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ASSISTED REPRODUCTION TECHNOLOGIES

The impact of paternal factors on cleavage stage and blastocyst development analyzed by time-lapse imaging—a retrospective observational study

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Abstract
Purpose Various time-lapse studies have postulated embryo selection criteria based on early morphokinetic markers. However, late paternal effects are mostly not visible before embryonic genome activation. The primary objective of this retrospective study was to investigate whether those early morphokinetic algorithms investigated by time-lapse imaging are reliable enough to allow for the accurate selection of those embryos that develop into blastocysts, while of course taking into account the correlation with the type of injected spermatozoa.
Methods During a period of 18 months, a total of 461 MII oocytes from 43 couples with severe male factor infertility and previous "external" IVF failures after cleavage-stage embryo transfer (ET) were fertilized by intracytoplasmic morphologically selected sperm injection (IMSI). Thereof, 373 embryos were monitored in a time-lapse incubator until ET on day 5. Blastocyst outcome in combination with three previously postulated MKc (cc2: t3–t2, 5–12 h; t3, 35–40 h; t5, 48–56 h) and the morphology of the selected sperm were analyzed.
Results A significant increase in the rate of blastocysts (54.0 vs. 36.3 %; $P<0.01$) and top blastocysts (25.3 vs. 10.8 %; $P<0.001$) was observed in the group of those meeting all three morphokinetic criteria (MKc3). However, MKc3 were only met in 23.3 % of all embryos. Moreover, TBR was influenced by the type of injected spermatozoa. In both groups, TBR decreased dramatically (MKc3, 35.0 vs. 17.0 %; MKc<3, 14.2 vs. 8.4 %) when class II/III sperm instead of class I were injected.
Conclusion Early morphokinetic parameters might give some predictive information but fail to serve as a feasible selective tool for the prediction of blastocyst development given the influence of the type of spermatozoa injected.

Keywords Time-lapse cinematography · Blastocyst · Morphokinetics · Paternal effect · Sperm · ICSI

Capsule Time-lapse imaging allows the detailed recording of embryonal morphokinetics until the day of transfer. However, by the use of time-lapse imaging, we were able to demonstrate that blastocyst development cannot be predicted accurately by a recently postulated time-lapse-based selection model in OAT patients with poor sperm morphology. Optimal sperm selection by IMSI and embryo selection at the blastocyst stage take into account the late paternal effect and, most probably, have a higher predictive value for the IVF outcome.

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Introduction

Nowadays, the progress made in IVF techniques allows prolonged embryo culture (PEC) until day 5/6 when the embryo has reached the blastocyst stage. Meanwhile, the transfer of blastocysts is suggested to be physiologically more associated with a better synchronization of the uterus and endometrium. Additionally, the extended culture allows a kind of natural pre-selection as not all embryos on day 2 or 3 reach the blastocyst stage and selecting them by an embryologist on the basis of morphological grading at the blastocyst stage has higher predictive value for implantation [1–3]. Moreover, embryos selected for transfer on day 5 are suggested to be at lower risk of aneuploidy—although chromosomal

abnormalities still cannot be excluded. The observations of higher implantation, pregnancy, and birth rates in day 5 transfers might be a combination of all these factors. Even though it has been shown that the prolonged culture and subsequent selection for single embryo transfer (SET) is still the most promising approach to increase pregnancy and life birth rates in IVF cycles [4, 5], the application of blastocyst transfers in the practice of assisted reproduction techniques (ART) is still highly controversial. There are multiple reasons for this. Besides the assumed higher costs and increase in the laboratory workload, PEC is often blamed to be associated with increased risks of congenital malformations, low birth weight, or pre-term birth [6]. However, recently published studies do not support these findings [7, 8]. Moreover, the fear of having to cancel transfer due to the lack of blastocyst development, as it is sometimes the case e.g. in advanced maternal age, or owing to restrictive legal situations in several countries, may lead to the fact that a huge number of IVF centers still perform embryo transfer (ET) on day 2 or 3.

Therefore, a variety of parameters for optimal embryo selection until day 2/3 were postulated. In fact, however, these are limited predictors of blastocyst quality on day 5 [9, 10]. These observations can be explained—amongst other factors—by the embryonic genome activation (EGA) on day 2/3 of human embryo development. The switch from a transcriptionally quiescent to an active embryonic genome displays chromosomal or genetic errors of i.e. paternal genes—an effect which is known as "late paternal effect" [11, 12].

