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Do age and extended culture affect the architecture of the zona pellucida of human oocytes and embryos?

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Summary

Advanced female age and extended *in vitro* culture have both been implicated in zona pellucida (ZP) hardening and thickening. This study aimed to determine the influence of (i) the woman's age and (ii) prolonged *in vitro* culture of embryos on ZP thickness and density using non-invasive polarized light (LC-PolScope) microscopy. ZP thickness and density (measured as retardance) were determined in oocytes, embryos and blastocysts in women undergoing intracytoplasmic sperm injection (ICSI) in two age groups (older, > 38 years; younger, ≤ 38 years). A total of 193 oocytes from 29 patients were studied. The younger group contained 100 oocytes and the older group 93 oocytes. The ZP was significantly thicker in metaphase II oocytes in the older group compared with the younger group (mean $\pm$ SD: 24.1 $\pm$ 2.5 μm vs 23.1 $\pm$ 3.3 μm; $p = 0.01$) but ZP density was equal (2.8 $\pm$ 0.7 nm). By day 2 of culture, embryos from the two groups had similar ZP thickness (22.2 $\pm$ 2.2 μm vs 21.7 $\pm$ 1.6 μm; $p = 0.28$) and density (2.9 $\pm$ 0.7 nm vs 2.8 $\pm$ 0.8 nm; $p = 0.57$). For the embryos cultured to blastocyst (older: n = 20; younger: n = 18) ZP thickness was similar in the two groups (19.2 $\pm$ 2.7 μm vs 19.1 $\pm$ 5.0 μm; $p = 0.8$) but thinner than on day 2. The older group had significantly denser ZP than the younger group (4.2 $\pm$ 0.5 nm vs 3.3 $\pm$ 1.0 nm, $p < 0.01$). Blastocysts from both groups had significantly denser ZP than their corresponding day 2 embryos (older: 4.2 $\pm$ 0.5 nm vs 2.9 $\pm$ 0.7 nm, $p < 0.001$; younger: 3.3 $\pm$ 1.0 nm vs 2.8 $\pm$ 0.8 nm, $p = 0.013$). It is concluded that there is little relationship between ZP thickness and its density as measured by polarized light microscopy. While ZP thickness decreases with extended embryo culturing, the density of the ZP increases. ZP density increases in both age groups with extended culture and, interestingly, more in embryos from older compared with younger women.

Keywords: Blastocyst, Extended culture, ICSI, PolScope, Zona pellucida

Introduction

A critical component for successful *in vitro* fertilization (IVF) and embryo transfer (ET) is achieving high-quality embryos capable of implantation and ongoing pregnancies. When good embryos can be selected and transferred, pregnancy rates increase and multiple pregnancies can be avoided by reducing the number of embryos transferred (Garside *et al.*, 1997). Prolonged exposure of human oocytes and embryos to artificial culture conditions is believed to alter their architecture and so impair their ability to implant (Jelinkova *et al.*, 2003), which may also reflect zona pellucida (ZP) hardening. ZP hardening occurs naturally after fertilization in order to ensure blocking of polyspermic fertilization and protection of the integrity of the preimplantation embryo during early embryonic development (Garside *et al.*, 1997). Implantation rates are inversely correlated with advancing female age (Gordon & Dapunt, 1993), possibly due to increasing ZP hardening.

A proportion of high-quality embryos with full developmental potential fail to implant, perhaps because of hatching difficulties. This may explain the fact that the implantation rate per embryo transfer in human IVF programmes varies from 8% to over 40% (Jelinkova *et al.*, 2003).

