, the zona pellucida was digested with carboxypeptidase X (Fluka, Buchs, Switzerland) 60 min at 36°C. The COC were collected at the spindle stage and replaced by the same culture medium in which HCG/EGF was present in culture medium, according to previously established conditions. Since oocyte cytoplasm was extracted before fixation, the spindle usually became attached with its long axis to the slide, and measurements of pole-to-pole distance did not require three-dimensional reconstruction of optical sections with this fixation protocol, as is necessary with most aldehyde-based fixation procedures. After dehydration in phosphate-buffered saline (PBS; Oxoid, Basingstoke, Hampshire, UK), slides were incubated with the first antibody (monoclonal mouse anti-alpha-tubulin antibody (Sigma; diluted 1:400 in PBS), followed by the second antibody [fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse antibody; Sigma], and the chromosomes were stained with 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma). Finally, slides were immersed in PBS with 20% glycerol containing 2 mg/ml dianinobicyclooctocane (DABCO; Sigma) as antifade. The slides were viewed with a Zeiss Axioskop microscope equipped with filter sets for FITC and DAPI fluorescence. Images were either recorded on Kodak T-MAX 400 negative film or Alpha-400ASA slide film. Images were later scanned and processed using Adobe Photoshop or PowerPoint software.

For confocal microscope analysis (Fluoview, Olympus, Hamburg, Germany) chromosomes were stained with propidium iodine instead of DAPI.

**Analysis of spindle size and chromosome alignment**

The centre of the two outermost microtubule organizing centres (MTOC) at each spindle pole were used to determine spindle width at poles. Figure 2 indicates how measurements were carried out using a micrometer on the fluorescent microscope, or on spindle images produced by the confocal laser microscope. Taking the middle of the imaginary line presented by the two polar plates as end points, the length of the spindle was determined. Spindle width was measured at the spindle equator and the relative quotient of spindle width at equator versus width at poles was determined as an additional parameter for spindle shape. Measurements performed independently by two investigators gave reproducible results.

Chromosome alignment was only determined in MII oocytes. Displacement of chromosomes was evaluated when an individual, whole chromosome was placed outside of the metaphase plate, but not when there was only a slight scattering of chromosomes along the spindle equator. According to this definition, spindles in which chromosomes were not forming a completely straight line at the equator were not regarded as abnormal.

**Statistical analysis**

Oocyte maturation data were compared by contingency analysis followed by χ² testing. A Student’s t-test was applied for comparison.
of means from oocyte diameters and quantitative assessment of spindle shape.

Results

Follicular development and oocyte maturation in pre-antral follicle culture

Pre-antral follicles (n = 548) were cultured for 12 days under normal oxygen tension (5% CO₂ in air). At HCG/rEGF treatment, follicles were divided into four groups for further in-vitro maturation at 20% or 5% oxygen tension for 16 or 20 h, respectively, to achieve oocyte nuclear maturation. The rate of survival of follicles in culture was high, and comparable in all treatment groups (94.2/98.2 and 95.6/95% respectively; Table I). Reduction of oxygen to 5% during the last 16–20 h of culture did not induce follicle degeneration or oocyte expulsion, as in continuous low oxygen tension throughout long-term culture (Smitz et al., 1996).

The diameters of the in-vivo-grown oocytes from 26-day or adult mice were significantly larger compared with all other experimental groups (when compared with 20% O₂ 16 or 20 h, 5% O₂ 16 or 20 h, in vitro 26-day, in vivo adult, in vitro adult; all P < 0.001). There was only a slight difference in size between the MII oocytes from adult compared with pubertal, 26-day-old mice. In-vitro-matured denuded oocytes obtained from large antral follicles of PMS-stimulated adult but not 26 day old mice were marginally but significantly larger (P < 0.001) compared with IVG oocytes, comprising the group with the smallest diameters (Table I).

