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The impact of endometriosis on early embryo morphokinetics: a case-control study

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ABSTRACT

The aim of this study was to evaluate the possible effects of endometriosis on early embryo development, by comparing the morphokinetic development of embryos obtained from women with clinically confirmed endometriosis with the ones obtained from tubal factor infertility cases. A total of 82 cycles/patients including 53 cycles with endometriosis and 29 cycles with tubal factor infertility were evaluated. A total of 439 embryos were scored for embryo morphokinetics. Age, body mass index, fertilization rates were similar within the groups. However, the number of previous ART trials was found to be higher ($p < 0.05$) in the study group. Also, the number of retrieved oocytes and M2 oocytes were found to be significantly lower in patients with endometriosis ($p < 0.01$). The duration of the first cell cycle (ECC1) and S2 (the time between t3 and t4) displayed significant distortions compared with embryos in the control group. All other analyzed early morphokinetic parameters (t2, t3, t4, t5, t6, t7, t8) and duration of events (VP, cc2a, ECC2, ECC3, S3) showed similar values between study and control groups, respectively. In the light of these findings, it is apparent that endometriosis predominantly affects the duration of the early morphokinetic events and cell cycles.

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Endometriosis; embryo morphokinetic; ICSI

Introduction

Endometriosis is one of the most common disorders that disturb the female reproductive system, affecting 0.5–5% of fertile women and 25–40% of women suffering from infertility (Houston et al. 1987; Ozkan et al. 2008). According to American Fertility Society classification, endometriosis can be divided into four categories: minimal (stage I), mild (Stage II), moderate (Stage III) and severe (stage IV) form ('Revised American Society for Reproductive Medicine classification of endometriosis: 1996,' 1997). Albeit the mechanisms in which it affects fertility remain poorly understood. The impact of endometriosis on *in vitro* fertilization (IVF) outcome has long been evaluated by numerous groups (Barbosa et al. 2014). In some cases, stage I/II endometriosis has been found to be associated with a significantly low fertilization rate, whereas in others, its effect on implantation and cycle outcome has been found to be similar to the cases of tubal infertility (Rock and Markham 1992; Barnhart et al. 2002; Hickman 2002; Suzuki et al. 2005; Harb et al. 2013; Barbosa et al. 2014; Polat et al. 2014). The presence of endometriosis was also found to significantly decrease the pregnancy rates and live birth outcomes (Opoien et al. 2012).

Due to its complex pathogenesis, endometriosis can therefore be suspected to have profound effects on oocyte/embryo quality as well as certain embryo development characteristics. Mechanisms responsible for these adverse effects are largely unclear, however, factors related to tubal dysfunction, disturbed follicular microenvironment and immunological defects are among the most suggested reasons (Gleicher et al. 1987; Garrido et al. 2000; Lyons et al. 2002; Matalliotakis et al. 2007; Reinblatt et al. 2011). Interestingly, the presence of endometriosis can create multidimensional alterations in the female reproductive system, however embryological and clinical outcome in different studies has shown conflicting results on fertilization, embryo development and implantation. Whether the presence of endometriosis has a detrimental effect on oocyte competence, embryo development, as well as its quality, is therefore still controversial.

Most efforts to evaluate embryo quality in endometriosis have so far been based on static observation of the embryo morphology (Garrido et al. 2000; Filippi et al. 2014).

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The recent development of time-lapse technology has given us the opportunity to monitor the embryo development and early morphokinetic changes in real time (Chen et al. 2013; Wong et al. 2013). By evaluating and comparing the morphokinetics of embryos whose implantation results are known, Meseguer and his colleagues were the first to establish a direct link and hierarchy between implantation potential and embryo evaluation by morphokinetics (Meseguer et al. 2011). Since then, numerous studies have been published in order to find an existing correlation between human embryo morphokinetics and numerous patient-specific, embryological and clinical parameters including obesity and polycystic ovary (PCO) syndrome (Ciray et al. 2014; Findikli and Oral 2014).

This study has been performed in order to analyze whether endometriosis affects early embryo development, by comparing the morphokinetics of embryos obtained from women with clinically confirmed endometriosis with the ones obtained from tubal factor infertility cases.

