A comparative analysis of the zona pellucida birefringence of fresh and frozen–thawed human embryos

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Abstract

The cryopreservation of human embryos is thought to induce alteration in the glycoprotein matrix and lead to zona change. However, this assumption has been full of controversies till now. The objective of this study was to evaluate the effect of cryopreservation on zona pellucida of human embryos. Fresh ($n=106$, from 40 patients) and frozen–thawed embryos ($n=123$, from 40 patients) were obtained from consenting patients who received conventional IVF and ICSI treatment. The birefringence of zona pellucida in human fresh and frozen–thawed embryos was imaged and quantitatively analyzed using polarized light microscopy before embryo transfer. There was no significant difference in retardance and thickness of the zona pellucida multilaminar structure between the two groups. Pregnancy and implantation rates of transferred fresh and frozen–thawed embryos were also compared. No significant difference was found in the rates of clinical pregnancy (47.5 vs 37.5%) and implantation (24.5 vs 23.2%) between the two groups. This study suggests that there is no significant change in the zona pellucida birefringence of human embryos before and after cryopreservation.

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Introduction

Zona pellucida is an extracellular matrix of mammalian oocyte and embryo, and plays different roles in fertilization and preimplantation development (Dean 1992). Zona pellucida is a multilaminar structure and composed of four glycoproteins (ZP1, ZP2, ZP3, and ZP4) in humans (Lefièvre *et al.* 2004), which are synthesized, secreted, and assembled during folliculogenesis (Wassarman 2008). These zona glycoproteins are assembled in a three-dimensional highly ordered filament structure in zona pellucida (Green 1997, Wassarman *et al.* 2004). During fertilization, sperm has to recognize and bind to zona, induce acrosome reaction, penetrate zona and fuse with oocyte membrane (Wassarman 2002). After fertilization, oocyte will release cortical granules (Sun 2003, Tsaadon *et al.* 2006) and induce the structure change in zona to prevent polyspermy (Moos *et al.* 1995). At the cleavage stage, the contact between the blastomeres is loose, so the main role of zona is to protect the embryos and maintain their integrity (Herrler & Beier 2000). When they reach the blastocyst stage, embryos have to hatch out of zona and implant in endometrium (Gonzales *et al.* 2001). *In vivo*, hatching procedure is the result of interaction

between blastocyst and uterus (Gonzales & Bavister 1995), while *in vitro*, it is because of the tension of the periodic contraction and expansion, and enzymatic digestion of trophoblast (Schiewe *et al.* 1995b, Montag *et al.* 2000).

Since the first successful pregnancy following transfer of human frozen–thawed embryos (Trownson & Mohr 1983), the cryopreservation of human embryos has been improved greatly and has become an efficient method to increase the accumulative outcome in human IVF programs (Hoffman *et al.* 2003). Carroll *et al.* (1990) suggested that the freeze–thawing process induced alteration in the glycoprotein matrix and led to zona change. Assisted hatching (AH) is an artificial method to drill a hole in the zona to help blastocyst hatching. Considering the ‘zona change’ assumption, AH is also proposed to improve the implantation rate in frozen embryo transfer (FET) cycles (Cohen *et al.* 1991). To date, most researchers suggest that selective AH may have benefits to embryo implantation (Cohen *et al.* 1992). However, it is still controversial whether the cryopreservation procedure will change the structure of zona pellucida and AH is necessary to improve the implantation ability of frozen–thawed embryos (Veiga *et al.* 2004). Some researchers suggested that

cryopreservation will induce zona change, so AH can increase the implantation of frozen–thawed embryos (Check *et al.* 1996, Tao & Tamis 1997, Gabrielsen *et al.* 2004, Balaban *et al.* 2006, Ge *et al.* 2008), while other researchers have opposing views (Edirisinghe *et al.* 1999, Primi *et al.* 2004, Ng *et al.* 2005, 2008, Sifer *et al.* 2006).

