The position of the metaphase II spindle cannot be predicted by the location of the first polar body in the human oocyte

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When performing intracytoplasmic sperm injection (ICSI) on human oocytes, the injection is traditionally made at the 3 o’clock position, with the first polar body (PB) at the 12 or 6 o’clock position. This has been based on the assumption that the second meiotic metaphase II (MII) spindle lies in close proximity to the first PB. The objective of this study was to document the actual spatial relationship between the first PB and the MII spindle both in in-vivo matured (fresh) human MII oocytes and in oocytes matured in vitro. We found that the MII spindle was, on average, not located directly adjacent to the PB. The in-vivo group (n = 54) showed a mean deviation of the MII spindle from the position of the PB of 41.7° and the in-vitro group 26.6° (n = 43). The difference between the angle of the two groups was statistically significant (P = 0.005), indicating that the lateral displacement of the first PB is only partly due to the denudation procedure during ICSI, because the in-vitro matured oocytes were denuded before extrusion of the first PB. The majority of the MII spindles in both groups were found in the same hemisphere as the first PB, suggesting that care should be taken to avoid damaging the MII spindle by inserting the ICSI needle in the other half of the oocyte.

Key words: human oocyte/ICSI/MII spindle/polar body

Introduction

Since the introduction of the intracytoplasmic sperm injection (ICSI) technique on human oocytes almost a decade ago (Palermo et al., 1992), it has been assumed that the second meiotic metaphase II (MII) spindle lies in close proximity to the first polar body (PB). This assumption has led to the accepted practice of injecting the spermatozoon at the 3 o’clock position (with the PB at the 12 or 6 o’clock position), in order to avoid damage to the MII spindle (Palermo et al., 1993). Before performing ICSI, the oocytes have to be denuded of their surrounding cumulus cells. This is achieved by incubating the oocytes in an enzyme solution and subsequently removing the cumulus mass mechanically by aspiration of the oocyte into a thin denudation pipette. During the ICSI treatment, a number of oocytes are damaged mechanically (9%) (Tarlatzis and Bili, 1998). Further, a proportion of the fertilized zygotes do not cleave, or cleave abnormally. This might partly be due to damage or manipulation of the MII spindle. Since the PB is the single most prominent landmark on the unfertilized oocyte, a better knowledge of the spatial relationship between the first PB and the second meiotic MII spindle might be of advantage when ICSI is performed on human oocytes, to optimize the ICSI method further.

The objective of this study was to document the actual spatial relationship between the first PB and the MII spindle in in-vivo matured human MII oocytes (freshly aspirated) and in oocytes matured in vitro. It has been speculated that an increased distance between the PB and the MII spindle might be brought about by the denudation procedure (Hewitson et al., 1999). By studying immature oocytes which were denuded before the extrusion of the first PB, we hoped to shed some light on why a difference, if any, was found between the first PB and the second MII spindle in the in-vivo matured oocytes.

Materials and methods

Source of human oocytes

Oocytes were obtained after informed consent from patients undergoing IVF at the Centre for Reproductive Medicine, Göteborg University. Ovarian stimulation was carried out by a desensitizing protocol using a short-acting gonadotrophin releasing hormone agonist preparation in combination with recombinant FSH. Follicular aspiration was performed 36–38 h after human chorionic gonadotrophin (HCG) administration using vaginal ultrasonography and follicle puncture. All the oocytes obtained from the follicular aspirates were treated with 80 IU/ml hyaluronidase (Hyase; Scandinavian IVF Science, Gothenburg, Sweden) for 30 s, followed by mechanical removal using a thin denudation pipette. This procedure removed most of the cumulus cell mass surrounding the cells. After this procedure, the oocytes were graded as GV if they had a well defined germinal vesicle and MII if the first PB had been extruded. The MII oocytes (in-vivo matured) were fixed at 3–4 h after the denudation process. The GV oocytes were cultured and checked for PB extrusion. Those GV oocytes which reached the MII stage the next morning were fixed 3–4 h later. The remaining GV oocytes were observed at 1–2 h intervals and fixed 3–4 h after the extrusion of the first PB in order to allow the second MII spindle to polymerize. These were classified as in-vitro matured oocytes. Immature oocytes that had not reached the MII stage 32 h after aspiration were considered to be arrested or degenerated and excluded from this study.