Results

This study includes a total of 439 fertilized and cultured embryos that were obtained in 82 consecutive IVF/ICSI cycles. The study group comprises 264 embryos (53 cycles) from cases with endometriosis, and the control group includes 175 embryos (29 cycles) obtained from cases that were diagnosed as a tubal factor infertility. Some of the baseline patient and cycle characteristics of these groups are shown in Table 1. It can be observed that age, body mass index, fertilization, duration of ovarian stimulation (OS), sperm concentrations and motility rates did not differ significantly within the groups. However, the mean number of previous ART trials was found to be higher in the study group than the control group (1.47 ± 0.5 and 1.34 ± 0.48, respectively ($p < 0.05$)). Also, the mean numbers of retrieved oocytes and M2 oocytes that were obtained is found to differ between the groups ($p < 0.01$). Overall, the clinical pregnancy rate for the study group was 41.5% (22/53) and for the control group was 44.8% (13/29), respectively.

Table 2 shows the embryo morphokinetic data according to time-lapse parameters of the study and the control groups. The following early morphokinetic variables were significantly different between the groups: time of detachment of the second polar body from the oolemma (tPB2) and appearance of the first pronuclei (tPNa), the duration of the first cell cycle (ECC1) and the time between t3 and t4 (S2) ($p < 0.01$). Whereas, all other morphokinetic parameters (t2, t3, t4, t5, t6, t7, t8) and duration events (VP, cc2a, ECC2, ECC3, S3) analyzed earlier showed similar values between the study and control groups. As a result, once we compare embryos from patients with endometriosis and the control group, significant disturbances were observed in the specific early morphokinetic time points, which can be interpreted as endometriosis having an influence on the duration of the early cell cycles as mentioned above.

The morphokinetic parameters of GOOD and POOR quality embryos were analyzed in both the study and control groups. The results showed that the percentage of good quality embryos was significantly lower in the study group ($p < 0.01$)(Table 2). These results might be due to endometriosis having an effect on the embryonic development. Moreover, the embryos were grouped as GOOD and POOR according to their morphological grading in order to evaluate differences in morphokinetic parameters depending on the quality of embryos in the study and control groups. Statistically, the only morphokinetic differences between the GOOD and POOR quality embryos in the study group were observed in tPB2, t2, t3, t4, VP, ECC1 and ECC2 ($p = 0.035$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p = 0.033$, respectively). On the other hand, only mean values of t5 and EEC2 were found significantly different between the GOOD and POOR quality embryos in the control group (Table 3). Furthermore, a significant difference was observed in tPB2, tPNa VP, ECC1 morphokinetic parameters of the GOOD quality embryos between the study and the control group albeit, there was no

Table 1. Patient demographics included in the study.

	Study Group	Control Group	*p*-value
Female age (years)	31.92 ± 2.75	32.13 ± 2.78	NS
BMI (kg/m^2)	21.64 ± 1.31	21.71 ± 1.29	NS
No. of previous ART cycles	1.47 ± 0.5	1.34 ± 0.48	$p < 0.05$
Oocytes collected	7.50 ± 3.58	8.44 ± 1.21	$p < 0.01$
M2 oocytes obtained	5.84 ± 3.24	7.2 ± 0.9	$p < 0.01$
Fertilization rate (%)	84.5	82.3	NS
Duration of OS (days)	10 ± 0.91	10.10 ± 0.72	NS
Sperm concentration (10^6/ml)	53.07 ± 16.44	51.93 ± 16.95	NS
Sperm Motility (%)	48.96 ± 7.72	53.34 ± 9.16	NS

Values are shown as mean±SD. Differences between means were tested by t-test for equality of means and differences in rates were tested by chi-square test. NS = not significant; OS = ovarian stimulation.

Table 2. Embryo morphokinetics data of the study and control groups, respectively.