Several techniques have been applied to identify this zona change. In the early stages, researchers selected the enzymatic digestion (α -chymotrypsin) method to quantitatively identify the change in human zona after fertilization (Schiewe *et al.* 1995a) and cryopreservation (Matson *et al.* 1997, Manna *et al.* 2001), but as the digestion time could be influenced by enzymatic activity and environmental factors (temperature and pH), its precision was limited. Transmission electron microscopy (TEM) was applied to illuminate the ultrastructure of human zona after cryopreservation (Nottola *et al.* 2007, 2008). While electron microscopy requires samples to be fixed in advance, which lose further development potential after fixation, it cannot provide quantitative data about this zona change. Sun *et al.* (2003) developed a microelectromechanical systems-based multi-axis cellular force sensor, characterized mouse zona mechanical properties, and quantified the mechanical property differences of the zona before and after fertilization. Other force sensors were subsequently developed and applied to measure the mechanical behavior of zona pellucida in mouse (Murayama *et al.* 2006), human (Wacogne *et al.* 2008), and bovine (Papi *et al.* 2009a, 2009b). However, these force sensors were not commercially available in the clinic.

A polarized light microscope was developed to study the birefringence of living cells (Oldenbourg & Mei 1995). The polarized light microscope uses novel electro-optical hardware and digital processing to image macromolecular structures in cells on the basis of their birefringence (an inherent physical property of highly ordered molecules; Oldenbourg 1996). There are two birefringent structures in human oocytes and embryos: spindle and zona pellucida. Polarized light microscopy has been employed for the noninvasive visualization of meiotic spindles in human oocytes (Wang *et al.* 2001a, 2001b), which proved no negative influence on the development potential of these oocytes and future embryos. Noninvasive polarized light microscope also provides an opportunity to investigate the structure of zona pellucida quantitatively in murine (Keefe *et al.* 1997, Silva *et al.* 1997) and human (Pelletier *et al.* 2004, Shen *et al.* 2005, Kilani *et al.* 2006, Rama Raju *et al.* 2007, Montag *et al.* 2008, Madaschi *et al.* 2009). To our knowledge, there is no quantitative analysis of zona pellucida birefringence of human embryos before and after cryopreservation to date.

Birefringence is an intrinsic property of human zona, which can be used to analyze the magnitude and

orientation of zona molecular order (Oldenbourg 1996). In this study, we tested the hypothesis that the cryopreservation will change the structure of zona pellucida in human embryos. To test this assumption, this study quantitatively analyzed zona pellucida birefringence of fresh and frozen–thawed human embryos using polarized light microscopy. Better understanding of the cryopreservation effect on human zona pellucida will clarify whether the procedure would reduce implantation of human embryos due to zona change.

Results

Multilaminar structure of human embryo zona pellucida

The polarized light microscope (Polscope) is able to distinguish the multilaminar structure of human embryonic zona pellucida, which is inconspicuous with conventional Hoffmann optics. Obvious variance was observed in zona retardance and thickness between human embryos, even from the same patient (Fig. 1). According to Polscope system analysis, the innermost layer (IL) is the most birefringent and thickest layer of the human zona pellucida, and the orientation of filaments in this layer is radial; the retardance and thickness of outermost layer (OL) are less, and the orientation of filaments is tangential; the thin middle layer (ML) exhibits minimal birefringent and the orientation of filaments is random (Table 1).

Figure 1 The birefringent zona pellucida of human embryos. (A) Zona pellucida of human embryos is inconspicuously imaged by conventional Hoffmann inverted microscope; (B) birefringent zona pellucida of human embryos can be imaged distinctly using polarized light microscopy, which is multilaminar and displays obvious variance between embryos; birefringent zona pellucida of fresh (C) and frozen–thawed (D) human embryos was analyzed using polarized light microscopy before embryo transfer. Scale bar = 10 μ m.

Table 1 Mean retardance magnitude and thickness of individual zone layers in human fresh and frozen–thawed embryos.

	Fresh embryos	Frozen–thawed embryos	P value
No. of patients	40	40	–
No. of embryos	106	123	–
Zona inner layer			
Retardance (nm)	3.18±0.65	3.12±0.72	0.569
Thickness (μm)	8.97±1.58	8.93±1.60	0.834
Zona middle layer			
Retardance (nm)	0.25±0.01	0.25±0.02	0.807
Thickness (μm)	3.34±0.55	3.28±0.51	0.398
Zona outer layer			
Retardance (nm)	0.79±0.19	0.79±0.20	0.963
Thickness (μm)	6.89±1.57	6.78±1.72	0.625
Zona total thickness (μm)	19.20±2.52	18.99±2.48	0.521

Values are mean ± s.d.

