Article

Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI cycles

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Abstract

A retrospective study recently showed that oocytes presenting with a high birefringence of the inner zona layer were more often associated with conception cycles. To further investigate these findings, a prospective study was conducted between September 2005 and September 2006 including intracytoplasmic sperm injection (ICSI) cycles presenting with at least two embryos for transfer. Using a polarization imaging system, oocytes were classified prior to ICSI treatment as having either a high zona birefringence (HZB) or a low zona birefringence (LZB) of the zona pellucida. Using zona birefringence as the only selection criterion, two fertilized oocytes, preferably derived from HZB oocytes, were selected for further culture and transfer. The required criteria were met by 135 ICSI cycles (124 patients; 34.9 ± 4.1 years of age). Embryos for transfer were used in 20 cycles derived from HZB/HZB oocytes, in 50 cycles from HZB/LZB oocytes and in 65 from LZB/LZB oocytes. The corresponding implantation ($P < 0.025$), pregnancy ($P < 0.005$) and live birth ($P < 0.025$) rates were significantly different between HZB/HZB and HZB/LZB versus LZB/LZB group. Embryo development was superior in embryos derived from HZB oocytes. This study concludes that oocyte zona birefringence is a good selection criterion and a good predictive criterion for embryo implantation potential.

Keywords: *human oocyte, ICSI, implantation, polarization microscopy, zona pellucida*

Introduction

An important factor for the success of any assisted reproduction technology is the potential of human oocytes to develop into implantation-competent embryos. Numerous attempts have been made to identify prognostic factors based on morphological characteristics of the oocyte that may allow a prediction of oocyte quality, fertilization rate and embryo development (e.g. De Sutter *et al*., 1996; Serhal *et al*., 1997; Xia, 1997; Balaban *et al*., 1998; Loutradis *et al*., 1999). The predictive value of the criteria used in these studies is still controversial.

The introduction of polarization light microscopy (Oldenbourg, 1996) allowed for the non-invasive visualization of subcellular structures in oocytes. Using this microscopic technique, new prognostic factors of oocyte quality were proposed, such as spindle imaging (Wang *et al*., 2001; Rienzi *et al*., 2003, 2004) and zona birefringence (Pelletier *et al*., 2004; Shen *et al*., 2005; Rama Raju *et al*., 2007). Several studies reported on the importance of the presence of a spindle in human metaphase II (MII) oocytes following retrieval in an assisted cycle. Some studies showed higher fertilization rates for oocytes with a visible spindle (Wang *et al*., 2001; Cohen *et al*., 2004) whereas others did not show a difference (Moon *et al*., 2003). The same controversy exists regarding the predictive value of spindle visualization of MII oocytes for embryonic developmental competence on day 3

(Wang *et al.*, 2001; Moon *et al.*, 2003; Rama Raju *et al.*, 2007; versus Cohen *et al.*, 2004). A possible explanation for these contradictory observations could be that the dynamics of spindle formation during oocyte maturation were not considered in all studies (Eichenlaub-Ritter *et al.*, 2002; Rienzi *et al.*, 2004; De Santis *et al.*, 2005; Montag *et al.*, 2006).

Besides the visualization of the spindle, zona imaging was proposed as another valuable predictive marker of oocyte/embryo quality. The underlying concept is that polarization microscopy allows the distinction of three layers within the zona pellucida of human oocytes. The inner layer exhibits the highest amount of birefringence (Pelletier *et al.*, 2004). In a retrospective study, Shen *et al.* (2005) observed that zona birefringence of the inner zona layer was different in conception versus non-conception cycles. Recently, Rama Raju *et al.* (2007) found a correlation between zona birefringence and the potential of an embryo to develop to the blastocyst stage. In order to prove the predictive value of zona imaging, a prospective study was conducted using zona imaging of unfertilized MII oocytes as the primary selection criterion for embryo transfer on day 3. The end-points of this study were implantation, pregnancy and live birth rate.

Materials and methods

Patients and study design

Between September 2005 and September 2006 all intracytoplasmic sperm injection (ICSI) cycles with two embryos available for embryo transfer were included in this study. Cycles with only one embryo or cycles where the patients insisted on the transfer of three embryos were excluded from further evaluations. All cycles performed in combination with polar body biopsy for aneuploidy testing were also excluded. In all cycles, male subfertility was the indication for ICSI. Zona imaging by polarization light microscopy was performed by the same investigator. All cycles that were performed in the absence of this investigator during the same time period received no zona evaluation and were used as controls (n = 68). As there was no intention to compare the results of zona imaging directly with controls (as this would have required another set-up) this data was not included in the **Results** section.