	Study Group	Control Group	*p*-value
No. of embryos	264	175	
tPB2	6.51 ± 9.07	3.71 ± 1.98	$p < 0.01$
tPNa	12.50 ± 7.87	11.13 ± 3.74	$p < 0.01$
tPNf	25.90 ± 6.31	25.30 ± 7.87	NS
t2	28.64 ± 5.24	28.25 ± 5.40	NS
t3	38.02 ± 6.87	37.67 ± 6.33	NS
t4	41.44 ± 7.35	40.19 ± 6.29	NS
t5	50.51 ± 9.86	49.76 ± 10.41	NS
t6	55.28 ± 10.14	53.77 ± 9.91	NS
t7	58.11 ± 10.14	58.33 ± 10.28	NS
t8	62.67 ± 11.80	61.45 ± 11.09	NS
t9	71.57 ± 13.37	69.62 ± 11.58	NS
VP (tPNf-tPNa)	13.25 ± 6.23	14.87 ± 7.79	NS
ECC1 (tPb2-t2)	22.19 ± 8.23	24.56 ± 5.66	$p < 0.01$
cc2a (t3-t2)	9.37 ± 5.08	9.42 ± 4.89	NS
ECC2	12.87 ± 5.47	12.02 ± 4.73	NS
ECC3	22.56 ± 9.46	22.03 ± 9.30	NS
S2(t4-t3)	3.40 ± 5.31	2.53 ± 4.24	$p < 0.01$
S3(t8-t5)	12.40 ± 9.20	12.59 ± 10.01	NS
GQE (%)	78 ± 41,2	93 ± 25,3	$p < 0.01$

Values are shown as mean±SD. Differences between means were tested by t-test for equality of means. NS = not significant. GQE = Good Quality Embryos

significant difference with regard to the POOR quality embryos between the study and control group (Table 4).

Discussion

The specific mechanisms involved in subfertility associated with endometrioma/endometriosis are still largely unknown. Although the role of pelvic organ adhesion/distorted pelvic anatomy can be the major cause of infertility in severe endometriosis cases, a possible decrease in fertility in minimal or mild cases with endometriosis may also be explained by the altered physiology of oogenesis, embryo development and implantation.

Since the majority of the studies published so far have focused on the clinical outcome per se, the possible impact of the disease on oocyte/embryo quality and development has not been clarified well (Barbosa et al. 2014). Recipients affected with severe endometriosis sharing sibling oocytes with control recipients without endometriosis in an oocyte donation program have been reported to have comparable implantation and live birth rates in a retrospective study, concluding that endometriosis is not detrimental to the endometrial receptivity (Diaz et al. 2000). On the other hand, in a similar treatment setting, a prospective cohort study in donors or recipients with endometriosis reported poor pregnancy and implantation rates with oocytes donated by donors with endometriosis in comparison to recipients without endometriosis, further implying that the possible negative effect of the disease on fertility might be related with the oocyte/embryo development (Hauzman et al. 2013).

Until recently, the possible impact of patient-specific or clinical variables on oocyte/embryo development could only be assessed by static observations under a conventional inverted microscope, at only a few specific time points during the course of *in vitro* culture. After the first introduction of time-lapse human embryo culture systems in routine assisted reproductive treatment cycles, the real time morphokinetic observation and analysis of embryos have now made the analysis of variables possible for a variety of different clinical as well as laboratory conditions (Gardner et al. 2015). With this approach, Wissing et al. and Bellver et al. has explored the possible effect of hyperandrogenic hormonal milieu, as well as obesity, on embryo development in PCOS and overweight patients, and documented the true effect of maternal metabolic and hormonal derangements on embryo development *in vitro* (Bellver et al. 2013; Wissing et al. 2014).

In this study, the comparison of embryo morphokinetic data between cases with endometriosis and tubal infertility displayed significant differences in the early post fertilization and first cleavage time points. Timely early cleavages may reflect good quality of the cytoplasmic and nuclear component of the embryo, as it is likely that activation events accompanying fertilization,

Table 3. Morphokinetics analysis of the GOOD and POOR quality embryos in study and control groups.