To overcome the possible problems with *in vivo* hatching of embryos and to assist embryos to escape

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from their zonae during blastocyst expansion, several techniques of assisted hatching and zona thinning have been developed using acids, micro-tools and non-contact lasers (Antinori *et al.*, 1996; Cohen, 1993). The clinical relevance of assisted hatching within an assisted reproduction programme remains controversial (Rufas-Sapir *et al.*, 2004; Sallam *et al.*, 2003). The lack of non-invasive techniques limited the assessment of the oocyte quality to morphological criteria. However, the latest breakthrough using polarized light microscopy (LC-PolScope, CRI, USA) allows the non-invasive analysis of the meiotic spindle in human oocytes (Wang *et al.*, 2001; Cooke *et al.*, 2003). The unique feature of the PolScope is that it alters the polarized light passing through an ordered structure, allowing qualitative and quantitative non-invasive assessment of any birefringent object in human oocytes (Wang *et al.*, 2001; Keefe *et al.*, 1997; Cooke *et al.*, 2003). Thus the PolScope in combination with the software has been used to analyse spindle morphology and texture as well as the structure of the ZP (Keefe *et al.*, 1997; Wang *et al.*, 2001). Silva *et al.* (1997) studied the effects of *in vitro* culture of mouse embryos on the architecture of the ZP and compared them with *in vivo* embryos. In this study Silva *et al.* managed to detect clearly the multi-laminar structure of the ZP. Recently, studies have analysed human oocyte zonae (Pelletier *et al.*, 2004; Shen *et al.*, 2005) by measuring the thickness and retardance. To date, the techniques used in performing these measurements have varied in each study. To our knowledge, in none of these studies have the authors validated their measurement technique. Therefore, in this study we validated our measurement technique by measuring inter-observer and intra-observer variation: as a result the most accurate technique was used in testing the hypothesis.

We tested the hypothesis that zona thickness and density of human oocytes are affected by the woman's age, and also investigated the changes that occur in ZP thickness and density during extended culturing of embryos.

Materials and methods

Trial design and exclusion criteria

This was a prospective cohort study with a group consisting of older women (age > 38 years) at the commencement of the treatment and another group of younger women (≤ 38 years). Women undergoing assisted reproductive technology (ART) and fulfilling the following criteria were included: (i) male factor as the only cause of infertility; (ii) intracytoplasmic sperm injection (ICSI) as the method of treatment used for fertilizing the oocyte; (iii) number of oocytes retrieved > 3 and < 15. Patients were excluded for the following reasons: (i) if they were donating oocytes; (ii) when surgically retrieved sperm was used for oocyte injection; (iii) failed fertilization. Only mature oocytes (metaphase II, MII) with clear presence of the first polar body (PB) were included.

Controlled ovarian stimulation

Controlled ovarian stimulation was achieved by a 'long downregulation' combination of a GnRH agonist and follicle stimulating hormone (FSH). The GnRH agonist was either a subcutaneous injection (Lucrin, leuprorelin acetate, Abbott, Kurnell, Australia) or a nasal spray (Syneral, nafarelin acetate solution, Searle, Rydalmere, Australia). Recombinant FSH was used (Gonal-F, Serono, Frenchs Forest, Australia; or Puregon, Organon, Lane Cove, Australia) to induce follicular growth, and 10 000 IU of hCG (Profasi, Serono, Frenchs Forest, Australia) was administered to achieve final maturation and 'trigger' ovulation when at least two follicles were 18 mm in average diameter as measured by transvaginal ultrasound. Thirty-six hours later, ultrasound-guided oocyte retrieval was performed using a single-lumen 17G ovum pick-up set (Cook, Eight Mile Plains, Australia). All oocytes were returned to the IVF laboratory in Hepes-buffered HTF (Sage BioPharma, Bedminster, USA) at 37 °C in a portable incubator (LEC-960, LEC Instruments, Scoresby, Australia).

Environment during oocyte injection, embryo culture and image analysis

Oocytes had their cumulus cells removed by a 30 s exposure to 80 IU/ml hyaluronidase (Sigma, Type VIII, Castle Hill, Australia) in Hepes-buffered HTF (Sage BioPharma, Bedminster, USA) followed by washing with Hepes-buffered HTF containing 5 mg/ml human serum albumin (Sage BioPharma, Bedminster, USA). The coronal cells were mechanically removed by means of 140 µl flexi-pipettes (Cook, Eight Mile Plains, Australia). Denuded oocytes were then assessed for their maturity and the presence of the first polar body (PB) and placed into culture in 0.8 ml fertilization medium covered with pre-warmed tissue culture oil (Sage BioPharma, Bedminster, USA), in a pre-equilibrated four-well Nunc dish (Nunclon, Medos, Lidcome, Australia) that had been pre-equilibrated at 37 °C in 5% carbon dioxide, 5% nitrogen and 90% oxygen.