Retardance and thickness of individual zona layers of human fresh and frozen–thawed embryos

Retardance and thickness in individual zona layers of 106 fresh embryos and 123 frozen–thawed embryos were quantitatively analyzed using polarized light microscopy. The results showed no significant difference in mean retardance of individual layers of zona pellucida ($P_{IL}=0.569$, $P_{ML}=0.807$, $P_{OL}=0.963$; Table 1) between the groups. Similar to the magnitude of retardance, the mean thickness of individual layers and total thickness did not differ significantly between the groups ($P_{IL}=0.834$, $P_{ML}=0.398$, $P_{OL}=0.625$, $P_{TOTAL}=0.521$; Table 1).

Pregnancy and implantation rate of human fresh and frozen–thawed embryos

In the fresh embryo group, 106 embryos from 40 patients were transferred, 19 patients became pregnant (19/40, 47.5%), and 26 embryos implanted (26/106, 24.5%).

Table 2 Comparison of the characteristics and clinical outcomes of patients undergoing fresh or frozen embryo transfer cycles.

	Fresh cycles	Frozen cycles	P value
No. of patients	40	40	–
Age (years)	29.8±4.1	29.1±3.5	0.419
Type of infertility (%)			0.179
Primary	16 (40.0)	22 (55.0)	
Secondary	24 (60.0)	18 (45.0)	
Cause of infertility (%)			0.612
Tubal	29 (72.5)	26 (65.0)	
Male	4 (10.0)	7 (17.5)	
Mixed	7 (17.5)	7 (17.5)	
Insemination procedure (%)			0.446
IVF	31 (77.5)	28 (70)	
ICSI	9 (22.5)	12 (30)	
Clinical outcomes (%)			
Clinical pregnancy	19/40 (47.5)	15/40 (37.5)	0.366
Implantation	26/106 (24.5)	19/82 (23.2)	0.829

Values are number (%) and mean ± s.d.

In the frozen–thawed embryo group, 82 embryos from 40 patients were transferred, 15 patients were pregnant (15/40, 37.5%), and 19 embryos implanted (19/82, 23.2%). No significant difference was found in pregnancy ($P=0.366$) and implantation rate ($P=0.829$) between the groups (Table 2).

Discussion

In this study, the cryopreservation effect on the zona pellucida birefringence was quantitatively analyzed. Our result did not support the ‘zona change’ hypotheses and suggests that there is no significant change in the zona pellucida birefringence of human embryos before and after cryopreservation.

A zona change was first raised based on the studies of oocyte cryopreservation (Carroll *et al.* 1990). The authors found that frozen–thawed mouse oocytes had a reduced rate of fertilization when compared with unfrozen controls and suggested that freeze–thaw cycle induced zona change. Previous studies also found that cryopreservation increased the zona digestion time of mouse (Matson *et al.* 1997) and human (Manna *et al.* 2001) oocytes. After TEM, Nottola *et al.* (2007, 2008) showed that the amount and density of cortical granules appeared to be abnormally reduced in frozen–thawed human oocytes, and this feature was frequently associated with an increased density of the inner zona, possibly related to the occurrence of zona change. Thus, it may not be suitable to extend these results from oocyte cryopreservation on embryos, since cortical granules exist in mature oocytes, and this zona modification could not occur in frozen–thawed embryos in theory. The study of Matson *et al.* (1997) also showed that cryopreservation of embryos was not associated with zona change in dissolution time or reduced implantation.

This study implied that the conventional cryopreservation procedure could not be sufficient to influence the magnitude and orientation of the zona molecular order and change zona birefringence. The freeze–thaw procedure induces various cryodamages (osmotic shock and ice crystal formation) in human embryos (Pegg 2002). The structure of zona pellucida is composed of glycoproteins (with low amount of water) and could be more tolerable to cryodamages, while blastomeres of human embryos contain high amount of water, which are vulnerable in freeze–thaw procedure. Van den Abbeel & Van Steirteghem (2000) reported that the incidence of zona pellucida damage was only 2.3% when embryos were frozen–thawed using plastic ministraw, although minor damage was not taken in consideration by these authors.

In fact, the term ‘zona change’ is a general description, and no method can measure each property of zona pellucida before and after cryopreservation. Enzymatic digestion method measures the zona solubility.

Electron microscopy provides the ultrastructural information of zona pellucida. Cellular force sensor quantifies the zona mechanical properties. Polarized light microscopy analyzes the density (measured as retardance) and thickness of individual zona layers.