The implementation of time-lapse cinematography (TLC) in ART now allows a more dynamic and detailed evaluation of the embryo. Instead of static observations of embryo morphology at specific timepoints, all alterations in the morphology of the embryo and the kinetic of the developing embryo until the transfer can be assessed. In consequence, various new morphological and morphokinetic markers (time to cleavage, duration of cell cycle, synchronization of division or the timepoint when the embryo has reached a certain stage of development) evaluated by TLC were associated with viability and competence of the embryo. Based on a large amount of data generated by time-lapse incubators, several (multivariable) prediction models for an optimal selection were published [13–17]. Those embryos that reveal a deviation of these selection criteria were regularly discarded.

TLC is recurrently hyped as the appropriate tool to select the embryo with the best capacity to further develop by those who perform SET on day 3.

However, to this day, there is still no general consensus about algorithms for embryo selection criteria before the onset of EGA. This, however, raises the question whether the current morphokinetic selection criteria on day 3 are predictive enough to choose those embryo(s) which have the highest capacity to develop to the blastocyst stage.

Besides the impact of oocyte competence on the resulting embryo quality, evidence of a paternal influence on early embryonic development and blastocyst formation in vitro has been reported [12]. A lack of sperm-specific activating protein and defects in the centrosome of the sperm can compromise early cell divisions in the human embryo (the so-called early paternal effect). However, sperm nuclear deficiencies are usually not detected before the eight-cell stage of embryo development, when sperm-derived genes start to be activated [18]. This late paternal effect is often suggested to be associated with DNA fragmentation and disorganization of the sperm chromatin.

Since the introduction of motile sperm organelle morphology examination (MSOME), enabling the evaluation of the subtle nuclear morphology of motile spermatozoa in real time at high magnification, more care has been taken not only to select normal spermatozoa which are defined by shape and size but also to select spermatozoa devoid of nuclear vacuoles located in the sperm head. The importance of the evaluation of the subtle sperm head morphology was already realized 14 years ago when the MSOME technique was implemented in IVF [19].

According to the growing body of literature, it is more and more obvious that large vacuoles are a sign of nuclear dysfunction, reflecting a failure of chromatin condensation and packaging [20–27]. It has been reported that the negative impact of large nuclear vacuoles (LNV) is perceptible after the onset of the EGA, leading to reduced blastocyst formation [28–31]. Moreover, LNV also affect ongoing pregnancy rates [31–34], miscarriage rates [31, 33, 35–37], and malformation in offspring [38, 39].

Bearing in mind the observation of a negative effect of LNV on the blastocyst rate, this raises the question if—at the moment—we are able to predict the blastocyst development based only on morphological and kinetic grading through continuous observation until day 3 without taking into account the quality of the fertilizing spermatozoa. The aim of the study was to analyze how spermatozoa influence the outcome in terms of development to the blastocyst stage of embryos that fulfill (or not) the morphokinetic criteria according to recently postulated embryo selection models [13, 14].

Material and methods

Patients

From June 2011 until January 2013, a total of 43 couples were included in the study. Inclusion criteria were patients with at least two previous "external" implantation failures after ET following ICSI and cleavage-stage embryo transfer and oligo-astheno-teratozoospermia (OAT) with severely impaired

sperm morphology. Detailed patient characteristics are given in Table 1.

All patients were informed about all therapies related to the IVF therapy, and their signed consent was obtained for publishing medical data. All activities performed were in concordance with the principles for medical research according to the WMA declaration of Helsinki. Ethical committee approval was not necessary as all the techniques used are standard techniques in reproductive medicine. A flow chart diagram of the study design is given in Fig. 1.

Ovarian stimulation and oocyte retrieval

Conventional controlled ovarian stimulation (cCOS) was performed using the gonadotrophin-releasing hormone (GnRH) long protocol [5]. Final oocyte maturation and the induction of ovulation were performed with 10,000 IU hCG (Ferring Arzneimittel, Vienna, Austria). Thirty-six hours after hCG injection, oocyte retrieval took place using transvaginal ultrasound-guided follicle aspiration.