	Study Group			Control Group		
	GOOD	POOR	*p*-value	GOOD	POOR	*p*-value
No. of embryos	207	57		163	12	
tPB2	7.12 ± 9.91	4.27 ± 4.24	$p < 0.05$	3,69 ± 1.98	3.89 ± 1.96	NS
tPNa	12.88 ± 8.56	11.10 ± 4.35	NS	11.15 ± 3.76	10.79 ± 3.48	NS
tPNf	25.51 ± 6.46	27.29 ± 5.53	NS	25.99 ± 8.11	26.05 ± 3.36	NS
t2	27.85 ± 3.76	31.47 ± 8.12	$p < 0.01$	28.22 ± 5.93	28.60 ± 4.07	NS
t3	37.10 ± 6.19	41.31 ± 8.15	$p < 0.01$	37.63 ± 6.20	38.07 ± 8.20	NS
t4	40.40 ± 6.60	45.18 ± 8.66	$p < 0.01$	40.03 ± 5.90	42.36 ± 10.36	NS
t5	50.43 ± 10.04	50.75 ± 9.25	NS	49.23 ± 9.24	56.74 ± 19.83	$p < 0.05$
t6	55.04 ± 10.17	56.46 ± 10.02	NS	53.54 ± 8.82	56.82 ± 19.82	NS
VP (tPNf-tPNa)	12.61 ± 6.41	15.69 ± 4.82	$p < 0.01$	14.83 ± 7.96	15.26 ± 5.15	NS
ECC1 (tPb2-t2)	21.18 ± 8.10	26.10 ± 7.60	$p < 0.01$	24.51 ± 5.75	25.15 ± 4.20	NS
cc2a (t3-t2)	9.24 ± 5.04	9.84 ± 5.23	NS	9.41 ± 4.77	9.47 ± 6.59	NS
ECC2	12.54 ± 5.32	14.55 ± 6.01	$p < 0.05$	11.81 ± 4.45	15.40 ± 7.48	$p < 0.05$
S2(t4-t3)	3.28 ± 5.18	3.87 ± 5.78	NS	2.40 ± 4.05	4.28 ± 6.20	NS

Values are shown as mean±SD. Differences between means were tested by t-test for equality of means. NS = not significant.

Table 4. Differences in morphokinetic data in control and study groups with respect to good and poor embryo quality.

	GOOD			POOR		
	Study group	Control group	*p*-value	Study group	Control group	*p*-value
No. of embryos	207	163		57	12	
tPB2	7.12 ± 9.91	3.69 ± 1.98	$p < 0.01$	4.27 ± 4.24	3.89 ± 1.96	NS
tPNa	12.88 ± 8.56	11.15 ± 3.76	$p < 0.05$	11.10 ± 4.35	10.79 ± 3.48	NS
tPNf	25.51 ± 6.46	25.99 ± 8.11	NS	27.29 ± 5.53	26.05 ± 3.36	NS
t2	27.85 ± 3.76	28.22 ± 5.49	NS	31.47 ± 8.12	28.60 ± 4.07	NS
t3	37.10 ± 6.19	37.63 ± 6.20	NS	41.31 ± 8.15	38.07 ± 8.20	NS
t4	40.40 ± 6.60	40.03 ± 5.90	NS	45.18 ± 8.66	42.35 ± 10.36	NS
t5	50.43 ± 10.04	49.23 ± 9.24	NS	50.75 ± 9.25	56.74 ± 19.83	NS
t6	55.04 ± 10.17	53.54 ± 8.82	NS	56.46 ± 10.02	56.82 ± 19.82	NS
t7	58.11 ± 10.12	58.32 ± 10.28	NS	58.25 ± 11.36	ND	
t8	62.66 ± 11.83	61.45 ± 11.09	NS	ND	ND	
t9	71.56 ± 13.37	69.62 ± 11.57	NS	ND	ND	
VP (tPNf-tPNa)	12.61 ± 6.41	14.83 ± 7.96	$p < 0.05$	15.69 ± 4.82	15.26 ± 5.15	NS
ECC1 (tPb2-t2)	21.18 ± 8.10	24.51 ± 5.75	$p < 0.01$	26.10 ± 7.60	25.15 ± 4.20	NS
cc2a (t3-t2)	9.24 ± 5.04	9.41 ± 4.77	NS	9.84 ± 5.23	9.47 ± 6.59	NS
ECC2	12.54 ± 5.32	11.81 ± 4.45	NS	14.55 ± 6.01	15.40 ± 7.48	NS
ECC3	22.25 ± 9.46	22.02 ± 9.29	NS	ND	ND	
S2(t4-t3)	3.28 ± 5.18	2.40 ± 4.05	NS	3.87 ± 5.78	4.28 ± 6.20	NS
S3(t8-t5)	12.47 ± 9.17	12.58 ± 10.09	NS	ND	ND	