Oocytes were microinjected 42 h after the ovulatory hCG injection. ICSI was performed, utilizing PolScope optics (Vital Diagnostics, Castle Hill, Australia), in a 5 µl droplet of Hepes-buffered medium in a

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sterile glass-bottomed dish (WPI-800865, Coherent Scientific, Australia). Oocytes were rotated four times around each axis until a full image of the meiotic spindle was obtained, then the image was captured. Captured images were saved as a CRI image on the computer software for later analysis. Microinjection was performed by holding the oocyte with the spindle at 12 o'clock and inserting the injection needle into the oocyte at the 3 o'clock position (Cooke *et al.*, 2003).

Following microinjection and imaging, the oocytes were transferred to equilibrated individually numbered 20 μl droplets for single culture in fertilization media in a sterile plastic dish (Nunc1-50288, Medos, 115 Lidcombe, Australia). All oocyte/embryo culture was performed in a mini-incubator (Minc-1000, Cook, Eight Mile Plains, Australia) supplied with a humidified triple gas mixture of (5% CO_2 + 5% O_2 + 90% N_2) at 37 °C.

Fertilization was assessed between 16 and 20 h (day 1) after microinjection. Fertilized oocytes were transferred to individual drop of Hepes for image capture. Zygotes were then washed in their respective cleavage medium (Sage BioPharma, Bedminster, USA) and each zygote placed in its respective numbered 20 μl droplet of medium for culture.

Cleavage was defined as the presence of a 2- or 4-cell embryo approximately 40 h (day 2) after ICSI. Images of embryos were taken in the same manner mentioned above and saved for later analysis.

For patients who had chosen to grow their embryos to blastocysts, the embryos were washed early on day 3 in their blastocyst medium and each embryo was cultured individually in 20 μl blastocyst medium covered with tissue culture oil. The growing embryos were checked at 120–124 h post-ICSI (day 5) for the formation of expanding blastocysts. Each blastocyst was imaged using the methodology.

A transcervical embryo transfer (ET) was performed, 41–44 hours after microinjection (or 120–124 h for blastocysts), utilizing a double-lumen catheter (K-Jets 7019, Cook, Eight Mile Plains, Australia). All patients received luteal phase supplementation of daily Crinone 8% vaginal gel (Serono, Frenchs Forest, Australia).

Measurement protocol used to determine zona pellucida thickness (ZPT)

The ZP was measured using the PolScope computer software, which measures the distance and ordered structure. Distance was measured in micrometres and structural order in nanometres. When light hits structure it bends, the degree of divergence of the light from its straight path being the 'retardance'. The more ordered the structure, or the denser it is, the more the light diverges and the greater the retardance (in nanometres).

The images were retrieved from their files and the measurement for quantitative analysis of thickness was performed by the same operator masked to the outcome of the patient. A line scan across the entire ZP was created. The line was then adjusted by using the computer mouse for measurement accuracy, starting with the outer zona and the retardance was equal to the background, and ending with the inner zona when the retardance dropped to that in the previtelline space (Fig. 1). The measurements were recorded in micrometres. For measurement accuracy all measurements were repeated on four different sites on the ZP (3, 6, 9 and 12 o'clock) while the spindle was always located at 12 o'clock. The average of the four measurements was used for data analysis.

To validate the methodology, samples of the oocytes were re-measured in two sets of the four sites by the same observer and the means used for calculating intra-observer variation. The same oocytes were measured by another observer who was masked to the objectives and outcome of the study and given only the measurement protocol to follow when performing the measurements. The means were used to calculate inter-observer variation (CV inter-observer 12.7% and intra-observer 15.3%).

Measurement protocol used to determine zona pellucida density (ZPD)

The ZP density was measured using the PolScope software by using the same line scan used for ZP thickness. Density was taken to be the retardance (in nanometres), as the denser an object, the greater its retardance. The graph representing the retardance of the three ZP layers has three peaks each representing the retardance of one layer. For this study the highest peak was used (Fig. 1). The measurement was repeated on the same four sites measured for thickness. The average was used for data analysis.