The maturation rate between oocytes from experimental groups (20% or 5% O₂) in pre-antral follicle culture was significantly different (P < 0.001) after 16 h or 20 h of harvest. Comparable with earlier observations (Cortvrindt et al., 1996, 1998), nearly half of all oocytes matured continuously in normal oxygen (20%) emitted a PB. There was nonsignificant increase in the rate of oocytes undergoing cytokinesis upon final culture for 20 h instead of 16 h (Table I). Some 34.2% of oocytes had still an intact germinal vesicle (GV) at 16 h (Table I). In addition, some oocytes of the 20% oxygen group appeared to be capable of resuming maturation and undergoing GVBD later, since the percentage of oocytes with GV decreased from 34.2% to 27.5% upon prolonged culture (Table I). Finally, 49.5% oocytes emitted a PB.

Culture of follicles in the presence of only 5% oxygen for the last 16 h or 20 h resulted in a significant reduction in the numbers of oocytes emitting a PB (less than one-fifth of the high-oxygen group; P < 0.001; Table I). There was a significant difference (P < 0.05) in the number of oocytes arrested in GV stage between the 5% and 20% oxygen group at 20 h post HCG/rEGF addition, but not at 16 h post HCG/rEGF addition (34.2 versus 37.4%). This indicates that the arrest in GV stage was irreversible in the low-oxygen group, while some oocytes cultured under normal oxygen conditions were able to resume maturation at a later time after addition of hormone. Over

Table I. Follicle survival, and oocyte growth and maturation in the presence of 5% CO₂ in air (20% O₂ group) or 5% CO₂/5% O₂/90% N₂ during the last 16 h or 20 h after addition of HCG/rEGF in pre-antral follicle culture (A), and diameter and maturation of oocytes retrieved from follicles of prepubertal (26-day) or sexually mature, adult mice, grown in vivo and matured in vivo or in vitro (B)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Oxygen</th>
<th>Time of HCG/rEGF</th>
<th>n</th>
<th>Diameter (µm)</th>
<th>Survival (%)</th>
<th>Diameter (µm)</th>
<th>Maturation</th>
<th>Aberrant/activated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCG/rEGF</td>
<td>20%</td>
<td>16 h</td>
<td>156</td>
<td>113.1</td>
<td>147 (94.2)</td>
<td>146</td>
<td>69.4d,<em>,e</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%</td>
<td>16 h</td>
<td>160</td>
<td>113.3</td>
<td>153 (95.6)</td>
<td>147</td>
<td>70.4d,e*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 h</td>
<td>119</td>
<td>112.7</td>
<td>113 (95.0)</td>
<td>111</td>
<td>69.4d,<em>,e</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maturation in vivo or in vitro</th>
<th>Age</th>
<th>n</th>
<th>Diameter (µm)</th>
<th>GV (%)</th>
<th>GVBD (%)</th>
<th>PB (%)</th>
<th>Aberrant/activated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes matured and ovulated in vivo</td>
<td>26 day</td>
<td>139</td>
<td>73.7a,b,c,e*,d,e*</td>
<td>0</td>
<td>0</td>
<td>139 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Adult</td>
<td>101</td>
<td>71a,b,c,c*,d,d*,e,e*</td>
<td>0</td>
<td>0</td>
<td>101 (100)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

| Denuded oocytes matured in vitro | 26 day | 123 | 70.4d,e* | 0 | 11 (8.9) | 123 (91.1) | 0 |
| Adult | 101 | 71a,b,c,d,d*,e,e* | 5 (5) | 5 (5) | 101 (90) | 0 |

χ² test for maturation, or t-test for diameter, significant differences:

- to 20% O₂, 16 h; P < 0.001
- to 20% O₂, 20 h; P < 0.01
- to 5% O₂, 16 h; P < 0.001
- to in vivo, 26 days; P < 0.001

GV = germinal vesicle; GVBD = germinal vesicle breakdown; HCG = human chorionic gonadotrophin; PB = polar body; rEGF = recombinant epidermal growth factor.
Spindles in oocytes from pre-antral follicle culture

The relative numbers of oocytes in prophase I with multiple microtubule asters and condensed chromosomes and of those with a bipolar prometaphase I spindle but still unordered chromosomes was similar at 16 h and 20 h of oocyte retrieval, while the numbers with metaphase I spindles increased (Table II). Overall, these observations suggest that some prophase I oocytes continued maturation to MII between 16 h and 20 h of culture after HCG/rEGF addition, and that some GV stages initiated spindle formation in the high-oxygen group, with a substantially delayed meiotic progression.

