Article

Effect of denuding on polar body position in in-vitro matured oocytes

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Abstract

The aim of this study was to determine whether the denuding procedure causes the polar body to move within the perivitelline space. Only those patients undergoing IVF who had unused in-vitro matured (IVM) oocytes were included in this study. IVM oocytes were initially viewed under a non-invasive, polarized light microscope. A laser was used to mark the location of the polar body on the zona. Oocytes were subjected to the denuding procedure with a 150 μm, 135 μm and 125 μm diameter pipette. After each pipetting, the oocytes were viewed again to determine whether the polar body had moved. After denuding, the oocyte was left to culture overnight and viewed 24 h later. After denuding with the 150 μm, 135 μm and 125 μm pipettes and after 24 h in culture, the average angle between the spindle and polar body was 15.4 ± 10.4°, 16.1 ± 10.1°, 20.9 ± 11.7°, and 26.7 ± 18.2°, respectively ($P = 0.0021$). Slight changes in angles were noted between denuding with the different diameter pipettes. The largest changes in angles were seen after 24 h in culture.

Keywords: *denuding, IVF, Oosight™, polar body, PolScope, spindle*

Introduction

During the first meiotic division in the oocyte, the first polar body is produced. The polar body is a small cellular body, located adjacent to the oocyte in the perivitelline space; its sole purpose is to house the chromosomes after a reduction division during meiosis (Gitlin *et al.*, 2003). Theoretically, the spindle (the structure within the oocyte responsible for the proper alignment of the chromosomes prior to meiosis) should re-form with the chromosomes aligned on the metaphase II (MII) plate, directly below the first polar body. Hence the reason intracytoplasmic sperm injection (ICSI) is performed with the polar body at the 12 or 6 o'clock position; preferentially when the first polar body is at the 6 o'clock position (Nagy *et al.*, 1995; Palermo *et al.*, 1996). These positions limit disturbances, such as cytoplasmic waves, that may be caused by the ICSI needle during the procedure (Payne *et al.*, 1997). Disturbances caused by the ICSI procedure may compromise oocyte competence and lead to diminished fertilization rates and embryo quality (Dumoulin *et al.*, 2001; Avery and Blayney, 2003).

Research suggests that the MII spindle may not always be located directly below the first polar body and the spindle could be displaced as much as 90° away from the first polar body (Silva *et al.*, 1999; Wang *et al.*, 2001a). The displacement of the spindle away from the polar body could make the spindle and cytoplasm more susceptible to disturbances caused by the ICSI needle. These disturbances may alter the cytoplasm by causing a shift in polarized molecules, therefore altering the position of these molecules within the cytoplasm (Garello *et al.*, 1999). This shift in position of polarized molecules could alter the course of embryo development (Cooke *et al.*, 2003).

Spindle 'dislocation' may also affect embryo development by interfering with polarization of the oocyte and the derived embryo. It has been demonstrated in different animal species that location of the spindle/first polar body determines cleavage planes. However, if the spindle and polar body are not aligned, it may lead to altered embryo development (Cooke *et al.*, 2003; Edwards, 2005).

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During IVF, when an oocyte is designated for ICSI insemination, the nuclear maturation of the oocyte must be graded. To do this, the oocyte must undergo a procedure known as denuding. This procedure subjects the oocyte to pipetting with pipettes of decreasing diameter in order to remove the outer cumulus cells and allow visualization and proper identification of oocyte maturity. The vigorous pipetting of the oocyte can sometimes cause the zona pellucida to fracture or even cause oocyte death. Aside from oocyte death, this pipetting can cause a large amount of stress on the oocyte, however, the extent of damage that this procedure can cause is unknown.

It is also unknown whether intrinsic or extrinsic factors are to blame for the dislocation of the spindle from the polar body. Research suggests that polar body movement may be caused by both types of factor. Hardarson *et al*. (2000) found that both in-vivo matured oocytes and in-vitro matured (IVM) oocytes had meiotic spindle displacement from the first polar body. It has been postulated that the denuding procedure causes the polar body to move within the oocyte. The aim of this study was to determine whether the mechanical removal of cumulus cells from the oocyte (denuding) causes the polar body to move within the perivitelline space.

Materials and methods

Oocytes that were to undergo ICSI were not used. Only oocytes that were determined to be metaphase I (MI) or germinal vesicle (GV) stage on day 0 and subsequently matured overnight to an MII (metaphase II) oocyte were used in the study. Informed consent for the study was obtained from all patients before the study started.

