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Effects of maternal ageing on ICSI outcomes and embryo development in relation to oocytes morphological characteristics of birefringent structures

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Summary

The aim of this study was to determine the morphological characteristics of the older reproductive aged women's oocytes and to reveal the influence of these characteristics on intra-cytoplasmic sperm injection (ICSI) outcomes. The oocytes of women older than 35 years of age were evaluated retrospectively. Non-invasive polarization microscopy (PolScope) examinations of mature oocytes were performed by measurement of meiotic spindles' length, area and retardance and zona pellucida thickness and retardance. Fertilization and conception competence and the correlation with the birefringent structures were assessed. Two hundred and thirteen mature oocytes from 54 women were evaluated with a PolScope. Length of the meiotic spindle was shown to be related to fertilization success of women with advanced maternal age. In conclusion, the PolScope is a useful device used to identify the oocyte quality. Quantitative measurements of meiotic spindle parameters may be valuable for the selection of high-quality oocytes that have the potential for embryo development in the *in vitro* fertilization (IVF) laboratory of women older than 35 years of age who are mostly poor responders.

Introduction

In the last decades the tendency to postpone childbearing for socio-economic reasons has spread globally, and normal age-related decline in fertility rates have contributed considerably to an increased incidence of subfertility (Mosher & Pratt, 1991).

Females are born with their complete complement of oocytes, although these oocytes can remain arrested for decades. During maternal ageing oocytes decline in quality or undergo programmed cell death. However, optimal female fertility is maintained until approximately 30 years of age and then decreases sharply. Therefore, the trend in delaying childbearing over the past 30 years has increased the risk of infertility. Additionally, in the average women of childbearing age, who reach the age of 37–38 years, the total number of oocytes reaches approximately 25,000 as a threshold, then the numbers decline rapidly. (Faddy *et al.*, 1992).

In vitro fertilization (IVF) provides the opportunity of having babies for women of advanced ages. However, it is well understood that advanced age in women is the main limiting factor for good fertility and reproductive outcomes (Tatone, 2008; Balasch, 2010). Women of older maternal age are candidates for poor IVF outcomes, poor embryo development and poor pregnancy outcomes (Thum *et al.*, 2008; Griffiths *et al.*, 2010; Liu & Case, 2011). Also, the incremental cost per IVF attempt, and additional treatment programmes increase with maternal age (Griffiths *et al.*, 2010).

Ovarian ageing, prior to controlled ovarian stimulation (COS), is utilized frequently to predict pregnancy outcome of IVF/intra-cytoplasmic sperm injection (ICSI) cycles. The ageing of oocytes in relation to maternal age has a greater risk of increasing aneuploidy

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rates, abnormal fertilization and developmental arrest, implantation failure and miscarriage (Te Velde *et al.*, 2002). The success of IVF depends upon the selection of a high-quality embryo that is capable of implantation and ongoing pregnancy.

The aim of the present study was to determine the morphological characteristics of oocytes in older reproductive age women and to demonstrate the influence of these characteristics on ICSI and embryo development stages.

Materials and methods

This study was retrospective, and conducted at the Gulhane Military Medical Faculty, IVF Center, Turkey between September 2010 and December 2011. Institutional review board approval was acquired from the local ethics committee, and written informed consent was obtained from all participants regarding the publication of cycle data. The patients who enrolled in this study were selected from women older than 35 years of age.

PolScope analysis is applied to all oocytes routinely during ICSI procedure at the Gulhane Military Medical Faculty IVF Centre.

Stimulation and oocyte retrieval

Controlled ovarian stimulation was achieved by a gonadotropin-releasing hormone analogue (Lucrin, Abbott, Turkey) in midluteal long protocol, short protocol or antagonist (Cetrotide; Serono, Turkey) combined with 150–450 IU recombinant follicle stimulating hormone (Gonal F, Serono, Turkey). Final maturation of the oocytes was triggered by using 10,000 IU of human chorionic gonadotropin (hCG) (Pregnyl, Schering-Plough, Turkey), and when at least two follicles reached 17 mm in mean diameter. Oocyte retrieval was carried out 36 h after the injection of hCG by transvaginal ultrasound guidance.