A clinical standard to describe the zona property could be the implantation of human embryos (Matson *et al.* 1997), which means that the embryos hatched from its zona and implanted in endometrium, although it is an indirect parameter. In this study, the implantation rate of human frozen–thawed embryos did not reduce after the freeze–thaw procedure. The result implied that cryopreservation did not affect human embryo hatching and implantation *in vivo*. Edgar *et al.* (2000) quantitatively analyzed the impact of cryopreservation on implantation potential of human early cleavage-stage embryos ($n > 5000$), and the results showed that full intact thawed embryos had the same implantation potential (11.3%) as their fresh counterpart (11.4%), but partial intact thawed embryos had lower implantation rate (6.2%). The authors concluded that blastomere loss was associated with the implantation potential reduction in human frozen–thawed embryos.

Although this study suggested that cryopreservation of human embryos did not overtly change zona birefringence, the results are not sufficient to answer whether AH is necessary for frozen cycles. The process of blastocyst hatching and implantation is the interaction of zona pellucida, trophoblast, and uterus *in vivo*. So considering whether AH is necessary for frozen–thawed embryos just based on the property of zona pellucida may bring biases. Cryodamages may lead to blastomere loss and reduce the cell number of trophoblast during blastocyst forming, and further impair the hatching ability of future blastocysts (Van Blerkom 1993).

This and other studies revealed multilaminar birefringent structure of human zona (Pelletier *et al.* 2004, Shen *et al.* 2005, Kilani *et al.* 2006, Rama Raju *et al.* 2007, Montag *et al.* 2008, Madaschi *et al.* 2009). Most data from this study were consistent with previous studies, except that the outer zona layer was thicker than that reported by Pelletier *et al.* ($3.7 \pm 1.4 \mu\text{m}$). We suppose that two reasons may lead to this difference: first, the outer zona layer is especially variable (Fig. 1B), so it is not easy to define the boundary in some cases (it may rely on the observer's experience); secondly, we cannot exclude the ethnic differences in zona pellucida.

This is still a preliminary study. There are two variables: the source of the embryos (from different patients) and the state of the embryos (fresh and frozen). It would be a better experimental design to analyze the zona structure of the same human embryo before and after cryopreservation, due to the significant variance of zona pellucida. However, this is not easy to achieve in a clinical setting, as it means that all embryos would have to be marked, cryopreserved, stored, and thawed individually, making the freeze–thaw procedure much

more complicated and which could have adverse influences on the clinical outcomes. Furthermore, the implantation rates could not be compared between paired samples in this experimental design. In future, we expect a randomized prospective experiment with larger sample size to obtain more reliable conclusions.

Materials and Methods

Source of human embryos and patients' selection

In this study, a total of 106 fresh embryos and 123 frozen–thawed embryos at the cleavage stage were respectively obtained from 40 fresh cycle patients and 40 frozen cycle patients, who received conventional IVF and ICSI treatment in Reproductive and Genetic Hospital of CITIC-XIANGYA. Patients for both fresh and frozen–thawed embryo transfer cycles were included if they met the following criteria: 1) normal baseline endocrine parameters; 2) tubal or male infertility; 3) < 37 years. The main characteristics of patients were not significantly different between the groups (Table 2), including age, type of infertility (primary or secondary), cause of infertility (tubal, male, or mixed) and insemination procedure (IVF or ICSI). Signed informed consent was obtained from these patients before performing the experiments. The study was approved by the ethics committee at the local hospital.

IVF and embryo culture

All patients underwent controlled ovarian stimulation induced by rFSH (Gonal-F, Serono), after pituitary downregulation by the administration of GnRH analogs (Leuprorelin, Takeda, Japan). GIII series culture system (Vitrolife, Göteborg, Sweden) was used for *in vitro* culture of human oocytes and embryos, according to the user's manual. On day 0, oocyte retrieval was performed by ultrasound-guided transvaginal aspiration 36 h after hCG (5000–10 000 IU; Profasi, Serono) administration. Cumulus–oocyte complexes (COCs) were collected in 1 ml G-FERT medium (Vitrolife) and cultured for 3–6 h to induce full maturation of oocytes.