Study endpoints

The primary endpoint of the study was the comparison of ZPT and ZPD in patients undergoing ICSI to determine whether the ZPT and ZPD differ on days 0, 2 and 5 according to the woman's age. The secondary endpoint was to study the changes that occur in ZPT and ZPD in oocytes and embryos during *in vitro* culture.

Statistical analyses

A two-tailed Student *t*-test was used for quantitative analysis of ZPT and ZPD. A value of $p < 0.05$ was considered significant. Inter-observer and intra-observer variation were calculated by measuring the coefficient of variation (CV) and the standard error (SE).

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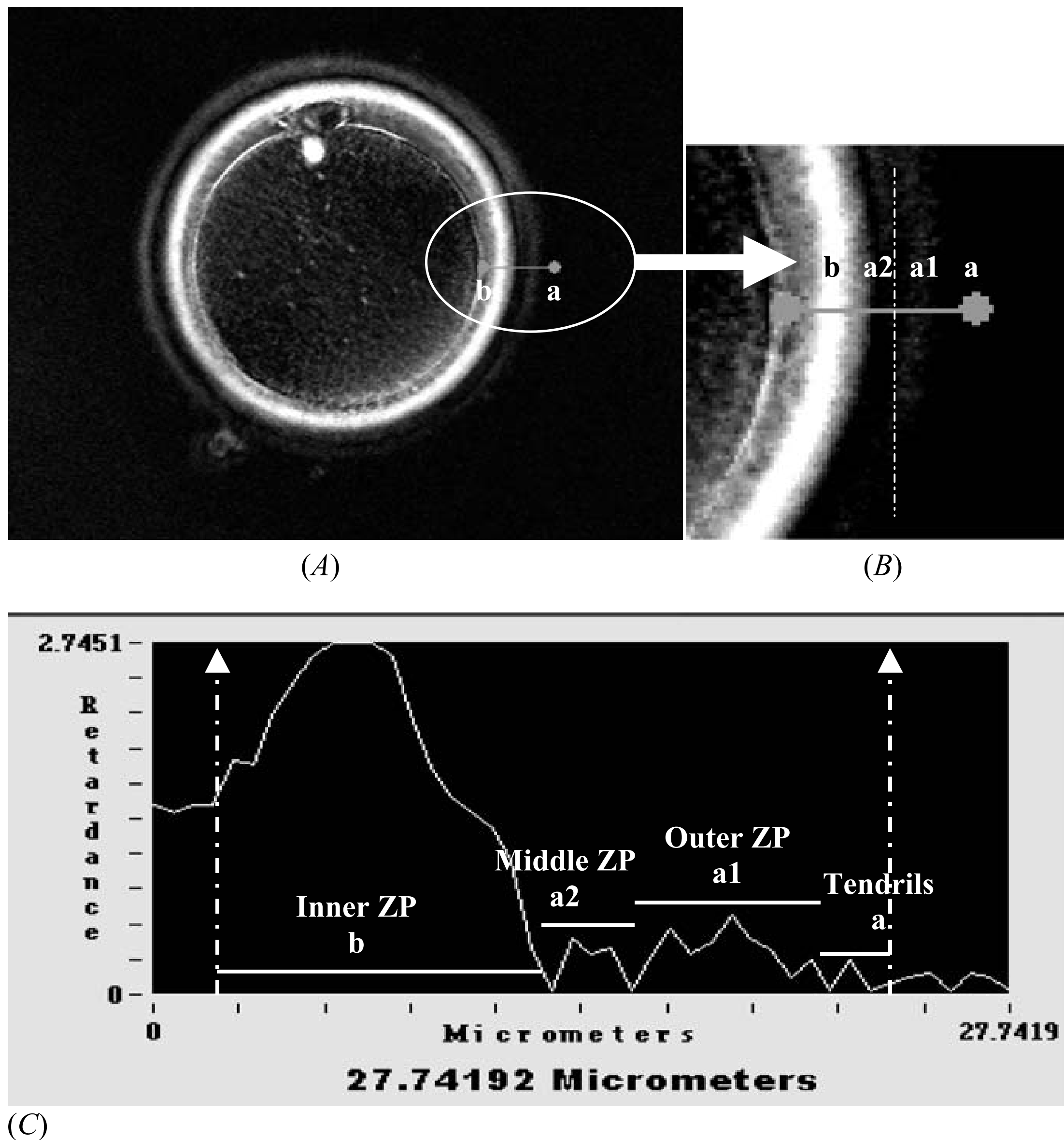