Oocyte–cumulus complexes were recovered from follicular aspirates using a stereomicroscope, washed, and cultured in HTF medium (Life Global, Ontario, Canada) at 37.0 °C and 5.8 % CO_2. Thirty-eight to 41 h post hCG injection, the cumulus cells were removed after enzymatic treatment for a maximum of 30 s in hyaluronidase (Life Global, Ontario, Canada).

Table 1 Patient's data

Characteristics	Study population (n=43 couples)
Female	
Mean age (years)	37.3±4.3
Primary infertility	26
Secondary infertility	17
Female infertility	6 (blocked tubes diagnosed)
Mean total stimulation dose (IU)	2645.4±216.3
Mean duration of ovarian stimulation (days)	11,5±1.2
MII oocytes retrieved (mean)	8.3±3.9
Male	
Mean age (years)	41.6±4.2
Sperm parameters	
Mean volume (ml)	1.8±0.7
Mean concentration (10^6/ml)	6.4±4.1
Mean progressive motility (%)	27.6±13.8
IMSI class I (mean %)	3.4±0.3
IMSI class II (mean %)	27.5±11.4
IMSI class III (mean %)	69.1±29.7

Values are mean±SD (n or %) unless otherwise stated

Preparation of the semen

Semen was collected by masturbation after an abstinence period of a maximum of 48 h. Sperm concentration and motility were assessed according to WHO criteria [40]. Motile sperm fractions were isolated after centrifugation (20 min, 600×g) on a three-layer gradient of PureSperm (Nidacon, Mölndal, Sweden). The 90 % fraction was washed with 10 ml of HTF media (Life Global, Ontario, Canada) and centrifuged for 7 min at 600×g.

Preparation of the dish and IMSI technique

According to the number of injectable oocytes, a minimum of two dishes were prepared. Two elongated polyvinylpyrrolidone (PVP; Life Global, Ontario, Canada) drops were placed into a glass-bottom dish (WPI): In the first one, the sperm suspension was deposited before selection [41].

In the second PVP drop, a picture of the spermatozoon was taken immediately after immobilization. Adjacent to the two drops of PVP, two drops of Global–HEPES (Life Global, Ontario, Canada) were placed. The drops were covered with sterile mineral oil (Cryo Biosystems, L'Aigle, France). One oocyte at a time was put into the dish to minimize the period of time outside the incubator.

A first selection of motile spermatozoa in the PVP drop was made at ×630–1000 magnification under a Normarski interferential Leica AM 6000 inverted microscope (Leica, Germany). If possible, morphologically normal spermatozoa were selected showing a normal oval head shape as well as the absence of both cytoplasmic extrusions and tail defects. Using a variable zoom lens (HC VarioC-mount; Leica, Germany), the morphology was re-evaluated on the monitor at a magnification of ×6600 after immobilization. A picture was taken for subsequent classification and the purposes of our study (see Fig. 2).

For each intracytoplasmic morphologically selected sperm injection (IMSI)–ICSI attempt, the selection policy consisted of attempting to select the best spermatozoa out of the prepared semen sample. The primary intention was to choose spermatozoa without vacuoles for injection into the oocytes. For all the patients included in this study, it was not possible to find class I spermatozoa for all the oocytes. Classification of vacuolization was done using the Vanderzwalmen criteria. Spermatozoa were classified as class I when showing normal shape, size, and no vacuoles or only small vacuole(s) (<4 % of the sperm's head). Class II encompasses spermatozoa with normal shape and size but with one or more vacuoles (>4 % of the sperm's head). Finally, class III included spermatozoa with abnormal shape and/or size with or without vacuoles (Fig. 2). In order to interpret the results more precisely, we distinguished between "good-quality spermatozoa" without or not more