Patients attending Reproductive Biology Associates clinic for IVF treatment underwent ovarian stimulation using recombinant FSH (rFSH, Gonal-F; Serono Laboratories Inc., Norwell, MA, USA) with gonadotrophin-releasing hormone (GnRH) agonist pituitary down-regulation (Lupron; TAP Pharmaceuticals, North Chicago, IL, USA) or GnRH antagonist (Ganirelix; Organon Pharmaceuticals, Inc., Roseland, NJ, USA) and human chorionic gonadotrophin (HCG, Profasi; Serono). Cycles were monitored with daily follicular ultrasound measurements and serum estradiol levels. HCG was given when two or more follicles had a diameter of ≥18 mm. Oocyte retrieval was conducted by a transvaginal ultrasound-guided puncture 34–36 h after HCG administration.

Oocyte retrieval, sperm and oocyte handling for ICSI procedures were performed according to Nagy *et al*. (1998). After oocyte retrieval, the oocytes designated for ICSI insemination were denuded by a short exposure to HEPES-buffered medium (Cooper/Sage, Bedminster, New Jersey, USA) with 20 IU/ml cumulase (Halozyme, San Diego, CA, USA). After exposure to cumulase, the oocytes were placed back into the incubator for 30 min. After incubation, the cumulus was completely removed by pipetting with a 150 μm diameter plastic pipette and then a 135 μm pipette (Stripper, MidAtlantic Diagnostic, Inc., Marlton, New Jersey, USA). The oocytes were then assessed for nuclear maturation (GV, MI or MII), and separated based on maturity. Those that were mature underwent ICSI.

Oocytes that were immature at the time of ICSI were left to culture overnight. Those oocytes that matured overnight in culture, which were not for patient use and for which the patients had signed the consent form, were used in the study. All oocytes used in the study were denuded on day 0, but were either a GV or MI at that time.

The oocytes were viewed under a non-invasive, polarized light microscope system (Oosight™; CRi, MA, USA). For the initial viewing, each IVM oocyte was viewed with the ×10 objective and then brought closer into view with the ×40 objective. The oocyte was rotated with an ICSI needle (Humagen, Charlottesville, Virginia, USA) until the polar body was in focus on the periphery of the oocyte. The IVM oocyte was rotated until the spindle was also visible on the periphery. A picture of the IVM oocyte with the spindle in view and the polar body in focus was taken with the microscope. A laser (Mid-Atlantic Diagnostics, Inc., Marlton, New Jersey, USA) was used to mark the zona pellucida directly above the polar body, so that the polar body, laser hole, and spindle were all within the same viewing plane (**Figure 1**). If the oocyte was rotated multiple times and the spindle was not visible, it was considered not to house a spindle and was not used in the study.

The initial angle of the spindle and polar body displacement in the IVM oocytes prior to denuding on day 1 was measured using the microscope program. After the initial angle was measured, each day 1 IVM oocyte was expelled 15 times for each diameter of pipette (150 μm, 135 μm and 125 μm) in consecutive order. After denuding with each pipette, the laser mark on the zona pellucida was realigned with the polar body and spindle in the same viewing plane and the angle of the polar body relative to the spindle was measured. Finally, after denuding with the 125 μm pipette, the final angle of spindle and polar body displacement was measured and the oocyte was cultured for another 24 h. After culture, the laser mark on the zona pellucida was aligned with the polar body and spindle in

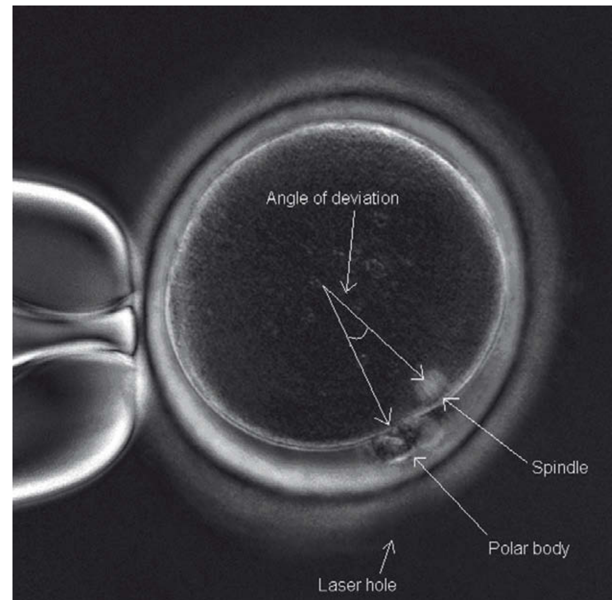

Figure 1. Oocyte with polar body, spindle and laser hole all present as viewed under the polarized light microscope, just prior to denuding.

the same viewing plane and the angle of the polar body relative to the spindle was measured. If no movement had occurred during denuding or culturing, then the polar body would be located directly below the laser mark and the angle between the polar body and spindle would not change.

Statistical analyses were performed using Kruskal–Wallis tests and Dunn's Multiple Comparison when appropriate. $P < 0.05$ was considered to be statistically significant.