Ovarian stimulation and oocyte retrieval and culture

Stimulation of patients, follicular puncture and denudation of oocytes, sperm preparation and ICSI were performed according to standard protocols (Montag *et al.*, 1997). The same stimulation protocol was used in all cycles. Following denudation, oocytes were placed individually into five 5-µl droplets of medium under oil in a glass bottom dish (WillCo Wells BV dish, MTG, Altdorf, Germany) and cultured for 1–2 h prior to zona imaging. Oocytes with vacuolization were excluded and neither used for zona imaging nor for ICSI. All media used for oocyte and sperm manipulation, as well as for embryo culture and transfer, were purchased from Cook (Mönchengladbach, Germany). Incubation was performed in a mini-incubator (Minc, Cook) using pre-mixed gas with low oxygen (6% CO_2, 5% O_2, 89% N_2) at 37°C.

Live zona imaging

Live zona imaging of individual oocytes was performed non-invasively on a Nikon Eclipse TE-2000 inverted microscope equipped with ×10, ×20 and ×40 Hoffmann interference optics, ×20 and ×40 stain-free objectives, a circular polarization filter and liquid crystal analyser optics. The birefringence analysis including autocalibration was fully controlled by a polarization imaging software module (OCTAX ICSI Guard™, OCTAX Microscience GmbH, Altdorf, Germany) implemented in an imaging software system (OCTAX Eyeware™). The microscope was further equipped with a motorized stage (OCTAX) containing a fully heated ceramic plate with a glass insert in the objective pathway. The temperature of the heated plate was adjusted with an external calibrated sensor to maintain 37.0 ± 0.5°C in a 5-µl medium droplet in the glass bottom dish during microscopic observation (Montag *et al.*, 2006).

Prior to zona birefringence analysis, oocytes were screened for the presence or absence of the first polar body by conventional light microscopy and change of focus to exclude immature oocytes (germinal vesicle, metaphase I). Individual images combining bright field (green) and birefringence (red) views were recorded online by the imaging software. Based on the intensity and uniformity of the birefringent inner zona layer, MII oocytes were classified as having a high zona birefringence (HZB) or low zona birefringence (LZB). More specifically, oocytes with a high-intensity birefringent inner zona layer where the birefringence was mostly uniform around the entire cell were classified as HZB and the highest priority was given to an evenly bright and very thick inner zona layer. Oocytes showing an uneven and/or low birefringence distribution around the cell were classified as LZB. The classification was based solely on subjective judgement and not supported by any measuring device. This allowed a rapid screening of 10 oocytes within 2 min. Due to the subjective nature of the screening, classification was always performed by the same investigator to eliminate interpersonal variation. The presence and location of the meiotic spindle was not a topic of this study and these data were therefore not included in the analysis.

ICSI, culture, embryo transfer and pregnancy

ICSI was performed within 1 h after zona imaging. Oocytes were kept in the same order as during zona imaging and thereafter cultured individually in 30-µl medium droplets under oil.

The selection of two oocytes for further embryo culture and transfer was performed 18 h after ICSI, at the pronuclear stage of those oocytes presenting with two pronuclei of equal size in close proximity and centrally located within the ooplasm. The primary criterion for selection was the intensity of zona birefringence. Preferably, two oocytes initially presenting with HZB were chosen for further embryo culture and transfer, followed by one HZB and one LZB oocyte or followed by the selection of two LZB oocytes in

the individual treatment cycles. In some cycles, HZB oocytes were available but did not fertilize and therefore LZB oocytes were chosen. This may account for minor discrepancies between data shown in **Tables 1** and **2**. Oocytes were cultured individually until embryo transfer on day 3. Embryo quality was assessed on day 2 and day 3 and an embryonic score was calculated as proposed by Steer *et al*. (1992). Embryo transfer was essentially performed using Sydney IVF transfer catheter (Cook). For calculation of the pregnancy rates, only cycles with visible gestational sacs, documented by ultrasound, were considered. The implantation rate was calculated from the number of gestational sacs divided by the total number of embryos transferred.