The adverse influence of reduced oxygen for oocyte maturation (as seen in the low number of oocytes with PB) was confirmed by immunostaining for spindles and scoring of the relative development according to spindle morphology. Thus, the number of oocytes incapable of meiotic resumption and spindle formation which still had a GV was increased in the 5% O₂ group as compared with the group matured for the last 16 h or 20 h in 5% CO₂ in air (Table II). Conversely, significantly fewer oocytes from pre-antral follicle culture matured to MII when an atmosphere with low oxygen was present during the last 16 h or 20 h of culture before oocyte retrieval (P < 0.001; Table II). There was no general shift from early to later stages in spindle formation in the oocytes from the low-oxygen group. Most oocytes of this group which had reached meiosis I at 16 h post HCG/rEGF addition appeared either irreversibly blocked in maturation, or aberrant, and possibly activated, with condensed chromat and an irregular microtubular cytoskeleton (Table II).

All IVM oocytes ovulated in 26-day-old mice and retrieved 14 h after stimulation by HCG had MII spindles. In adult mice, one anaphase I and one telophase I stage were detected among 111 ovulated oocytes. Therefore, in-vivo maturation of oocytes appears to be generally completed at 14 h after stimulation by gonadotrophin.

Among 123 IVM oocytes obtained from 26-day-old mice and matured outside of a follicle, only 9% were still in prometaphase I/metaphase I stage, while the rest (91%) had MII spindles 15 h after isolation and culture in M16 medium. Similarly, 90% of oocytes obtained from adult mice matured spontaneously in vitro after isolation from follicles possessed a MII spindle. Moreover, 5% had a GV and the rest of this group were in meiosis I. Therefore, maturation in vivo appeared to be highly synchronous and completed in most oocytes of 26-day-old mice matured in vitro for 15 h when oocytes were obtained from PMS-primed cycles.

Spindle morphology in oocytes from follicle culture and in-vitro and in-vivo-matured oocytes

Spindle morphology appeared normal in most oocytes retrieved from follicles after continuous culture in an atmosphere with 20% O₂. Oocytes in metaphase I possessed typical anastral, barrel-shaped spindles. Condensed chromosomes were congressed at the spindle equator at the end of the culture. Small, cytoplasmic asters could often be discerned (red arrowheads in Figure 3), as in ovulated MII oocytes (arrowhead in Figure 4B) oocytes possessing late anaphase I or telophase I spindles, there was no evidence for lagging of chromosomes in the equatorial, central part of the spindle in the group of oocytes matured under normal oxygen conditions (Figure 3B, C).
**Figure 3.** Spindles in oocytes from pre-antral follicle culture. (A–D) Oocytes matured for 16 h after stimulation by HCG and rEGF in presence of high oxygen (20% O$_2$ group). (E–H) Oocytes matured for the last 16 h in the presence of 5% CO$_2$/5% O$_2$/90% N$_2$. (A) Cytoplasmic asters (red arrowheads) in a maturing oocyte at metaphase I. Normal appearing spindles (green) at metaphase I (A') with well-aligned chromosomes (blue), at late anaphase I (B), and at late telophase I (C) with visible constriction of the interpolar spindle and separated chromosomes (blue). Metaphase II spindle of oocyte from pre-antral follicle culture (D) with characteristic barrel-shape, flat, broad spindle poles, a rectangular to square appearance, some cytoplasmic asters (red arrowheads) and well-aligned chromosomes (blue) at spindle equator (D'). Frequently observed prophase I spindle in an oocyte of the low oxygen group with unordered asters of microtubules (E) assembled in the vicinity of the bivalent chromosomes (E'). The latter are still in the circular bivalent configuration after germinal vesicle breakdown (GVBD), 16 h past hormonal stimulation by HCG and rEGF. Characteristic bipolar prometaphase I spindle observed in high- and low-oxygen groups (F) in which chromosomes (blue) are still in the process of congression. Tripolar telophase I spindle (green) with lagging chromosomes in the interpolar region (arrow) at 16 h after initiation of resumption of maturation (G) in oocyte of the low-oxygen group from a culture omitting LH. Oocyte of the low-oxygen group in metaphase II with normal barrel-shaped spindle (H) and unordered chromosomes (H'), with individual chromosomes located outside of the equator (arrow). Green staining = spindle immunofluorescence; blue staining = DAPI-stained chromosomes. Red arrowheads = cytoplasmic asters. Scale bar in (A) = 4 μm; scale bar in (B–H) and (A'–H') = 20 μm.