Figure 1 The three ZP layers and retardance of each layer presented as peaks. (*A*) Metaphase II oocyte with a line scan pulled from the outer layer of the ZP (a) up to the inner layer (b). (*B*) Section of the ZP magnified showing the three layers of the ZP starting from tendrils (a, a1, a2 and b). (C) Graph showing the three ZP peaks using the PolScope computer software. The *x*-axis shows the ZP thickness and the *y*-axis shows the highest ZP density.

Results

Effect of age on ZPT and ZPD

Twenty-nine patients had their oocytes collected in this trial: 17 from the older group and 12 from the younger group. The average age in the older group was 43.1 years (range 41–48 years) and in the younger group was 32.9 years (range 22–38 years).

The older group produced 93 oocytes and the younger group produced 100 oocytes. On day 0, the average ZPT was significantly higher in the older group compared with the younger group (24.1 ± 2.5 μm vs 23.1 ± 3.3 μm, $p = 0.01$). The average ZPD was not significantly different between the groups (2.8 ± 0.6 nm vs 2.8 ± 0.7 nm, $p = 0.6$) (Table 1).

On day 2 there were 43 embryos from the older group and 52 embryos from the younger group. The average ZPT for the embryos in the older group was higher than that in the younger group but the difference did not reach significance (22.2 ± 2.2 μm vs 21.7 ± 1.6 μm, $p = 0.29$). ZPD was also not significantly different between the two groups (2.8 ± 0.7 nm vs 2.9 ± 0.6 nm, $p = 0.57$) (Table 1).

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Table 1 Effect of the woman's age and time in culture on zona pellucida thickness (ZPT) and zona pellucida density (ZPD)

Culture day		Study group (older women)	Control group (younger women)	p (effect of woman's age)
0	ZPT	24.1 ± 2.5 μm[a]	23.1 ± 3.3 μm[a]	0.01
0	ZPD	2.8 ± 0.6 nm[b]	2.8 ± 0.7 nm[c]	0.6
2	ZPT	22.2 ± 2.2 μm	21.7 ± 1.6 μm	0.3
2	ZPD	2.8 ± 0.6 nm	2.9 ± 0.6 nm	0.57
5	ZPT	19.2 ± 2.7 μm	19.1 ± 5.0 μm	0.57
5	ZPD	4.4 ± 0.5 nm[b,d]	3.6 ± 0.7 nm[c,d]	0.009

Student t-test significant at $p < 0.05$.
[a]ZPD was significantly thicker in older women's day 0 oocytes than younger ones.
[b]ZPD in older women's blastocysts was significantly denser than 0 oocytes.
[c]ZPD in younger women's blastocysts was significantly denser than 0 oocytes.
[d]ZPD on day 5 was significantly denser in older women than younger ones.

Of the 29 patients studied, 11 chose to culture their embryos to the blastocyst stage, resulting in 20 blastocysts from the older group and 18 from the younger group. On day 5, ZPT was not different between the groups (19.2 ± 2.7 μm vs 19.1 ± 5.0 μm; $p = 0.81$). However, ZPD was significantly higher in the older group than the younger group (4.4 ± 0.5 nm vs 3.6 ± 0.7 nm, $p = 0.009$) (Table 1).

Effect of extended culturing on ZPT

The ZP in both the older and younger groups was significantly thinner in day 2 embryos compared with day 0 oocytes (older group: 22.2 ± 2.2 μm vs 24.1 ± 2.5 μm, $p = 0.01$; younger group: 21.7 ± 1.6 μm vs 23.1 ± 3.3 μm, $p = 0.0017$). On day 5, ZPT in both the older and younger groups was significantly thinner compared with day 2 embryos (older group: 19.2 ± 2.7 μm vs 22.2 ± 2.2 μm, $p < 0.001$; younger group: 19.1 ± 5.0 μm vs 21.7 ± 1.6 μm, $p < 0.001$) (Table 1).