Values are shown as mean±SD. Differences between means were tested by t-test for equality of means. NS = not significant. ND = No data

occurred correctly, that mitochondria provide sufficient amounts of energy, and that the cytoskeleton is functional (Milewski and Ajduk 2017)

Interestingly, such observations could not be detected by any static embryo observation/scoring criteria that have been published so far, indicating the power of time lapse morphokinetic analysis as a diagnostic/research tool in human ART. These findings can also explain why significant differences exist in different studies, aimed to document the impact of endometriosis on the cycle outcome. During the last decade, many reports have been published about the optimal time ranges for morphokinetic events (Meseguer et al. 2011; Storr et al. 2015). In a recent study, Aguilar and colleagues have investigated the correlation between early fertilization/cell cycle characteristics and implantation potential in a total of 1448 transferred embryos and found that there are optimal time ranges for the early time points that distinguish embryos implanted from those not- implanted (Aguilar et al. 2014). Previous mouse and bovine model studies reported that, the presence of follicular fluid from cases of endometriosis can be associated with a decreased oocyte quality and cause multiple polar body- and spindle-associated abnormalities (Mansour et al. 2010; Da Broi et al. 2014; Cohen et al. 2015). Although our study has not been designed to measure implantation and clinical outcome, significant disturbances observed in the polar body extrusion, as well as a delay in the early cell cycle events, correlate with previous findings, leading to the extrapolation that human oocytes from endometriosis patients can also be prone to lower implantation potential and higher chromosomal errors.

Follicular fluid is an active microenvironment which has critical role in oocyte growth and maturation. These two processes, on the other hand, are crucial for the embryo ability to develop (Pasqualotto et al. 2004). The alterations in hormonal levels, especially estradiol concentrations in women with endometriosis, can cause or have a causative effect on the difference in the early morphokinetic parameters. It is well established that endometriosis is characterized by changes in the intrafollicular environment and increased levels of certain cytokines such as TNF-a and ROS, which may affect oocyte and embryo development (Mansour et al. 2009). Aromatase, a rate-limiting enzyme for conversion of androgens to estrogen, is one of the major players in steroidogenesis. Estrogen also stimulates cyclooxygenase-2 enzyme that results in an elevated level of prostaglandin E2 (Bulun et al. 2004). Aromatase expression in endometriotic tissue and endometriomas has long been investigated and Noble et al. and Smuch et al. have reported that such expression was increased in these tissues as compared to disease free peritoneal endometriotic implants and in the eutopic endometrium (Noble et al. 1996; Smuc et al. 2009). On the other hand, studies using granulosa cell culture from women with and without endometriosis submitted to OS for IVF/ICSI reported decreased aromatase activity in woman with endometriosis, and recent publications by Delvoux et al. and Colette et al. failed to confirm aromatase expression in endometriotic lesions (Harlow et al. 1996; Colette et al. 2009; Delvoux et al. 2009).

Majority of the morphokinetic parameter values seen in the study and control groups are similar (Table 2), albeit the duration of the first cell cycle (ECC1) became noticeably shorter in the study group while tPB2, tPNa and S2 is seen to be significantly shorter in the control group. The kinetics of embryos derived from the endometriosis and control groups was evaluated according to the embryo quality. Comparison between morphokinetic

parameters in the GOOD control versus the GOOD endometriosis embryos revealed that tPB2 and tPNa times were significantly longer in the endometriosis group. Endometriosis seems to affect time of the second PB extrusion (tPB2) and the pronuclei appearance (tPNa), which affects duration of the first cell cycle (ECC1) and PN duration (VP) (Table 4). Timing of tPB2 has been previously linked with quality of the embryos and implantation by Agiular et al., (2014). It is also known that the first cleavage, or t2, timing has been surveyed widely and is accepted as a good parameter of embryo development, with the early cleavage being preferable to the late one.