In conventional IVF programs, the COCs were inseminated with prepared sperms (final concentration about $10^5/\text{ml}$) and cultured in fresh G-FERT medium (2–3 COCs/1 ml) to the next day. In ICSI program, the COCs were briefly rinsed in 80 IU/ml hyaluronidase (Sigma Chemical Co.), and cumulus cells were removed by repeated gentle aspiration. Denuded oocytes were washed twice in fresh G-MOPS (Vitrolife) handling medium and performed ICSI according to standard protocol (Van Steirteghem *et al.* 1993). After ICSI, inseminated oocytes were cultured in G-FERT (10 $\mu\text{l}/\text{drop}$) till next day.

After fertilization evaluation on day 1, embryos were cultured in G1.3 (Vitrolife) medium (50 $\mu\text{l}/\text{drop}$) in groups (1–3 embryos/drop), according to the pronuclear scoring till day 3.

All culture media were supplemented with 5% human serum albumin (HSA; Vitrolife), covered under paraffin oil (Vitrolife), and pre-equilibrated overnight. All oocytes and embryos were cultured at 37°C , 6% CO_2 , 5% O_2 .

Assessment and selection of fresh embryo

On day 1 (16–18 h postinsemination), fertilization assessment was performed according to the number, shape, and distribution of pronuclei and nucleoli (the pronuclear scoring system; Scott *et al.* 2000). Abnormal fertilization (NPN, 1PN, or ≥ 3 PN) may be as a result of zona dysfunction (Tarin *et al.* 1999), so all embryos in this study were normal fertilized zygotes (2PN).

On day 3 (66–68 h), morphology of fresh embryo was assessed, and fresh embryos were selected for transfer according to the conventional criteria (De Placido *et al.* 2002) in this study:

- 1) Normal fertilization;
- 2) Number of blastomeres is at least four;
- 3) Shape of blastomere is almost the same;
- 4) Fragments of embryos do not exceed 20%;
- 5) Blastomere is transparent without serious cytoplasmic inclusion or vacuoles.

On day 3, 2–3 fresh embryos with highest morphology scores were selected for transfer. The other embryos accorded with these criteria were cryopreserved.

Cryopreservation and thawing of human embryos

The procedure of cryopreservation and thawing of human embryo were based on the method of Testart *et al.* (1986).

Cryopreservation

On day 3, fresh embryos (≥ 4 -cell, fragments $\leq 20\%$) were cryopreserved using a programmable freezer (Kryo 360-1.7, Planer Products Ltd, Sunbury-On-Thames, UK) with Embryo Freeze-Kit 1 (Vitrolife) as cryoprotectant.

The components are as follows:

Cryo-PBS = PBS with 25 mg/ml HSA.

Freeze solution 1 (FS1) = 1.5 M PrOH in Cryo-PBS

Freeze solution 2 (FS2) = 1.5 M PrOH + 0.1 M sucrose in Cryo-PBS

Briefly, all components were pre-equilibrated at room temperature (25 °C). Embryos were rinsed in 0.5 ml Cryo-PBS and kept in FS1 for 10 min. Embryos were transferred into FS2, loaded (3 embryos/straw) into straws (Minitube, Tiefenbach, Germany) immediately, and the 0.1 ml FS2 containing embryos was sealed with air bubbles. The freezing program for embryos in our unit was as follows: starting temperature: 25 °C; rate of cooling: -2 °C/min from 25 to -7 °C; soak at -7 °C for 5 min; manual seeding; hold the temperature at -7 °C for 10 min; rate of cooling: -0.3 °C/min from -7 to -30 °C; rate of cooling: -50 °C/min from -30 to -140 °C. The frozen straw was quickly transferred from the freezing chamber to a reservoir of liquid nitrogen (-196 °C).

Thawing

Embryo Thaw-Kit 1 (Vitrolife) was used in the thawing procedure. The components are as follows:

Thaw solution 1 (TS1) = 1.0 M PrOH + 0.2 M sucrose in Cryo-PBS

Thaw solution 2 (TS2) = 0.5 M PrOH + 0.2 M sucrose in Cryo-PBS

Thaw solution 3 (TS3) = 0.2 M sucrose in Cryo-PBS.

Briefly, all components were pre-equilibrated at room temperature (25 °C). Frozen embryos were thawed on the FET day at room temperature (25 °C) for 30 s and then at 30 °C in a water bath for 30 s. Subsequently, the cryoprotectant was removed by washing the embryos successively through TS1, TS2, TS3, and Cryo-PBS for 5 min respectively. And thawed embryos were transferred into G2.3 medium and cultured for 2–3 h before transfer.