Statistics

The cycles were sorted into three groups according to whether oocytes were classified as both HZB (HZB/HZB) or LZB (LZB/LZB) or one of each (HZB/LZB). Chi-squared test was used for comparing implantation rate and pregnancy rate between the HZB/HZB, HZB/LZB and LZB/LZB groups and number of top quality embryos on day 3 in the HZB and LZB groups. Analysis of variance (ANOVA) was performed for comparing maternal age, number of MII oocytes between the HZB/HZB, HZB/LZB and LZB/LZB groups and embryonic score on day 2/3 between HZB- and LZB-derived embryos.

Results

A total of 1029 MII oocytes were obtained in 135 ICSI cycles from 124 patients. Polarization microscopy enabled the differentation of oocytes based on the intensity and brightness of the inner layer in the birefringence image of the zona pellucida (red colour channel in the combined brightfield/birefringence image). Oocytes showing a high intensity and uniform brightness were classified as HZB (**Figure 1a**) and oocytes showing an uneven and/or low brightness were classified as LZB (**Figure 1b**). Owing to these criteria, it was possible to decide within seconds whether an oocyte showed LZB or HZB. The majority of oocytes (832/1029, 80.9%) showed LZB. In 44 treatment cycles (43 patients), all available oocytes were classified as LZB (**Table 1**). The mean age of patients in this group was not different to patients presenting with only one HZB oocyte. There was a significant difference between both groups regarding the total number of MII oocytes available ($P < 0.05$), but not between fertilization rates. In cycles with at least one HZB oocyte, the percentage of HZB oocytes varied from 6.7% to 62.5%, the mean being 26.5% (corresponding to a mean of 2.16 HZB oocytes per cycle out of a mean of 8.16 MII oocytes per cycle).

The primary selection criterion for further culture and subsequent embryo transfer was zona birefringence. Two embryos derived from HZB oocytes (HZB/HZB) were available in 20 cycles, HZB/LZB embryos were available in 50 cycles and LZB/LZB embryos in 65 cycles. For all cycles, the overall implantation and pregnancy rates were 21.9% and 40.0%, respectively. Mean maternal age and fertilization rates were not different between groups. In the HZB/HZB group, significantly more MII oocytes were available compared with the other two groups ($P < 0.005$). There was no difference in the number of MII oocytes between the HZB/LZB and LZB/LZB group (**Table 2**). When analysed according to the relevant subgroups, significantly higher implantation and pregnancy rates were observed with transfer of HZB/HZB- (40.0% and 65.0%, respectively; $P < 0.025$) and HZB/LZB- (26.0% and 50.0%, respectively; $P < 0.005$) derived embryos compared with transfer of LZB/LZB-derived embryos (13.1% and 24.6%). The miscarriage rate was not different between the groups; however, the live birth rate was significantly different ($P < 0.025$) between HZB/HZB (60%) and HZB/LZB (40%) groups versus LZB/LZB group (20%).

Based on the calculation of embryo score, embryo development on day 2 was not different between embryos derived from HZB or LZB oocytes (12.5 ± 3.5 versus 12.3 ± 4.0). However, on day 3 the embryo score in the HZB group differed significantly from the LZB group (26.8 ± 7.9 versus 21.5 ± 9.3; $P < 0.025$). Accordingly, the percentage of high-quality embryos (8-cell grade A) on day 3 was higher in the HZB group compared with the LZB group (41.7% versus 24.4%; $P < 0.025$).

Table 1. The number of metaphase II (MII) oocytes and fertilization rates in relation to oocyte birefringence.

Parameter	*Only LZB oocytes*	*At least one HZB oocyte*	P-*value*
No. of cycles	44	91	–
No. of patients	43	87	–
Mean maternal age ± SD (years)	35.6 ± 4.5	34.3 ± 3.8	NS
No. of MII oocytes	286	743	0.05
Mean no. of MII oocytes/cycle ± SD	6.5 ± 3.2	8.2 ± 3.4	
Fertilization rate (%)	204/286 (71.3)	510/743 (68.6)	NS

HZB = high zona birefringence; LZB = low zona birefringence; NS = not statistically significant.

Table 2. Cycle outcome in relation to oocyte zona birefringence.