Results

A total of 50 IVM oocytes from 21 patients were included in this study. Four oocytes were not included because the spindle disappeared after 24 h in culture; one oocyte was not used because the polar body was ejected through the hole made with the laser during the denuding procedure; and five oocytes were fractured during the denuding procedure. Each oocyte that was denuded and had a spindle after extended culture was used. Oocytes underwent five stages: (i) measurement of the initial angle of deviation between the polar body and spindle; (ii) denuding with the 150 μm pipette; (iii) denuding with the 135 μm pipette; (iv) denuding with the 125 μm pipette; (v) culture for 24 h after denuding with the 125 μm pipette. All IVM oocytes were denuded with each diameter pipette.

The average initial angle between the spindle and polar body was 15.4 ± 11.1°. After denuding with the 150 μm, 135 μmand 125 μm pipettes and after 24 h in culture, the average angle between the polar body and spindle was 15.4 ± 10.4°, 16.1 ± 10.1°, 20.9 ± 11.7°, and 26.7 ± 18.2°, respectively (**Table 1**; Kruskal–Wallis test; $P = 0.0021$). Statistical significance was found between stage 1 (15.4 ± 11.1°) and stage 5 (26.7 ± 18.2°) (**Table 1**; Dunn's Multiple Comparisons test; $P < 0.05$). Statistical significance for the average angle that the polar body was away from the spindle was also found between stage 2 (15.4 ± 10.4°) and stage 5 (26.7 ± 18.2°) (**Table 1**; Dunn's Multiple Comparisons test; $P < 0.05$).

Because the polar body can move closer or further away from the spindle, the absolute value of the difference in angle afterdenuding must be used. Taking into account the absolute value, the average difference in angle between the polar body and spindle for the stages was statistically significant (**Table 1**; Kruskal–Wallis test; $P < 0.0001$). The average differences were: 2.1 ± 1.6° between stages 1 and 2; 3.2 ± 4.5° between stages 2 and 3; 6.5 ± 7.0° between stages 3 and 4; and 10.7 ± 9.1° between stages 4 and 5 (**Table 1**; Kruskal–Wallis test; $P<0.0001$).

Discussion

Research suggests that the spindle is not always directly beneath the polar body (Calarco, 1995; Silva *et al.*, 1999; Hardarson *et al.*, 2000). The present data indicate that the spindle of IVM oocytes can be displaced as much as 40.0° from the polar body and on average the spindle was 15.4 ± 11.1° away from the first polar body. This large variation makes it difficult to establish a meaningful control for the experiment. One possible control would be to leave IVM oocytes that were not denuded in culture for 24 h. However, the data show that a large variation of angles exist between the polar body and spindle and this large variation makes controlling for the experiment extremely difficult.

The IVM oocytes in this study were subjected to the denuding procedure as immature oocytes, prior to the ejection of the polar body, and therefore the spindle should be directly below the polar body. The data indicate that even without extrinsic factors such as denuding, the polar body is still able to move within the perivitelline space. Gardner (1997) suggests that the spindle does not move, but rather the polar body moves along the plasma membrane of the oocyte. He explains that this can only occur if the cellular bridges that connect the polar body to the oocyte are damaged or non-existent. These cellular bridges are composed of microtubules and actin filaments, which can be damaged by vigorous pipetting of the oocyte during denuding, manipulation of the oocyte prior to ICSI, or by the physical stress involved in the puncturing of the plasma membrane by the ICSI needle. The presence of microtubules that anchor the spindle to the plasma membrane supports Gardner's suggestions (Compton, 2000). Although the present data show that denuding does not significantly alter polar body positioning within the IVM oocyte, slight changes in polar body positioning were observed. These changes could be attributed to the visualization system. After each trial, even though there was a laser mark on the zona, aligning the oocyte so the exact same plane was in focus was impossible.

External and internal factors may influence polar body positioning within the oocyte. All oocytes used in this study were IVM oocytes. These oocytes may behave differently to in-vivo matured oocytes and therefore it may not be possible

Table 1. Average angle of deviation between spindle and polar body after denuding of in-vitro matured oocytes ($n = 40$) with pipettes of decreasing diameter.