Assessment and selection of frozen–thawed embryos

After thawing, frozen embryos were examined for the number and integrity of blastomeres and the degree of fragmentation, and graded according to the criteria of Veeck (1988). Embryos were classified as fully intact (100% blastomeres survived), partially damaged ($\geq 50\%$ blastomeres survived), or degenerated ($< 50\%$ blastomeres survived). Frozen–thawed embryos were considered to have survived if $\geq 50\%$ of blastomeres were intact. Two or three survived frozen embryos with highest morphology scores (fully intact embryos were preferable) were selected for transfer. In this study, 123 embryos were thawed, 97 embryos (78.9%) survived, including 52 fully intact embryos (42.3%) and 45 partially damaged embryos (36.6%), and 26 embryos (21.1%) were degenerated.

Embryo transfer and implantation confirmation

On day 3, selected fresh embryos or frozen–thawed embryos were loaded in catheters (Wallace 1816N, Hythe, UK) with 20–25 μ l G2.3 culture medium and transferred into patients' uteruses. Clinical pregnancy and implantation were confirmed by ultrasonic monitor (pregnant sac and fetal heart beat) 4 weeks after embryo transfer.

Imaging and analysis of zona pellucida using polarized light microscopy

To avoid the potential influence of *in vitro* culture on zona structure, all embryos were imaged on the day of embryo transfer, so the culture duration of both fresh and frozen–thawed embryos was 3 days.

To image zona pellucida, each human embryo was placed in a 5 μ l drop of G-MOPS (Vitrolife) handling medium covered with warm paraffin oil in a glass-bottomed (0.17 mm thick) Petri dish (20 mm diameter; Delta TPG dish, Biotech, Butler, PA, USA). Embryos were examined with an inverted microscope (Nikon TE-2000U, Tokyo, Japan) equipped with Hoffman interference optics 20 $\times$ objective lenses equipped with LC Polscope filters, analog video camera (COHU, Cambridge Research and Instrumentation, USA), and a personal computer (Dell, Round Rock, TX, USA) running image analysis software (LC-Polscope pro 4.4; Cambridge Research and Instrumentation, Woburn, MA, USA). Dishes were maintained at 37 °C during examination.

with a thermal plate (IVF-1200, Pacific Contrast Co., San Diego, CA, USA) and objective heater (HT300, Minitübe). Alignment of the microscope and calibration of the software were performed before zona imaging. Zona pellucida was imaged five times by focusing at an equatorial plane of each embryo to ensure the accuracy of image representation of zona layers. Digital images were saved to a compact disk, and best images were selected for subsequent analysis.

Owing to significant variance in retardance and thickness of human zona, we used the analysis method of Pelletier *et al.* (2004) to ensure the accuracy and reproducibility. Briefly, eight points distributing evenly on zona (1–12 clock position) were sampled to measure the retardance and thickness of each layers (try to avoid the cumulus cells, sperms, and abnormal area on zona). We drew chords extending from the inner boundary of the zona outward, and collected retardance magnitude (R_i , as retardance) at the midpoint and length (T_i , as thickness) of each chord for each layer. Mean retardance ($\bar{R} = (\sum_{i=1}^8 R_i)/8$) and thickness ($\bar{T} = (\sum_{i=1}^8 T_i)/8$) for each layer were calculated and compared statistically.

All measurements were collected by the same observer. The duration of zona imaging was 2–5 min in each sample. The accuracy of thickness measurement in the Polscope system was calibrated with standard scale slide (Ronchi slide, 50 $\mu\text{m}/\text{scale}$, Edmund Industrial Optics, Barrington, NJ, USA) prior to the experiment. To confirm the reproducibility and reliability of the image capture and analysis system, nine embryos from two patients were imaged and analyzed twice, and the results from two measurements showed highly correlated (the middle layer retardance was constantly as low as background value, which showed weak linear correlation).

Statistical analysis

The reproducibility test for Polscope system was performed using paired samples *t*-test. The characteristics of patients were compared between the groups using two-tailed Student's *t*-test and χ^2 test where appropriate. The retardance and thickness of individual zona layers between the groups were quantitatively analyzed using two-tailed Student's *t*-test. The pregnancy and implantation rates between the groups were compared using χ^2 test. Significance was considered as $P < 0.05$.

Declaration of interest

All authors have no potential conflict of interest to disclose.

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