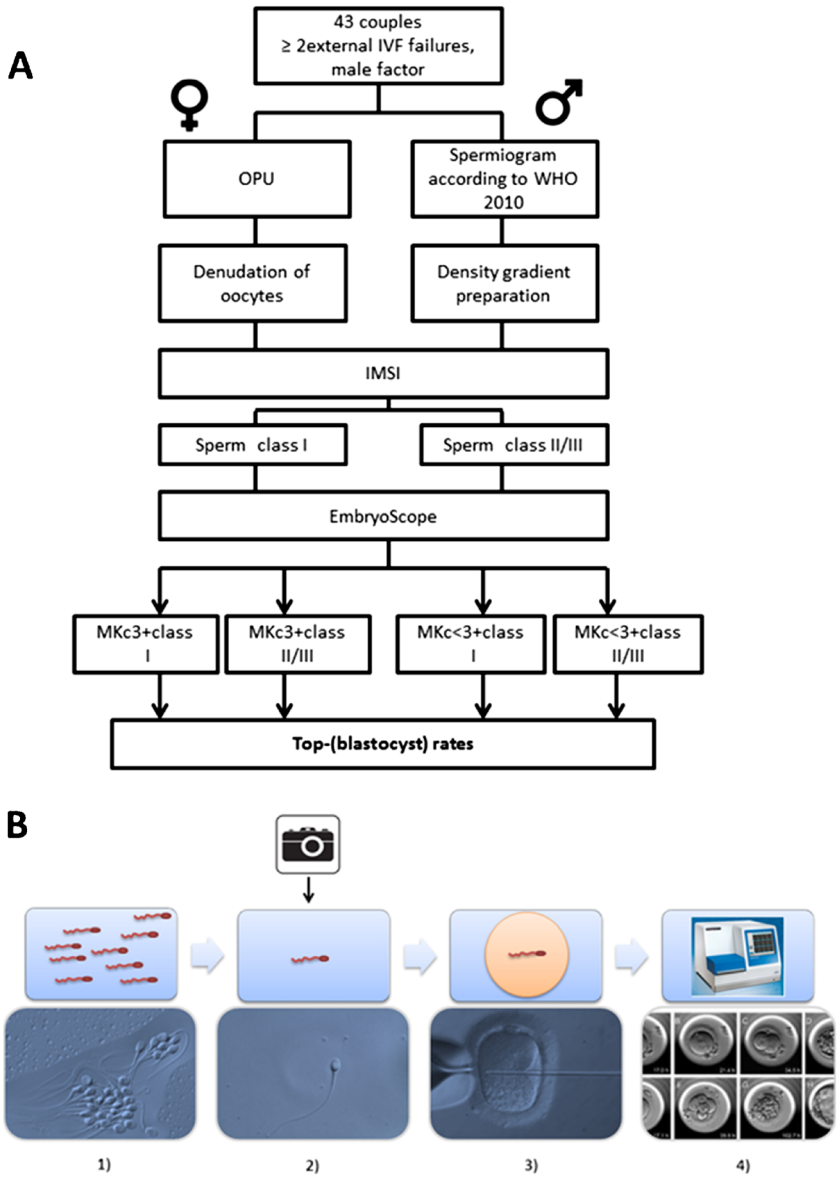

Fig. 1 Flow chart diagram of the study design. **a** Experimental design. **b** Chronology of the working steps: *1)* MSOME selection, *2)* immobilization and picture of the selected spermatozoon, *3)* IMSI, *4)* incubation in the time-lapse incubator

than two small vacuoles (IMSI class I) and "poor-quality spermatozoa" (IMSI class II and III) [28]. In regard to oocyte quality, injection into oocytes was done in a randomized manner.

Embryo culture and incubation

Following IMSI, the injected oocytes were placed individually on a special culture slide (EmbryoSlide®, Unisense Fertilitech, Aarhus, Denmark) which was then placed in a time-lapse incubator (EmbryoScope™, Unisense Fertilitech, Aarhus, Denmark) under oil at 37 °C in 5.8 % CO_2 without humidification. Out of 461 injected MII oocytes, a total of 373 normally fertilized zygotes (2PN) were monitored by means of pictures taken every 20 min from the injection onwards until day 5. On day 3, the culture medium of each microwell (20 μl) was exchanged. Embryos were cultured until transfer on day 5.

Morphokinetic analysis

Embryo development was retrospectively analyzed in correlation to sperm quality (Fig. 1). The duration of the second cell cycle (cc2, 5–12 h) was defined as the time interval between a two-cell-stage embryo and a three-cell embryo (t3–t2). The period between IMSI and the three-cell stage was defined as t3 (35–40 h), and the period between IMSI and the five-cell-stage embryo as t5 (48–56 h) [14]. According to these three criteria, embryos were divided into two groups: one group, where all the applied criteria were fulfilled (MKc3), and another group, where at least one criterion was not met (MKc<3).