Immunostaining for tubulin

In order to assess the spatial relationship between the first PB and the second MII spindle, a method employing double fluorescence staining was used. The oocytes were fixed for 20 min at 37°C in a microtubule stabilizing buffer (0.1 PIPES, pH 6.9, 5 mmol/l
The MII spindle in relation to the first polar body

**Figure 1.** A schematic illustration of how the MII spindle was ‘moved’ relative to the cortex as if the oocyte was rotated on an axis going through the PB (top) and the centre of the oocyte. Illustration by H.Samuelsson.

MgCl₂.6H₂O, 2.5 mmol/l EGTA containing 2.0% formaldehyde, 0.5% Triton X-100, 1 μmol/l taxol), then washed three times in a blocking solution of phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA), 2% powdered milk, 2% normal goat serum, 0.1 mmol/l glycine and 0.01% Triton X-100. The oocytes were attached to poly-L-lysine coated glass slides. For microtubular visualization, the oocytes were incubated in anti-α tubulin monoclonal antibody (Amersham International, Amersham, Buckinghamshire, UK) for 1 h at 37°C, at 1:1000 in PBS containing 0.1% BSA and 0.02% sodium azide (PBS + sodium azide). The slides were washed for 1 h at room temperature in blocking solution and further incubated in a 1:100 solution of Cy3 conjugated goat anti-mouse immunoglobin G (IgG; Amersham International) for 1 h at room temperature. After this step, the oocytes were washed three times in PBS + sodium azide and chromosomes identified by counterstaining with 4’,6’-diamidino-2-phenylindole (DAPI) (500 ng/ml; Sigma, St Louis, MO, USA) diluted in mounting medium (Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA) for 10 min at room temperature. The oocytes were examined using a Nikon epifluorescence microscope equipped with appropriate filters. Images were transferred via a video camera using a data imaging program (Applied Imaging, Scotswood Road, Newcastle upon Tyne, UK) and stored in a computer. Although a simpler method of staining the oocytes would have sufficed, data for this publication were drawn from another parallel study in which oocyte fixation and staining of the spindle apparatus for visualization was necessary.

Although the oocytes were attached to the glass with their PB perpendicular to the observer, not all the spindles had a cortical position, i.e. when observed, they appeared to be positioned somewhere inside the oocyte. Since the MII spindle has been shown to be attached to the inner cell surface (Johnson et al., 1975; Longo and Chen, 1985; Szöllösi et al., 1986), it should be possible to measure the angle between the PB and the MII spindle in a two-dimensional view. Therefore, the oocytes which were observed with their MII spindles ‘inside’ the ooplasm were ‘rotated’ relative to the cortex (Figure 1). This was achieved by drawing an imaginary line through the PB and the centre of the oocytes and then moving the MII spindles by rotation of the oocytes perpendicularly around this axis until the spindles were situated close to the cortex. By this procedure, all the angle measurements were made with the MII spindle close to the cortex of the oocyte.

For statistical analysis, Student’s t-test was used to compare the mean angle between the two groups and confidence interval calculations were used to find out if the in-vitro and in-vivo groups’ mean angles were significantly different.

**Figure 2.** MII risk zones. A schematic illustration of how we propose that the spermatozoon should be injected into the oocyte. The closer to the first PB (top), the greater the risk of MII-spindle damage (indicated by the red colour). Illustration by H.Samuelsson.

**Figure 3.** Examples of the relationship observed between the MII spindle and the first PB. (A) An oocyte with the MII spindle directly under the PB. (B) An oocyte with its MII spindle at 90° from the first PB.
we have shown in this study that: (i) the first PB is generally performed at a 90° angle away from the MII spindle, which is the main reason why sperm injections are delayed. It has been presumed that the MII spindle is positioned close to the first PB in 100% and 93% of instances respectively (Table I).