Sperm samples

Ejaculated spermatozoa were obtained by masturbation after 3–5 days of ejaculatory abstinence. Sperm samples were prepared using standard gradient gravity technique (Isolate, Irvine Scientific, Santa Ana, California USA) after liquefaction of semen at room temperature. Semen samples were incubated at 37°C for 1 h, allowing spermatozoa to move from the seminal plasma to the over-layered culture medium.

Preparation of oocytes

Retrieved oocytes were incubated for 2 h at 37°C and in 6% CO_2 in air, and then denuded enzymatically in an 80 IU hyaluronidase solution (Hyase, VitroLife, Sweden) before mechanic denudation. Coronal cells were removed manually using a finely drawn glass Pasteur pipette.

Meiotic spindle imaging

For meiotic spindle observation and ICSI procedures, the oocytes were placed in a 10-μl drop of MOPS-buffered medium (G-MOPS, VitroLife) covered with mineral oil (Ovoil, VitroLife) in a glass-bottomed culture dish (Willco Wells, Amsterdam, The Netherlands), which was maintained at 37°C on a heated stage (Tokai, Japan). The meiotic spindle visualization was performed at ×200 magnification in a PolScope system (Spindle View; Cambridge Research & Instrumentation (CRI), Woburn, MA, USA) attached to an inverted microscope (Olympus IX 70, Japan) and its controller unit, combined with a computerized image analysis system (Spindle View software). For this purpose, the oocyte was immobilized with a holding pipette, and rotated with the use of the injection pipette until the meiotic spindles were clearly focused in it. After imaging, ICSI procedure is routinely performed.

The exclusion criteria of the study were non-visualization of the meiotic spindle or zona pellucida under PolScope. The inclusion criterion was the presence of properly recorded PolScope images. Only mature oocytes (metaphase II; MII) with clear presence of the first polar body (PB) were included.

The PolScope images from 54 cases were appropriate for analysis. PolScope images of the oocytes were analysed using the Spindle View program. Spindle observation and analyses were carried out without the awareness of fertilization, cleaving and implantation success of the oocytes. The spindle length was measured in the barrel-shaped long axis, the area of the spindle was calculated and retardance was analysed using the PolScope software (Figure 1). In addition, thickness of inner zona pellucida was measured and retardance was analysed and recorded for whole oocytes of all the patients.

Fertilization, cleaving rates of the oocytes and implantation success of the transferred embryos were recorded for each oocytes. Implantation success was defined as a clinical pregnancy with the visualization of an intrauterine gestational sac.

Statistical analysis

Statistical analyses were performed using Statistics Package for Social Sciences version 15.0 (SPSS, Chicago, IL, USA). Each variable was tested to check the normality distribution using the Kolmogorov–Smirnov test. All values were presented as mean ± standard deviation (SD) unless stated