There is evidence that in mammals alterations in the follicular ovarian microenvironment may be associated with a presence of epigenetic chromatin-modifying enzymes such as arginine methyltransferases. Up and down regulation of these enzymes affects the chromatin environment. The changes in the chromatin environment caused by arginine methyltransferases might predispose to a potential transgenerational inheritance of abnormal epigenetic modifications established during the oocyte growth (Baumann et al. 2015). Endometriosis may influence follicular microenvironment and the embryo morphokinetics. However, the effect of microenvironment on follicular growth and embryo morphokinetics should be evaluated in detail. A nonoptimal metabolic status of the oocytes due to alterations in microenvironment reflects the status of the embryo development (Leese 2002).

According to the best of our knowledge, this is the first study in which the possible effects of endometriosis on the early embryo morphokinetics were analyzed and the significant differences have been found. However, our study has two limitations: Firstly, due to the strict patient selection criteria, it has been carried out with a relatively small sample size. Secondly, since the authors' clinics routinely seek to apply a deferred embryo transfer protocol in the routine IVF/ICSI cycles, most of the embryos that were included in the study were later pooled and vitrified on day 3. Since subsequent warming, culture and transfer are conducted with the pooled samples, individual morphokinetics and implantation data could not be assessed.

Finding that the number of oocytes retrieved was lower in the study group is also in agreement with the current literature (Barbosa et al. 2014). Although this finding may seem to create a potential bias on the number of embryos analyzed, there is yet no reported data showing that poor ovarian response has any effect on embryo morphokinetics. Furthermore, since only embryos with the superior quality are selected for embryo transfer, the morphokhinetic parameters of rest of the embryos in the cohort do not reflect the cycle outcome. Current clinical outcome data on endometriosis also supports this result (Barbosa et al. 2014). Further investigations using a larger cohort of patients and a prospective study design are needed to confirm these results.

In conclusion, this study has found significant differences in the early morphokinetic time points, such as tPB2, tPNa, ECC1 and S2 in embryos from endometriosis patients and embryos from patients having tubal factor infertility. Our data indicate that endometriosis predominantly affects the duration of the early morphokinetic events and cell cycles. Other early development parameters from t2 to t7 were found to be similar in all groups analyzed. Due to the limitation of the study protocol, a possible correlation of these changes with implantation and clinical outcome could not be analyzed; however a prospective study on the impact of such alterations on the clinical outcome is currently underway.

Materials and methods

The study is a retrospective observational study which was performed at Bahceci Umut and Fulya IVF Centres. The study was approved by Bahceci Health Group Ethics Committee (reference 32, May 2015) and patients were included in the study after providing them with written consent followed by written and verbal explanation. Cycles performed between March 2011 and May 2015 was included in the analysis.

Study population

A total of 82 consecutive cycles including 53 cycles with endometriosis and 29 cycles with tubal factor infertility were evaluated. A total of 439 embryos were scored for embryo morphokinetics.

The diagnosis of endometriosis was confirmed by laparotomy/laparoscopy (in 27 patients) or by transvaginal USG (in 26 patients) since the diagnostic power and validity of transvaginal ultrasonography (TVUSG) is very high (Savelli 2009). After the laparoscopic surgery the diagnosis of endometriosis was confirmed by expert pathologists. Grade 3–4 endometriosis patients were included in the study the control group consisted of 30 women with laparoscopically confirmed tubal factor infertility and undergoing their first IVF attempt at our center; all control patients were also confirmed not to have endometriosis or hydrosalpinx at the time of laparoscopy. Clinical pregnancy was confirmed by visualization of gestational sac and fetal heartbeat by using TVUSG after 1–2 weeks of serum βhCG measurement.

In all cycles, ejaculated spermatozoa were used. Women who were >40 years of age, having partners

with the male factor infertility, displaying structural or numerical chromosomal errors necessitating preimplantation genetic diagnosis, with uterine anomalies or polycystic ovary syndrome were excluded from the study.