MII, most oocytes had square, anastral spindles and mostly well-aligned chromosomes (Figure 3D, D').

Oocytes in the early stages of meiosis were observed shortly after GVBD, predominantly in the group retrieved from culture at 5% CO$_2$/5% O$_2$/90% N$_2$ (Figure 3E, E'). Some oocytes of the low- (Figure 3F) and the high-oxygen groups had asymmetric bipolar spindles. Although the relative numbers of oocytes reaching meiosis II in pre-antral follicle culture with reduced oxygen was low (Table II), those which were able to progress to meiosis II all possessed anastral and bipolar spindles (Figures 3H and 4F).

Metaphase II presents the end point of development before fertilization in oocytes matured either in vivo or in vitro.
Spindles in oocytes from pre-antral follicle culture

Figure 4. Characteristic metaphase II spindles and chromosome behaviour in in-vivo-matured oocytes obtained from: adult (A, A′) or 26-day-old (B, B′) mice; in oocytes matured denuded in vitro for 14 h (C, C′) after retrieval from ovary of 26-day-old mouse (IVM-oocytes; see Figure 1); or from pre-antral follicle culture isolated 16 h (D, D′, F, F′) or 20 h (E, E′) after hormonal stimulation and matured for the last period of culture in the presence of high (D, E′) or low (F, F′) oxygen. Rectangular and compact shape of metaphase II spindle in in-vivo-matured oocytes of adult mice (A) with well-aligned chromosomes (A′). Pointed, narrow and more astral poles (B) in oocyte of 26-day-old mouse after in-vivo maturation. Long, slender spindle of an oocyte matured in vitro without follicle (C). Metaphase II spindle of oocyte from pre-antral follicle culture with polar asters and microtubule organizing centres and astral microtubules extending into the cytoplasm (D) providing for a cushion-like and slightly asymmetric spindle shape. Barrel shape of spindle in ‘aged’ oocyte after culture for 20 h post HCG/rEGF addition in presence of high oxygen (E). Relatively short and square-appearing spindle in oocyte of low-oxygen group isolated at 16 h post HCG/rEGF addition (F). Well-aligned chromosomes in metaphase II oocytes matured in vivo, or in pre-antral follicle culture (A′, B′, D′ respectively). Chromosomes of an in-vitro-matured oocyte which are slightly scattered along the equator (C′), but without displacement of an individual chromosome from the metaphase plate. Displaced chromosomes (arrows) in spindles of an ‘aged’ oocyte from pre-antral follicle culture retrieved at 20 h post HCG/rEGF addition (E′) and in an oocyte of the low-oxygen group (F′). A–F = anti-tubulin; A′–F′ = DAPI-stained chromosomes. Arrowhead in (B) = cytoplasmic asters. Scale bar in (A–F′) = 9 µm.

Table II. Stages of meiotic progression as assessed by the state of spindle formation at 16 or 20 h post HCG/rEGF addition, and unaligned chromosomes in metaphase II oocytes from pre-antral follicle culture

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Stages of meiosis</th>
<th>Alignment of chromosomes</th>
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<tbody>
<tr>
<td>Oxygen</td>
<td>Time of HCG/rEGF</td>
<td>n</td>
</tr>
<tr>
<td>20%</td>
<td>16 h</td>
<td>110</td>
</tr>
<tr>
<td>20 h</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>5%</td>
<td>16 h</td>
<td>128</td>
</tr>
<tr>
<td>20 h</td>
<td></td>
<td>62</td>
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</table>

bSignificant difference to 20% O2; P < 0.001.
Significant difference to 20% O2; P < 0.01.
*Displaced chr. in MII: displaced chromosomes in metaphase II spindles; †MII with aligned chr. of all: metaphase II oocytes with aligned chromosomes of all oocytes.
GV = germinal vesicle; HCG = human chorionic gonadotrophin; rEGF = recombinant epidermal growth factor.