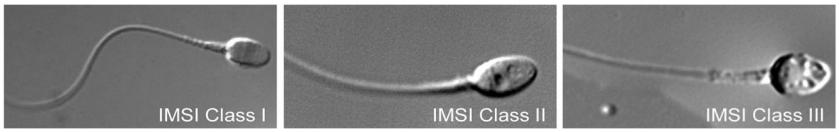

Fig. 2 Example of the sperm classification according to intracytoplasmic morphologically selected sperm injection (IMSI). Classes II and III were summarized in the poor-sperm-quality group

Embryo grading

On day 5, blastocyst quality was recorded and assessed according to the degree of blastocoele expansion and the quality of both the inner cell mass (ICM) and the trophectoderm. Blastocysts with a degree of expansion of 4–5 and with A grading for inner cell mass and trophectoderm or a combination of A and B grading were classified as top blastocysts. All other blastocysts were grouped together as non-top blastocysts [1].

Statistical analysis

The chi-squared test was used to analyze the rate of blastocysts and top blastocysts according to the MKc and the sperm quality. P values <0.05 were considered as statistically significant.

Results

Oocytes were injected with different classes of spermatozoa (IMSI classes I–III). Due to the severely impaired quality of semen samples in our patient cohort, it was not possible to inject all patients' oocytes with morphologically normal spermatozoa. In fact, even after extensive selection, insufficient numbers of class I spermatozoa were available. One hundred sixty-two embryos (43.4 %) derived from injected oocytes with normal sperm (class I); 211 embryos derived from injected sperm with LNV (class II [90; 24.2 %] and class III [121; 32.4 %]).

Interestingly enough, we could not find any significant differences in early morphokinetics in correlation to the grade of vacuolization of the sperm's head (data not shown).

Rate of blastocysts and top blastocysts in relation to MKc

A detailed retrospective analysis of morphokinetics (t3, t5, cc2) was made for 373 embryos, of which 87 (23.3 %) met all three morphokinetic criteria (MKc3). At least one criterion (MKc<3) was not fulfilled in a group of 286 embryos (76.7 %). A significant increase in the rate of blastocysts (54.0 vs. 36.3 %; P<0.01) and top blastocysts (25.3 vs. 10.8 %; P<0.001) was observed in the group of those that fulfill all the MKc in the first 3 days of development compared to the group where at least one criterion was not met (data not shown).

Rate of blastocysts and top blastocysts in relation to MKc and sperm quality

In the group of embryos that fulfill all three MKc on day 3, no significant difference in the blastocyst rate was observed when oocytes were injected either with a sperm of normal fine morphology or class II/III sperm (62.5 vs. 46.9 %; n.s.). However, a significant increase in the rate of top blastocysts was found when oocytes were injected with a morphologically normal spermatozoon as compared to the injection of a spermatozoon with LNV (35.0 vs. 17.0 %; P<0.05) (Table 2). In the group of embryos in which at least one criterion was not met (MKc<3), a trend to higher blastocyst and top blastocyst rates was found when IMSI class I was used for fertilization (BR, 40.8 vs. 33.1 %; TBR, 14.2 vs. 8.4 %; Table 2). However, this was without any significance and probably due to the low number of cases.

Discussion

Currently, several selection models and algorithms are postulated based on morphological criteria and kinetic parameters assessed by TLC (summarized in Kirkegaard et al. 2015) [42]. In the presented study, we used the algorithm for selection presented by Meseguer in 2012. According to this selection model, it was postulated that embryos having complied with the three morphokinetic criteria (cc2, 5–12 h; t3, 35–40 h; and t5, 48–56 h) had a 77 % chance of reaching the blastocyst stage [14].

In our study cohort including patients with severe male infertility and recurrent implantation failures, we observed that more than three quarters of all embryos did not fulfill all three propagated morphokinetic criteria. However, out of these 286 embryos with deviant morphokinetic patterns, 104 (36.3 %) reached the blastocyst stage and still 29.8 % of these blastocysts were designated as top blastocysts.