Controlled OS

Patients from both the endometriosis and control groups received controlled OS with GnRH antagonist protocol. Recombinant FSH (150–225 IU, Gonal-F; Serono) and hMG (75 IU, Menogon; Ferring) were administered on day 2 of the menstrual period. Starting on the sixth day of controlled OS, ovarian response was monitored with serial TV-USG and serum estrodiol and progestrone levels. Daily 0.25 mg of GnRH antagonist (Cetrotide; Serono) usage was started until the day of hCG, when the leading follicle exceeded 13 mm in diameter. In both protocols, 250 mcg hCG (Ovitrelle, Serono) was administered when at least two follicles reached 18 mm in diameter.

Oocyte retrieval, intracytoplasmic sperm injection (ICSI) and embryo culture

An oocyte pick-up procedure was performed 34–36 h after hCG administration under an ultrasound guidance. Collected oocytes were washed and placed into 50 µl drops of a continuous single culture complete with gentamicin and HSA (CSCM; Irvine Scientific, CA, USA). After oocyte retrieval, oocyte cumulus complexes were kept in an incubator for 2 h at 37°C, 6.5% CO2 and 5% O2 until denudation. Enzymatic removal of granulosa cells was performed by hyaluronidase treatment (Hyaluronidase Solution; Irvine Scientific, CA, USA). Semen samples were prepared with an isolate sperm separation medium (Irvine Scientific, CA, USA) and sperm pellet was washed two times using a sperm washing medium (Irvine Scientific, CA, USA). Intracytoplasmic sperm injection (ICSI) procedure was performed within an hour following the denudation in mHTF containing HEPES. After this, the microinjected oocytes were cultured in a pre-equilibrated EmbryoSlide culture dish (EmbryoSlide, Vitrolife, Sweden) until day 3 of the *in vitro* embryo development. The EmbryoSlide™ dish has 12 individual wells and these wells were filled with 30–35 µl of CSCM, covered with 1,4 ml of mineral oil (Irvine Scientific), in order to prevent evaporation, and equilibrated overnight before use. Oocytes/embryos were cultured in a time-lapse incubator (EmbryoScope®, Vitrolife, Sweden) at 37°C, 6.5% CO2 and 5% O2 for at least 72 h. It is a tri-gas incubator that has a built-in microscope, captures images automatically up to 72 individual embryos every 10 min from seven different focal planes during embryo development.

Analysis of morphokinetic parameters and embryo morphology

Time-lapse morphokinetic assessments of the second polar body extrusion timing, pronuclei appearance, pronuclear fading and duration of VP (tPNf-tPNa), ECC1 (t2- tPB2), ECC2 (cc2a) (t3-t2), cc2b (t4-t2), ECC3 (t8-t4), S2 (t4-t3), and S3 (t8-t5) were evaluated as proposed in the guidelines by Ciray and colleagues (Ciray et al. 2014). Other parameters that include the early cleavage morphokinetic timings of 2- (t2), 3- (t3), 4- (t4), 5- (t5), 6- (t6), 7- (t7), 8- (t8) and 9-cell (t9) stages were analyzed as described in our previous study (Serdarogullari et al. 2014). Embryos were grouped as GOOD and POOR quality depending on their morphological evaluation as described in (Ciray et al. 2012) to investigate the morphokinetic characteristics in the study and control groups. Analysis of the morphokinetic parameters was performed by only one embryologist in order to maintain a reliability/quality assurance associated with annotation practice.

Statistical analysis

Data were analyzed using Student's *t*-test for comparison of timings and chi-squared test for comparison of the proportions. GraphPad InStat version 3.10 for Windows; GraphPad Soft- ware, San Diego, CA, USA. A two-tailed p-value < 0.05 was considered significant.

Disclosure statement

No potential conflict of interest was reported by the authors.

Authors' contributions

Fazilet Kubra Boynukalin Substantial contributions to design, acquisition, analysis and interpretation of data, and drafting the article: FKB, MS, MG; critical revision of manuscript and approval of final version of the manuscript: FKB, MS, MG, OC, NF, MB.

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