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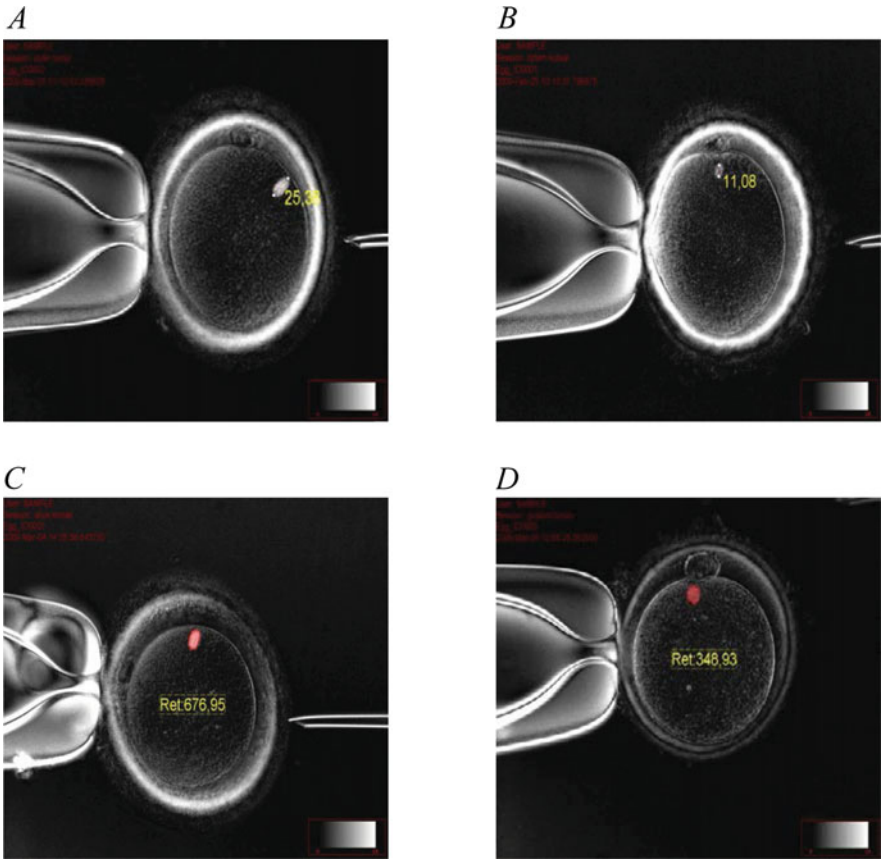

Figure 1 Meiotic spindle of human oocytes imaged by PolScope. (*A*) Barrel-shaped meiotic spindle with a length of 25.38 nm on the long axis. (*B*) A short meiotic spindle, length is measured 11.08 nm. This oocyte was not fertilized. (*C*) Normal retardance of meiotic spindle. (*D*) Weak retardance.

otherwise. Differences between the groups were examined with independent samples *t*-test, Mann–Whitney *U*-test and chi-squared test when appropriate. Pearson correlation analysis was used to estimate the correlation between parameters. A *P*-value <0.05 was considered to be statistically significant.

Results

Fifty-four patients with mean ages 37.3 ± 2.3 (range 35–45) were enrolled in this trial. In total, 369 oocytes were retrieved (6.8 ± 3.8 per patient), 57.9% of which were mature (5.1 ± 2.6 per patient) such that 213 *in vivo* matured oocytes were used in the study. The characteristics of the patients and outcomes of the cycles are given at Table 1.

The treatment indications were: unexplained infertility, 16 (29.6%); reduced ovarian reserve, 17 (31.5%); tubal factor, 4 (7.4%); male factor, 10 (18.5%); and secondary infertility, 7 (13.0%). Treatment protocols were: short protocol, 40 (74.1%); antagonist protocol, 11 (20.4%); luteal long protocol, 3 (5.6%). Twenty-three patients became pregnant (42.6%).

Quantitative analysis of meiotic spindle and inner zona pellucida of the whole mature oocytes by using spindle length, area, retardance and zona pellucida thickness and retardance is given in Table 2.

We observed a 75.1% (160/213) fertilization rate after ICSI. Spindle images of fertilized and non-fertilized oocytes were compared. The length of the fertilized oocytes' meiotic spindle was significantly longer than in non-fertilized oocytes (respectively 20.99 ± 3.82 and 19.85 ± 2.74) ($P = 0.013$). However, spindle area, retardance and inner zona pellucida thickness and retardance values were not significantly different. After embryo culture, at a mean time of 3.1 ± 0.9 days, 74 embryos of good quality were transferred. Mean number of transferred embryos per woman was 1.9 ± 0.5 and the range differed to between 1–3 embryos. Conception occurred in 36 of the 74 transferred embryos.