Of the 112 human oocytes included in this study, it was possible to determine the spatial relationship between the first PB and the second MII spindle in 97. The oocytes were donated at random by 53 women (mean 1.8 oocyte per woman). The in-vitro matured group consisted of 43 oocytes, while we obtained 54 images from the in-vivo matured MII oocytes. The spatial relationship between the first PB and the MII spindle is summarized in Table I and Figure 2. The MII spindle was, on average, not found directly adjacent to the PB (Figure 3). The in-vitro group showed a mean deviation of the MII spindle from the position of the PB of 26.6 ± 3.3° (SEM) and the in-vivo group 41.7 ± 4.0° (SEM). There was a statistically significant difference in this angle between the in-vitro and the in-vivo matured oocytes (P = 0.005). Furthermore, the average position of the MII spindle in both groups was found to be significantly different (P < 0.05) from a ‘null position’, i.e. situated directly under the first PB.

Upon calculating the cumulative percentage, we found that in the in-vitro and the in-vivo oocyte group the MII spindle was situated in the same hemisphere of the oocyte as the first PB in 100% and 93% of instances respectively (Table I).

### Results

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### Discussion

It has been presumed that the MII spindle is positioned close to the PB, which is the main reason why sperm injections are generally performed at a 90° angle away from the PB. However, we have shown in this study that: (i) the first PB does not accurately predict the position of the MII spindle in human oocytes and (ii) the distance between the PB and the MII spindle cannot be explained solely as being a result of lateral displacement during denudation before ICSI. Why, then, is the MII spindle not positioned adjacent or very close to the first PB? The in-vivo group showed a significantly greater deviation of their MII spindles from the PB, than the in-vitro group. One of the reasons for this difference might be that the ICSI denudation procedure causes lateral displacement of the PB in relation to the spindle by affecting those polar bodies that lie directly above the MII spindle. Arguments against such an explanation are observations made by Garello et al. (Garello et al., 1999) where they found that the angle between the first and second PB had the same profile in oocytes from IVF and ICSI. Another explanation might be that the displacement of the PB is a time-dependent process, indicating that not all the oocytes had reached their ‘final’ PB/MII angular difference at the time of fixation. This would especially hold true for the in-vitro matured oocytes, assuming that they were on average fixed closer to the time of first PB extrusion than the in-vivo oocytes. Finally, it cannot be excluded that the delayed maturation of the GV oocytes involves an unknown mechanism causing the difference between the two groups. The fact remains that the in-vitro matured group also showed a significant deviation of the PB from the MII compared with the null position, which cannot be explained by the denudation process during ICSI as the in-vitro group oocytes were denuded before extruding their first PB.

In order to build up the MII spindle, the oocyte has to complete its first meiosis, extrude the first PB, enter the second meiosis and arrest in the second metaphase. This means that the microtubulins that make up the first metaphase spindle have to break down and a new spindle has to be formed. It has been suggested that these events are organized by so-called microtubular organizing centres (MTOC) (Battaglia et al., 1996). These MTOC seem to govern the microtubular organization until the appearance of the sperm aster after fertilization and are found mainly in the cortical area of the oocyte during both MI and MII. It is not known whether the MTOC remain stationary during both MI and MII and it is possible that after completion of the first meiosis, the MII spindle can be built at a new location. Another explanation for how the distance between the first PB and the MII spindle arises might simply be the dynamic movements of the oolemma during and after the first PB extrusion, which has been documented by using time-lapse video recording (own unpublished data). The underlying process might possibly involve the microfilaments in the cortical area of the oocyte.