When compared to spindles in the oocytes of pre-antral follicle culture (Figure 4C),

To determine whether there were consistent differences in spindle shape between IVG, IVM and ovulated MII oocytes, spindle length (X), width at the equator (Y′) and width at both poles (Y) were measured, taking the outermost centre of microtubular asters at poles as reference points (see Figure 2).
Table III. Spindles in oocytes from 26-day-old or 3-month-old (adult) mice matured in vivo to metaphase II, or matured without follicle in vitro for 16 h, as compared to spindles in oocytes obtained from pre-antral follicle culture with resumption of maturation inside of a follicle after HCG/t-EGF addition for 16 h or 20 h within an atmosphere of 5% CO₂ in air (20% O₂) or 5% CO₂/5% O₂/90% N₂ (5% O₂)

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>n</th>
<th>Length (µm) (pole to pole)</th>
<th>Width (µm) (equator)</th>
<th>Width (µm) (pole)</th>
<th>Width at equator/width at pole</th>
</tr>
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<tbody>
<tr>
<td>20% O₂</td>
<td>16 h</td>
<td>31</td>
<td>21.3 ± 1.8</td>
<td>14.2 ± 2.1</td>
<td>10.9 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>20 h</td>
<td>25</td>
<td>20.9 ± 2.1</td>
<td>16.5 ± 2.29</td>
<td>12.5 ± 1.95</td>
</tr>
<tr>
<td>5% O₂</td>
<td>16 h</td>
<td>14</td>
<td>20.7 ± 2.14</td>
<td>15.5 ± 2.29</td>
<td>11.6 ± 1.95</td>
</tr>
<tr>
<td></td>
<td>20 h</td>
<td>10</td>
<td>2.47 ± 1.3</td>
<td>14.2 ± 2.1</td>
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<tr>
<td>In vivo</td>
<td>26-day</td>
<td>108</td>
<td>24.5 ± 2.35</td>
<td>12.8 ± 1.12</td>
<td>7.6 ± 2.21</td>
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<tr>
<td></td>
<td>Adult</td>
<td>111</td>
<td>22.7 ± 1.59</td>
<td>14.5 ± 1.46</td>
<td>11.1 ± 2.0</td>
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<tr>
<td>In vitro</td>
<td>26-day</td>
<td>110</td>
<td>27.5 ± 2.30</td>
<td>12.5 ± 1.53</td>
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<td></td>
<td>Adult</td>
<td>114</td>
<td>25.9 ± 2.25</td>
<td>12.8 ± 1.61</td>
<td>6.4 ± 2.01</td>
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</table>

Values are mean ± SD.
Statistical significance: t-test, P < 0.001:
α: to 16 h, 20% O₂  
β: to 16 h, 5% O₂  
γ: to in vivo, adult  
δ: to in vitro, adult

The dimensions of spindles from in-vivo-matured and ovulated oocytes differed between adult and 26-day-old mice (Figure 4A, B). Oocytes from adult mice had very compact, barrel-shaped spindles, the width of spindles at the poles and the equator being similar. In contrast, oocytes from 26-day-old mice were on average slightly and significantly longer, and had narrower spindle equators and poles (Figure 4C). Quantitative analysis confirmed that both oocytes from adult and 26-day-old mice matured in vitro were significantly longer and had exceptionally small poles (6 and 6.4 µm respectively) when compared with those of oocytes of the adult females matured in vivo (11.1 µm) and those of IVM oocytes from follicle culture (10.9−12.5 µm; Table III). As a result, the ratio of equator width to pole width was about 2, but it was much lower (1.22−1.38) in the spindles of oocytes matured in vivo in adult mice or such from pre-antral follicle culture. The average ratio was 1.58 in oocyte spindles of prepubertal mice matured and ovulated in vivo (Table III).