The total number of embryos that reached blastocyst stage was 29 (18.1%), but no statistically significant differences were observed ($P > 0.05$) compared with the cleavage stage embryos in terms of meiotic spindle length, area, retardance and zona pellucida (ZP) characteristics of precursor oocytes. Seven of those

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Table 1 Features of the patients and treatment outcomes

	Mean ± standard deviation (SD)	Range
Age	37.3 ± 2.3	35–45
Follicle stimulating hormone (FSH) levels	10.2 ± 5.1	3.7–25
Cycle length	9.6 ± 2.0	6–15
Gonadotropin dosage (IU)	3322.2 ± 1788	700–10,000
Total number of oocyte	6.8 ± 3.1	1–13
Germinal vesicle	0.8 ± 1.2	0–5
Metaphase I oocyte	0.9 ± 1.4	0–7
Metaphase II oocyte	5.1 ± 2.6	1–11
Fertilization	3.9 ± 2.4	0–10
Embryo transfer day	3.1 ± 0.9	0–5
Number of transferred embryo	1.9 ± 0.5	1–3

Table 2 Quantitative analysis of meiotic spindle and inner zona pellucida

	Mean ± standard deviation (SD)	Minimum	Maximum
Spindle length	20.70 ± 3.61	5.54	31.14
Spindle area	283.96 ± 82.38	65.51	748.53
Spindle retardance	1.98 ± 0.70	0.73	7.19
Zona thickness	9.43 ± 2.46	1.53	18.82
Zona retardance	2.09 ± 0.64	0.56	3.80

Table 3 Comparison of the meiotic spindle and zona pellucida characteristics in relation to fertilization, cleaving and implantation successes

		Meiotic spindle			Zona pellucida	
Oocytes	No.	Length (μm)	Area (μm^2)	Retardance (nm)	Thickness (μm)	Retardance (nm)
Fertilized	160	20.99 ± 3.82[a]	287.02 ± 87.61	1.94 ± 0.44	9.34 ± 2.23	2.10 ± 0.62
Non-fertilized	53	19.85 ± 2.74[a]	274.73 ± 63.92	2.09 ± 1.16	9.68 ± 3.06	2.07 ± 0.68
Transferred	74	20.56 ± 3.51	284.42 ± 85.68	1.93 ± 0.47	9.44 ± 2.03	2.11 ± 0.61
Non-transferred	86	21.35 ± 4.06	289.25 ± 89.67	1.95 ± 0.42	9.26 ± 2.40	2.09 ± 0.63
Conception	36	20.46 ± 3.42	288.14 ± 103.84	1.93 ± 0.49	9.56 ± 2.13	2.11 ± 0.58
Non-conception	38	20.67 ± 3.64	280.48 ± 62.31	1.92 ± 0.45	9.32 ± 1.94	2.12 ± 0.64

Values with the same superscript letter are significantly different [a] $p < 0.05$.

blastocyst staged embryos were transferred and three pregnancies were achieved.

Twenty-three out of 54 IVF/ET programmes resulted in pregnancy and the pregnancy rate was 42.6%. Table 3 shows the comparisons of the meiotic spindle and ZP morphological traits of fertilized versus non-fertilized oocytes, transferred versus non-transferred and conceived versus non-conceived embryos. No difference was found in the characteristics of meiotic spindles between transferred and non-transferred, conceived and non-conceived embryos.

Discussion

Women of an advanced age have a reduced chance of being fertile due to depletion in the number and quality of the oocytes because of programmed cell death (Broekmans *et al.*, 2009). Artificial reproductive technology (ART) promises hope for women who are willing to have babies at an older reproductive age. Therefore, the success rate for IVF-involved women at an advanced age indicates an age-related reduction in the developmental competence of the human oocyte. Also, the yield of oocytes in reproductively aged women was reduced after COS. Although morphology, fertilization rate and rate of embryo transfer are not significantly different, pregnancy rate is reduced dramatically in comparison with younger patients (Wood *et al.*, 1992).

In this study we aimed to analyse the morphologic characteristics of oocytes from advanced aged women and compared these traits with fertilization, cleaving and implantation competence.

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Oocyte quality is a major determinant of embryo quality and of subsequent success in fertility treatment. Thus, to improve the outcomes of assisted reproduction procedures, it is important to identify non-invasive parameters to evaluate oocyte quality. Among these parameters, examination of egg phenotype using optical microscopy is the most commonly used method.