The fact that the PB of a denuded MII oocyte is not necessarily positioned above or very near the second MII spindle might be cause for concern when performing ICSI. Possible consequences of inserting the ICSI needle into or close to the MII spindle may include total or partial disruption of the spindle or displacement of the spindle from the oolemma. Total disruption of the spindle would eventually lead to cell death, and it is possible that these events could be avoided if care is taken to ensure that the needle is inserted as close as possible to the PB without damaging the spindle.
death and thus higher damage and lower fertilization rates. Partial disruption might result in perturbation of chromosomal segregation and subsequent aneuploidy, as meiosis in the mammalian female does not seem to have rigorous spindle check-points, thus making the gamete prone to errors during meiosis (Fulka Jr et al., 1997; LeMaire-Adkins et al., 1997). It has been shown that children born after ICSI run a slightly higher risk of chromosomal aneuploidy mainly involving the sex chromosomes (Tournaye et al., 1995). However, it seems unlikely that only the sex chromosomes would be affected by a possible negative effect of the injection method. A higher prevalence of Klinefelter syndrome and translocations in the population of infertile men needing ICSI seems a more likely explanation (Chandley and Hargreave, 1996).

Although the fertilization rate after ICSI is fairly good, reaching 60–70% in most clinics, this leaves us with the fact that a significant proportion (30–40%) of the oocytes are potentially wasted. It has been reported (Flaherty et al., 1995) that in an ICSI programme a majority of the unfertilized MII oocytes contained a swollen sperm head. They also found that in 4% of the unfertilized oocytes the swollen sperm heads were located among the metaphase chromosomes and concluded that the first PB must have moved from the spindle region at the time of injection as they had taken great care in aligning the first PB at the 12 o’clock position before injecting at the 3 or 9 o’clock position. Although a 100% fertilization rate will presumably never be accomplished, the fertilization and survival rates after ICSI might be further increased by altering the position of the injection to the hemisphere opposite to that of the PB.

Recent data, based on hamster (Silva et al., 1999) and rhesus monkey oocytes (Hewitson et al., 1999), are in accordance with our observation on human oocytes that the PB does not always reside close to the spindle. The clinical validity of the hamster experiments for humans is limited, however, since there does not seem to be any regularity as to where the MII spindle is positioned in relation to the first PB. It has been shown (Hewitson et al., 1999) that the MII spindle could be displaced up to 68° from the PB in in-vivo matured rhesus monkey oocytes, with a mean difference of 19.8 ± 23.3° (n = 19), which is lower than our findings. Furthermore, they found that in human oocytes matured in vitro (n = 3) and in IVF failures (n = 5) the mean displacement between the PB and the spindle was 12 and 11° respectively. When examining denuded human oocytes intended for ICSI, they found that the displacement had increased to an average of 56 ± 27.5° (n = 8), which is much higher than we found. Although based on a low number of observations, these results do correlate to our findings. In the mouse oocyte, a time-dependent displacement of the MII chromosomes in relation to the first PB has been documented (Kono et al., 1991). Interestingly, they found that 2 h after the PB extrusion only 10% of the chromosomes were still located directly under the PB and the remaining chromosomes distributed over the whole oocyte, which was not found in our study of the human oocyte.

Recently, some interest has been shown in the polarity of the oocyte/zygote and the possible advantages of this when choosing embryos for transfer (Edwards and Beard, 1997; Fulka Jr et al., 1998. Garello et al., 1999). These studies base their morphological evaluation of polarity partly on the position of the polar bodies and the pronuclei. In the study by Garello et al. the placement of a spermatozoon in a fixed plane relative to the first polar body (ICSI) did not result in an altered pronuclear/polar body orientation relative to IVF (Garello et al., 1999). However, these IVF zygotes were found to have a higher degree of eccentric pronuclei and higher incidence of irregular cleavage or cleavage failure. If the first PB is indeed an important landmark of zygotic polarity, the denudation method used in relation to the ICSI method and the subsequent displacement of the PB might introduce an error, making the validity of this zygote screening questionable.

It is important to point out that in 93% of the oocytes in our study the MII spindle was actually located in the same hemisphere as the PB (the animal pole). Therefore, it might be advantageous when performing ICSI to inject the spermatozoon on the vegetal side of the midline between the two hemispheres of the oocyte to avoid damage to the MII spindle (Figure 2). Furthermore, the opening of the ICSI needle should be pointed towards the animal pole so that the spermatozoon is ejected in the direction of the MII spindle, shown by other investigators to be of benefit for embryonic development (Blake et al., 2000) and pregnancy rates (Van der Westerlaken et al., 1999).

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