Metaphase II spindles in denuded oocytes matured to MII in vitro were also examined. Initially, they appeared similar in shape to those from oocytes obtained from 26-day-old mice after in-vivo ovulation. IVM oocytes of 26-day-old or adult mice also had a rather slender shape and relatively pointed poles (Figure 4C). Quantitative analysis confirmed that both oocytes from adult and 26-day-old mice matured in vitro were significantly longer and had exceptionally small poles (6 and 6.4 µm respectively) when compared with those of oocytes of the adult females matured in vivo (11.1 µm) and those of IVM oocytes from follicle culture (10.9−12.5 µm; Table III). As a result, the ratio of equator width to pole width was about 2, but it was much lower (1.22−1.38) in the spindles of oocytes matured in vivo in adult mice or such from pre-antral follicle culture. The average ratio was 1.58 in oocyte spindles of prepubertal mice matured and ovulated in vivo (Table III).

Chromosome alignment at MII in oocytes matured within or outside of a follicle

The behaviour of chromosomes in those oocytes reaching MII after maturation in follicle culture under different conditions was also compared. In fact, most oocytes of the ambient oxygen group had well-aligned chromosomes (Figures 3D, D’ and 4D, D’; Table I). Of the 108 oocytes ovulated in vivo in 26-day-old mice, and the 111 oocytes from adult females, all (100%) had all chromosomes aligned (Figure 4B, B’). Similarly, all oocytes matured in vitro outside of a follicle from the 26-day-old mice had well-aligned chromosomes. In only 1.8% of those oocytes obtained from adult mice was a slight scattering of chromosomes at the equator detected (Figure 4C, C’); this was not scored as chromosome displacement according to the criteria used.
Similar to the situation in in-vivo-matured or denuded IVM oocytes, in only 13% of all the MII oocytes from pre-antral follicles cultured continuously in an atmosphere of 5% CO₂ in air (20% O₂ group) were individual chromosomes located outside of the equator (Table II, right column). However, this rate increased to 27% upon culture to 20 h post HCG/rEGF addition (Table II). The percentage of MII oocytes from follicle culture with displaced, unordered chromosomes was significantly higher in the low-oxygen as compared to the normal-oxygen group, with 34.8% unaligned chromosomes at 16 h and 50% at 20 h retrieval of oocytes (Table II; Figure 4F, F'). When the rate of oocytes at MII with well-aligned chromosomes from all oocytes obtained in pre-antral follicle culture was calculated, about 40% of all oocytes from the normal-oxygen group belonged to this high-quality group. In contrast, only 11.7 and 11.3% of oocytes with this high quality were collected in the low-oxygen group after final maturation for 16 or 20 h respectively (Table II).

Discussion
The present study focused on an analysis of spindle formation and chromosome behaviour in mammalian oocytes obtained from pre-antral follicle culture. The current observations confirm previous results which show that a large percentage of oocytes in pre-antral follicles of prepubertal mice are capable of growing and reaching meiotic competence during a 12-day culture period on the condition that 20% oxygen tension was present throughout the entire culture period (Cortvrindt et al., 1996, 1998; Smitz et al., 1996, 1998). Oocytes cultured in vitro for 12 days reached diameters comparable with those of isolated oocytes (grown in vivo) from large pre-ovulatory follicles, but remained marginally smaller (2–3%). This observation confirms previous data from this group (Cortvrindt and Smitz, 1998) and others (Eppig et al., 1992).

The present study provides evidence that oocytes from pre-antral follicle culture form functional, normal-appearing meiosis I spindles and progress to MII with well-aligned chromosomes. Spindle shape at MII in oocytes from in-vitro meiosis I spindles and progress to MII with well-aligned chromosomes from pre-antral follicle culture form functional, normal-appearing spindles with signiﬁcantly smaller diameters at the spindle pole compared to the former groups. This observation suggests that it is not only cytoplasmic maturity that is obtained during follicle growth, but also signalling and paracrine interactions between the oocyte and its somatic compartment and milieu at and prior to the resumption of maturation which influence spindle formation in oocytes. Molecules and signals obtained by granulosa cells, as well as factors produced inside of the oocyte in response to such signalling events, might act directly on cellular organization.