	Before denuding	*Denuding pipette diameter (µm)* 150	135	125	*After 24-h culture*	P-*value*[a]
Mean angle (°) ± SD	15.4 ± 11.1[b]	15.4 ± 10.4[c]	16.1 ±10.1	20.9 ± 11.7	26.7 ± 18.2[b,c]	0.0021
Absolute value of mean angle deviation after denuding	NA	2.1 ± 1.6[d,e]	3.2 ± 4.5[f]	6.5 ± 7.0[d,g]	10.7 ± 9.1[e–g]	<0.0001

[a]Kruskal–Wallis test; [b–g]Within each row, values with the same superscript letter are significantly different ($P < 0.05$, Dunn's Multiple Comparison test).

to apply the results directly to day-0 oocytes. However, reports show that the polar body can be displaced from the spindle of in-vivo matured oocytes, indicating that the polar body moves even without the forces from the denuding procedure (Silva *et al.*, 1999; Wang *et al.*, 2001a). Since a majority of the IVM oocytes exhibited spindle displacement from the polar body, it would be expected that intrinsic factors play a large role in polar body movement. In order to answer this question, the IVM oocytes were viewed 24 h after denuding. Most polar body movement was observed to occur after 24 h in culture (10.7 ± 9.1°). This could be an indication that oocyte ageing may play a role in polar body displacement.

The underlying mechanism that allows the polar body to move is unknown. In this study, a laser was used to mark the positioning of the polar body within the oocyte. It is possible that the latent heat emitted by the laser could damage the microtubules that anchor the polar body to the oolemma, thereby making the polar body more susceptible to movement while in culture or undergoing the denuding procedure. Payne *et al.* (1997) described cytoplasmic waves travelling throughout the oocyte. It may be possible that these waves also cause the spindle or polar body to move along the oolemma. However, in this study, all spindles, even after extended culture, were shown not to move. This indicates that cytoplasmic waves may not have affected spindle positioning and indeed the polar body and not the spindle is the cause of these angles of deviation.

Table 2. Oocytes were grouped together based on the initial angle of deviation between the spindle and polar body (A: initial angle of deviation 0°–9.9°, $n = 13$; B: initial angle of deviation 10.0°–24.9°, $n = 19$; C: initial angle of deviation >24.9°, $n = 8$).

Category	*Absolute value of mean angle deviation after denuding (°)*	*P-value*[a]
150 μm		
A	2.0 ± 1.8	NS
B	1.8 ± 1.5	
C	3.0 ± 1.3	
135 μm		
A	2.3 ± 2.3	NS
B	2.7 ± 2.0	
C	6.0 ± 9.1	
125 μm		
A	9.0 ± 9.3	NS
B	5.6 ± 5.8	
C	4.8 ± 4.3	
24-h culture		
A	7.8 ± 7.3[b]	0.0457
B	9.8 ± 8.4	
C	17.5 ± 10.8[b]	

[a]Analysis of variance; [b]$P < 0.05$, Dunn's Multiple Comparison test; NS, not statistically significant.

In order to observe if IVM oocytes with larger angles between the spindle and polar body were more susceptible to further movement, the oocytes were grouped by their initial spindle and polar body angle of deviation. Group C, the group with the largest initial angle deviation (>24.9°), also had the largest polar body movement after 24 h of culture (17.5 ± 10.8°) (**Table 2**). The cause of this initial angle of variation between the spindle and polar body is unclear. The denuding procedure itself, regardless of the size of the pipette, did not cause any significant increase in the spindle/polar body angle of deviation between group A and group B. Perhaps a larger angle of deviation can be used as an oocyte quality indicator. Rienzi *et al.* (2003) found no difference in fertilization rates if the spindle was within 90° of the polar body. However, the authors did find a lower incidence of fertilization when the spindle was located >90° from the polar body. The findings of Rienzi *et al.* (2003) support those of Abdelmassih *et al.* (2002) who found that fertilization was diminished when a larger angle of deviation between the spindle and polar body was present. Recent research indicates that oocytes without a spindle have a diminished fertilization rate but similar embryo quality on day 3, when compared with oocytes that house a spindle (Wang *et al.*, 2001b; Moon *et al.*, 2003; Cohen *et al.*, 2004). In this study, ICSI was not performed; therefore, fertilization and embryo development could not be analyzed.

The spindles of five IVM oocytes disappeared after 24 h in culture. This represents 10% of the oocytes, which is consistent with the figure of 7–25% day 0 oocytes without a spindle reported in the literature (Cooke *et al.*, 2003; Konc *et al.*, 2004). No spindles disappeared during the denuding procedure, indicating that the temperature was maintained properly during the study. Furthermore, the overall goal of the study was a quantitative measurement of spindle presence. If a study of the qualitative aspect of spindle presence was to be undertaken, a stricter way of maintaining the temperature at precisely 37°C would be required, as the spindle is extremely sensitive to temperature fluctuations.

This research shows that a physically aggressive denuding procedure can slightly alter the position of the polar body within the perivitelline space. However, the data also show that IVM oocytes have large angles of deviations prior to denuding. It may be that the cellular bridges of oocytes become compromised with extended culture or ageing. Both extrinsic and, to a larger extent, intrinsic factors play a role in spindle and polar body dislocation.

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