Parameter	*HZB/HZB*	*HZB/LZB*	*LZB/LZB*	P-*value*
No. of cycles	20	50	65	–
Mean maternal age (years) ± SD	33.9 ± 3.6	34.6 ± 4.0	35.4 ± 4.1	NS
Mean no. of MII oocytes ± SD	10.9 ± 3.9[a,b]	7.6 ± 3.4[a]	6.7 ± 2.7[b]	< 0.005
HZB oocytes (%)	73/218 (33.5)[a]	88/378 (23.3)[b]	36/433 (8.3)[a,b]	< 0.001
Fertilization rate (%)	162/218 (74.3)	256/378 (67.7)	296/433 (68.4)	NS
Implantation rate (%)	16/40 (40.0)[a]	26/100 (26.0)[b]	17/130 (13.1)[a,b]	< 0.025
Pregnancy rate (%)	13/20 (65.0)[a]	25/50 (50.0)[b]	16/65 (24.6)[a,b]	< 0.005
Miscarriage rate (%)	1/13 (7.7)	5/25 (20.0)	3/13 (23.1)	NS
Live birth rate (%)	12/20 (60.0)[a]	20/50 (40.0)[b]	13/65 (20.0)[a,b]	< 0.025

HZB = high zona birefringence; LZB = low zona birefringence; MII = metaphase II; NS = not statistically significant.
[a,b]Within rows, values with the same superscript letter are significantly different. All other comparisons were not significantly different.

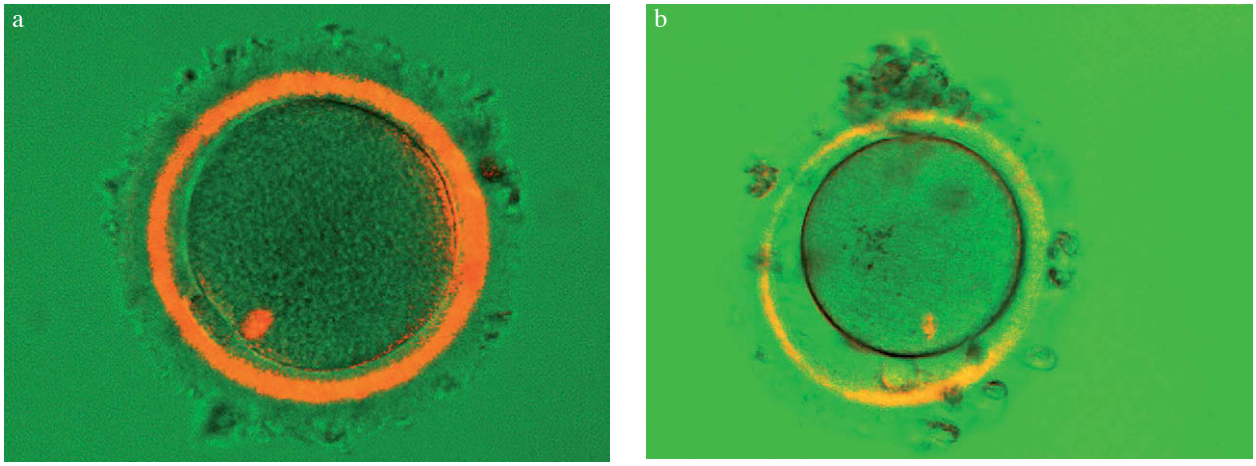

Figure 1. Two human metaphase II oocytes are shown. (**a**) Oocyte characterized by a uniform and very intense birefringence of the inner zona layer, representative of high zona birefringence. (**b**) Oocyte representative of low zona birefringence with a weak and irregular birefringence of the inner zona layer.

Discussion

Reliable parameters for the assessment of oocyte quality and the predictability of embryo development are currently among the most important topics in assisted reproduction. Most studies performed so far have focused on morphological cytoplasmic markers such as the presence of vacuoles, granules and refractive bodies (De Sutter *et al.*, 1996; Serhal *et al.*, 1997; Balaban *et al.*, 1998; Loutradis *et al.*, 1999; Ebner *et al.*, 2003, 2006). However, the results of the different studies are inconclusive. Conflicting results were also reported for evaluation of the presence and location of the meiotic spindle after visualization by polarization microscopy (Wang *et al.*, 2001; Eichenlaub-Ritter *et al.*, 2002; Moon *et al.*, 2003; Rienzi *et al.*, 2003, 2004; Cohen *et al.*, 2004; De Santis *et al.*, 2005; Rama Raju *et al.*, 2007).