Effect of extended culturing on ZPD

The same 29 patients were studied for density changes in the ZP between days 0 and 5. ZPD in both the older and younger groups was not significantly different in day 2 embryos compared with day 0 oocytes (older group: 2.9 ± 0.7 nm vs 2.7 ± 0.6 nm. $p = 0.26$; younger group: 2.8 ± 0.6 nm vs 2.8 ± 0.7 nm, $p = 0.99$). However, ZPD was significantly higher in day 5 compared with day 2 embryos (older group: 4.4 ± 0.5 nm vs 2.9 ± 0.7 nm, $p < 0.001$; younger group: 3.6 ± 0.7 nm vs 2.8 ± 0.8 nm, $p = 0.03$) (Table 1).

Discussion

Nawroth *et al.* (2001) and Pelletier *et al.* (2004) reported the average ZPT for metaphase II human oocytes as 13.07 ± 1.35 μm and 19.5 ± 2.2 μm, respectively. In this study, the average ZPT was 23.1 ± 3.3 μm. The sequence of ZPTs for each layer measured in this study for the same patient group agrees with those of Keefe *et al.* (1997) using polarized light on hamster oocytes and Pelletier *et al.* (2004) using polarized light on human oocytes.

Most of the early studies used tools for ZPT measurement that involved images captured using white light, which unlike polarized light lacks the ability to show fine structures of the ZP such as the 'tendrils' (Fig. 1). These are parts of the ZP involved in signalling between the ZP and the coronal layer (Fig. 1). The use of polarized light in this study, in combination with the unique image analysis software, can achieve ZP measurements more accurate and reproducible than previous techniques. This study also used a defined start and endpoint of measurement, enabling more accuracy than in previous studies.

In the study of Pelletier *et al.* (2004) the culture medium, protein supplementation and oxygenation were different from those used for this study (20% Irvine + 6% SSS vs 5% Sage + 5% HSA), but whether these differences in culture conditions had an effect on ZPT and ZPD is an issue which needs further investigation.

We observed that oocytes retrieved from older women have thicker zonae on day 0, but in oocytes retrieved in both older and younger women the zona thickness was the same on day 2 and day 5 of culture (Table 1). However, while all zonae become denser from day 0 to day 5, this is more apparent in oocytes retrieved from older women compared with those from younger women. There is controversy in the literature regarding this aspect, as some studies suggest a significant increase in ZPT with age (Nawroth *et al.*, 2001) whilst others have shown the opposite (Garside *et al.*, 1997).

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ZPD is a novel variable investigated in this study. The ZP is composed of protein filaments, which rearrange after fertilization and during embryo development (Silva *et al.*, 1997). The result of these rearrangements is to block sperm entry after fertilization and protection of the embryo until the hatching process. These changes might best be measured as density. The ZP hardness is difficult to quantify because of the need to fix the oocyte/embryo or use acids to dissolve it; in both cases, the oocyte/embryo will be damaged. We have assumed that ZPD reflects ZP hardness, but acknowledge this requires verification.

Our results showed that ZPD does not differ significantly between day 2 embryos of older and younger women, but the difference between the two age groups was significant on day 5. There is controversy in the literature regarding the relationship between ZP thickness and its hardness and it is said that the thicker the zonae the harder they are (Cohen *et al.*, 1990). In this study we did not find this.

Significant thinning of the ZP in this study was detected in day 2 embryos when cultured to day 5. This finding contradicts previous studies, which suggest that the ZP starts thinning on day 5 by the action of lysins to prepare the embryo for the hatching process (Gabrielsen *et al.*, 2001; Garside *et al.*, 1997). Pelletier *et al.* (2004) showed significant thinning of the ZP from day 3.

In conclusion, using PolScope technology we have studied ZP changes which may be useful in furthering our understanding of the events that occur prior to implantation. Extension of these preliminary observations may lead to methods enabling better selection of embryos for transfer in clinical IVF.

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