Chromosomes in vertebrate oocytes are able to recruit MTOC (Eichenlaub-Ritter et al., 1988a; Messinger and Albertini, 1991), but organization of the oocyte spindle is dependent on the expression of distinct classes of microtubule-motor proteins (Heald et al., 1996; Walczak et al., 1998; Mountain et al., 1999). Differences in expression patterns were reported between human oocytes resuming maturation in vitro, and in-vivo-matured ones (Anderiez et al., 1998). Differences may also exist in the expression, and hence in the availability, of spindle-associated or motor proteins between oocytes actively initiated to progress into M-phase of first meiosis by their contact and interactions with follicle and theca cells and those matured in vitro in this study; such differences might be responsible for the altered spindle shape.

In agreement with the concept of a crucial role of cell–cell interactions and a balanced hormonal environment, the present observations therefore suggest that in-vitro maturation conditions with oocytes inside of a follicle produce oocytes in which spindle formation is more comparable to in-vivo controls than to oocytes obtained from IVM (devoid of normal somatic interactions).

There appears to be a correlation between aberrant spindles and unaligned chromosomes at MII, and errors in chromosome segregation and induction of aneuploidy due to post-ovulatoy ageing or in response to chemical exposures of oocytes (Eichenlaub-Ritter et al., 1986; Yin et al., 1998b). Unordered chromosomes in a MII-arrested mammalian oocyte may present the result of predivision of homologous chromosomes at metaphase I or failures of kinetochores to attach properly to spindle fibres and congress at the equator at MII (Angell, 1997; LeMaire-Adkins et al., 1997; Yin et al., 1998a,b). Prolonged arrest at MII increases the risk for untimely segregation of chromatids and errors in chromosome segregation at anaphase II (Eichenlaub-Ritter, 1998; Mailhes et al., 1998).
and, in consequence, trisomy in the embryo (Eichenlaub-Ritter, 2000).

Prolongation of oocyte culture by only 4 h did not visibly affect spindle structure in a consistent fashion in this study, unlike that found in spontaneously ovulated oocytes post-ovulatory aged in the ampullae in vivo for extended times (Eichenlaub-Ritter et al., 1986). However, the percentage of MII oocytes with displaced, unordered chromosomes was increased in both the normal- and low-oxygen groups as in in-vivo-aged oocytes. There was no consistent alteration in spindle shape associated with such chromosome displacement. Only subtle disturbances in spindle structure could have been responsible for the loss of attachment of kinetochores to spindle fibres and displacement of chromosomes from the equator during ageing in culture. Mammalian oocytes possess a cell cycle checkpoint causing meiotic arrest in the absence of a spindle (Eichenlaub-Ritter and Boll, 1989; Brunet et al., 1999). However, oocytes may fail to sense chromosome displacement (LeMaire-Adkins et al., 1997; Yin et al., 1998b) such that defects in chromosome congression on the first or second meiotic spindle may have profound effects on the fidelity of chromosome segregation. To prevent such risks, it appears therefore advisable to harvest the oocytes from pre-antral follicle culture already at 16 h after the addition of HCG and rEGF (or even earlier), although this may reduce the total yield of MII oocytes. Hormonal homeostasis modulates the kinetics of in-vivo maturation in mouse oocytes (Polanski, 1986). Using follicle culture, it is now feasible to determine precisely the influence of LH and FSH on maturation kinetics, spindle formation and chromosome alignment in oocytes.