A more reliable and probably quantitative marker was proposed by Shen *et al.* (2005). These authors observed in a retrospective study that the embryos transferred in conception cycles derived primarily from oocytes with high magnitude of light retardation caused by the inner layer of the zona pellucida (Pelletier *et al.*, 2004). A key feature in the study by Shen *et al.* was the measurement of retardation and thickness of the inner zona layer. Measurements of the zona were performed on stored images from human oocytes on three different locations. This approach, however, is time-consuming and may not be applicable in a busy routine IVF laboratory requiring a fast and efficient laboratory technique. Therefore, an automatic measuring device that could instantaneously record and analyse all relevant data would certainly be an advantage. With this in mind, a prospective study to investigate the usefulness of zona imaging was initiated to ascertain whether this technique is reliable. As the main parameter in Shen's work was intensity

and uniformity of the zona inner layer retardance, it was decided to use these parameters too. In this study, the analysis was based on a red/green composite picture resulting in a simultaneous view of oocyte morphology and polarization image. Red/green images allow for a better visual distinction of picture characteristics compared with black-and-white polarization-only image. Altogether, this approach allows a very fast subjective zona judgement within minutes (for the entire set of oocytes from one patient) without the need for multiple measurements and additional calculations.

The results of this prospective study are in accordance with the findings of Shen *et al*. (2005) and show clearly that the selection of embryos for transfer based on zona imaging at the oocyte stage is predictive for successful implantation, pregnancy and live birth rate. Compared with cycles without zona imaging (n = 68), the overall implantation and pregnancy rates after zona imaging were significantly higher (21.9% and 40.0% versus 16.2% and 30.9%, respectively; $P < 0.01$).

A unique observation made in this study is that embryos derived from HZB oocytes showed a better embryo development on day 3 but not on day 2 compared with LZB oocytes. This may lead to the impression that extended embryo culture followed by selection at the blastocyst stage may substitute for zona imaging. However, it should be kept in mind that a quarter of the LZB-derived embryos were of top quality on day 3. Out of 11 cycles in the LZB/LZB group with transfer of at least one top quality day-3 embryo, four resulted in a pregnancy and seven did not. Therefore, one may speculate that, in patients presenting with LZB oocytes and without a top quality embryo on day 3, extended embryo culture would not be beneficial and transfer on day 3 would probably be a better option.

A still unresolved question of zona imaging is related to the biological cause of the variation of birefringence. The multilaminar structure of the zona pellucida revealed by polarization microscopy (Keefe *et al*., 1997; Pelletier *et al*., 2004; Shen *et al*., 2005) is directly linked to the paracrystalline network structure of the zona (Wassarman *et al*., 2004), which is formed during the follicular maturation by the oocyte (Nikas *et al*., 1994) and to a lesser extent by the granulosa cells (Sinowatz *et al*., 2001; Bogner *et al*., 2004; Gook *et al*., 2004). A high birefringence of the inner zona layer appears to be primarily an indication of an optimal formation of this ordered structure during oocyte maturation. Secondly, it may show that a HZB oocyte had better conditions during follicular growth and maturation compared with a LZB oocyte with an 'unordered' zona structure. One may speculate that a regular structural integrity of the zona pellucida may reflect an optimal cytoplasmic potential of an oocyte and its various cellular and molecular structures, with HZB oocytes having the best developmental competence for embryonic growth and implantation. Whether or not other exogenous factors (Herrler and Beier, 2000) have an influence on oocyte competence is still open to question (for discussion see Shen *et al*., 2005). Interestingly, assessment of the zona pellucida by conventional microscopy, without the information provided by polarization microscopy, cannot be used as a predictive factor for the success of ICSI (Ten *et al*., 2007).

In conclusion, the data of this prospective study show that selection of oocytes based on zona imaging by a non-invasive single observation can significantly improve implantation and pregnancy rates. This positive predictive parameter of oocyte and subsequent embryo quality may also be used to reduce the number of embryos transferred to avoid multiple gestations. Because of problems arising from a subjective analysis, an automatic scoring module has been developed that allows for user-independent zona imaging. Relevant results from an ongoing study will be presented in the near future. Furthermore, studies are currently underway in the authors' IVF programme to evaluate the effect of hormonal stimulation protocols on zona birefringence in order to optimize the quality of ovarian stimulation in assisted reproduction.

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