The success of ICSI depends upon the nuclear and cytoplasmic maturation of the oocyte (Van De Velde *et al.*, 1998). Inverted light microscopy can detect the extracellular and intracellular morphological characteristics of the oocytes. PolScope provides an analysis of the birefringence structures, such as the meiotic spindle and the ZP as prognostic markers.

Numerous studies have demonstrated how the presence of meiotic spindles in human oocytes can predict a higher fertilization rate (Wang *et al.*, 2001a, 2001b; Moon *et al.*, 2003), and have a positive correlation with pregnancy and implantation rates (Madaschi *et al.*, 2008). To date, the shape and birefringence of the meiotic spindle have been investigated. Birefringence of the spindle was correlated positively with pronuclear score after ICSI (Konc *et al.*, 2004) and also pregnancy rates (Fang *et al.*, 2007). Recently, the area, length and retardance of the spindle have been determined to be higher in early cleaving oocytes (Tomari *et al.*, 2011).

Meiotic spindle is an essential cellular organelle, composed of cytoskeletal microtubules, and plays a crucial role in chromosome segregation during meiotic division. Spindle morphology and kinetics can be influenced by maternal age and environmental conditions that lead to disorganization of the microtubules and ultimately to aneuploidy of the zygotes (Eichenlaub-Ritter *et al.*, 2002).

Battaglia *et al.* (1996) have shown that age-related disturbances of meiotic spindles predispose oocytes to disorganization of chromosomal segregation and successful aneuploidy, maturation arrest and the corresponding lower fertilization rates.

Shen *et al.* (2006) compared the mean retardance and length of the meiotic spindle and maternal age in groups who were either ≤30 years, 31–35 years or ≥36 years in age. There was an age-related reduction in mean retardance value of the transfer oocytes from older patients. Rama Raju *et al.* (2007) observed a decrease in retardance and length of the spindle with increase in women's age. Additionally, De Santis *et al.* (2005) have shown the association between low retardance and poor embryo quality in aged patients.

The zona pellucida (ZP) is composed of protein filaments that are rearranged after fertilization and during embryo development (Silva *et al.*, 1997). The human ZP has the properties of birefringence that can indicate the formation of the ordered bimolecular structures in the ZP, during oocyte maturation. The birefringence of ZP was proposed to identify competence of blastocyst formation (Madaschi *et al.*, 2009), and implantation (Ebner *et al.*, 2010). Madaschi *et al.* (2009) reported higher implantation and pregnancy rates with higher ZP birefringence.

Our study included poor responder patients of advanced maternal ages. We compared the meiotic spindle characteristics as length, area and retardance between fertilized–non-fertilized, transferred–non-transferred and conception–non-conception oocytes. As a result we discovered that spindle characteristics are related to fertilization rates. The oocytes that are barrel shaped, and have longer meiotic spindle can fertilize and progress to the cleavage stages, but the oocytes with shorter meiotic spindles have significantly lower fertilization competence.

The PolScope provides visualization of the meiotic spindle and a non-invasive analysis of the spindle characteristics. This method can provide the assessment of high-quality oocytes before fertilization and indicates that the oocyte is more likely to progress to cleavage, and have implantation success. Embryo selection that is based on birefringent spindle detection leads to improved embryo competence, as confirmed by our clinical outcomes. However, we were unable to demonstrate the relationship between cleavage, implantation rates and spindle characteristics.

The most important challenge for assisted reproduction success is the improvement in oocyte quality, plus the identification of embryos with high implantation competence. Advanced maternal age is a fundamental factor in the failure of IVF treatment and in determination of the consequential role that high-quality oocytes play in achieving success. Our findings indicate that spindle length can be an important tool to predict improved fertilization potential and embryo development. Embryo selection for transfer may be based not only on embryo development but also on oocyte morphologic characteristics.

In conclusion, quantitative measurement of meiotic spindle parameters may be a valuable option for the selection of the higher quality oocytes that have a potential for embryo development in the IVF laboratory with poor responder patients.

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