In order to test for beneficial or adverse influences of unphysiologically high oxygen tensions during IVM, maturation rate, spindle formation and chromosome behaviour were examined in oocytes placed into an atmosphere with a more physiological (i.e. reduced) oxygen concentration. Indeed, in the Fallopian tube—where freshly ovulated oocytes remain shortly before actual fertilization—oxygen tension is low. However, the reduction in oxygen tension interfered severely with maturation of oocytes under our culture conditions, since low numbers of oocytes emitted a polar body. Earlier studies (Epig and Wigglesworth, 1998) using different follicle culture techniques for oocyte growth and maturation in vitro suggested that an atmosphere of 5% CO₂ in air (20% O₂) may significantly reduce the rate of oocyte maturation and the developmental capacity of the obtained embryos. There may be a direct link between increases in ROS and H₂O₂ in oocytes and embryos, and developmental arrest (Nasr-Esfahani and Johnson, 1991; Kwon et al., 1999) and induction of apoptosis (Yang et al., 1998). From our previous experience, denuded mouse oocytes are capable of developing to MII in an atmosphere of 5% CO₂/5% O₂/90% N₂, so the meiotic arrest in pre-antral follicle culture with reduced oxygen tension during the last day prior to oocyte retrieval was unexpected.

There are two possibilities to explain the meiotic block in oocytes from our pre-antral follicle culture with low oxygen during the last 16 or 20 h. It may be primarily the follicle and theca cells which are dependent on sufficient oxygen supply for their metabolism during in-vivo and in-vitro folliculogenesis, especially at resumption of maturation (Boland et al., 1994). Since availability of oxygen depends entirely on diffusion in follicle culture, reduced oxygen tension could interfere with activity of follicle and theca cells, particularly when HCG and rEGF are added, and thus prevent initiation of resumption of maturation.

The very efficient depletion of oxygen by the large number of follicle cells (15–30×10³ cells) in the microdroplet may also have caused indirectly the meiotic arrest of oocytes such that oocytes had perhaps to resume maturation under near-anoxic conditions.

Oxygen supply and ATP appear to be important determinants of meiotic and developmental competence in in-vivo maturing oocytes. Antioxidants reversibly inhibited the spontaneous resumption of meiosis in mammalian oocytes (Takami et al., 1999), and chemicals which affected mitochondrial function and the intimate association and accumulation of mitochondria with the oocyte spindle caused meiotic arrest or delay, and predivision or non-disjunction of chromosomes (Yin et al., 1998a). Under reduced oxygen concentrations, the activity of motor proteins and other regulatory factors in spindle formation (Huang et al., 1999; Mountain et al., 1999) and cell cycle-regulating proteins may be affected in oocytes, when mitochondrial functions are compromised and little ATP provided. Under such conditions, synchrony in cytoplasmic and nuclear maturation, spindle formation and fidelity of chromosome segregation could be severely affected.

Under an ambient oxygen tension as is used routinely (Cortvrindt et al., 1996), diffusion in pre-antral follicles appears sufficient to provide for this basic and essential oxygen supply, without inducing visible damage. Preliminary observations on blastocyst formation rates from oocytes derived from culture under high oxygen did not provide evidence for an adverse influence of a normal oxygen tension (20%) under the presently chosen culture conditions. Oocytes express antioxidant genes and proteins (El Mouatassim et al., 1999), and antioxidants and proteins suppressing ROS are also present in follicular fluid and granulosa cells (Briggs et al., 1999; Jozwik et al., 1999) such that even high concentrations of ROS may be tolerated. Oestradiol which is produced normally in this culture system (Cortvrindt et al., 1996) may have an additional protective activity to prevent damage by ROS (Lund et al., 1999). The present study supports the notion that oxygen supply is a critical parameter during pre-antral follicle culture, particularly during resumption of maturation in oocytes when spindle assembly and chromosome alignments are occurring.

In summary, the observations made in this study stress the importance of appropriate interactions between oocytes, their somatic compartment and the culture medium composition, and the requirement for sufficient oxygen in oocyte maturation, spindle formation, and high fidelity of chromosome segregation. Growth and maturation of oocytes within normally differentiating somatic cells in a system such as described herein provides the opportunity to study the effects of culture environment on ultrastructural dynamics involved in chromosome segregation. Further studies are required to determine the significance of these effects on embryo quality and the ability to develop to term.
Acknowledgements

The study was supported by the EU (ENV4-CT97-0471), by grants from the Fund for Research Flanders (FWO grant G.301.93N) and by an STWW/IWT grant (No. 980343). Ares Serono International is greatly acknowledged for donation of recombinant hormones.

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Spindles in oocytes from pre-antral follicle culture
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Received on August 17, 2000; accepted on January 2, 2001