With the introduction of TLC in ART, great hope was initially placed in the use of an optimal non-invasive tool to select embryos at an early stage of their development according to a specific algorithm that takes the morphology and several kinetic key points into account. Several retrospective studies on TLC reported correlations between distinct

Table 2 Blastocyst rate and top blastocyst rate originating from day 3 embryos in correlation to morphokinetic criteria (MKc<3/MKc3) and sperm morphology (IMSI class I vs. IMSI class II/III

Morphokinetic embryo selection criteria[a]	IMSI class[b]	No. of blastocysts/d3 embryos (%)	No. of top blastocysts/d3 embryos (%)
MKc<3	IMSI class I	49/120 (40.8 %)*	17/120 (14.2 %)*
	IMSI class II/III	55/166 (33.1 %)*	14/166 (8.4 %)*
MKc3	IMSI class I	25/40 (62.5 %)*	14/40 (35.0 %)**
	IMSI class II/III	22/47 (46.9 %)*	8/47 (17.0 %)**

Values are numbers or %

**P* value not significant; ***P*<0.05

[a] Morphokinetic embryo selection criteria were used according to Meseguer et al. 2011 [13] and Meseguer 2012 [14]

[b] The definition of IMSI classes was done according to Vanderzwalmen et al. 2008 [28]

morphokinetic parameters and blastocyst development [15, 16, 43–47]. But we also have to consider that a model for the prediction of further embryo development must guarantee high sensitivity and specificity. Meanwhile, the euphoria has clearly waned, giving way to a more disillusioned view. A recently published review has correctly stated that a blastocyst prediction model should be used for ranking rather than selection to avoid the risk of discarding usable embryos [42].

The prediction of the embryonic fate even with the time-lapse technology at the moment of early cleavage stages remains a difficult task. We have previously demonstrated that embryos that do not fulfill the current morphological or morphokinetic criteria still have the potential to develop to a blastocyst and lead to the birth of a healthy baby [48]. Such observations are supported by other studies showing that even vitrification of blastocysts derived from fair- to poor-quality cleavage-stage embryos can produce high pregnancy rates after warming [49, 50].

Irrespective of morphokinetic parameters, we observed a significant increase in the rate of top-quality blastocysts when spermatozoa devoid of large nuclear vacuoles were selected for injection. Little is known about the paternal impact on embryo morphokinetics, of note in correlation to subtle sperm morphology. Recently, Knez and colleagues demonstrated that the kinetics until the blastocyst development is affected by the type of sperm evaluated by MSOME. They found that blastocysts derived from morphologically normal spermatozoa free of vacuoles required the shortest mean time for all developmental events in comparison with blastocysts originating from poor-quality spermatozoa. Interestingly enough, there was a significant difference in accomplishing the second cell division when embryos derived from the best and poorest sperm quality were compared [18]. For our patient cohort, however, we could not confirm these observations.

It is well known that dysfunction in the sperm negatively affects the development of embryos [12, 51, 52]. The present study supports the hypothesis that the impact of male infertility may be related not only to zygote and early cleavage-stage morphological abnormalities (early paternal effect) but also to poor developmental competence which in turn might lead to implantation failure due to disorganization of the chromatin and/or a high level of sperm DNA fragmentation. With regard to the reproductive outcomes in terms of fertilization, embryo development, pregnancy, and abortion rates, the importance of selecting normal spermatozoa becomes obvious when comparing oocyte injections performed with morphologically normal sperm to those injections with spermatozoa exhibiting different subcellular defects. Based on several studies, it is well documented that fertilization with spermatozoa revealing large nuclear vacuoles and/or abnormal shape of spermatozoa reduces the percentage of good-quality blastocysts on day 5 [18, 28–30]. However, the main limitation of the here-presented study is the low case number which is due to our policy to avoid fertilization with poor-quality sperm and the neglecting of the implementation of further female characteristics. Thus, we cannot exclude that these findings apply only to a certain patient clientele and different results might be achieved in couples with substantially younger female age or certain infertility diagnosis.

Conclusion

Our results confirm that at the moment, it is not (yet) possible to use the time-lapse technology to predict the development potential of an early cleavage-stage embryo on day 2 or 3 in a reliable manner. According to this study, we have recognized that approximately a quarter of all embryos meet all three criteria, which in turn means that the remaining three quarters of day 3 embryos would be disposed of, although even in this group a notable rate of blastocysts and top blastocysts was obtained. Moreover, there is no universal algorithm that takes the importance of the sperm factor into account. In fact, the recent literature shows that various other factors have an impact on the embryonic development, making the establishment of a reliable algorithm extremely difficult. Often, studies suggest that TLC might be an appropriate tool for embryo selection. However, evidence-based data is often lacking

[47, 53, 54]. We found higher (top) blastocyst rates when class I spermatozoa were used, irrespective of whether or not the MKc3 criteria have been met. Moreover, we could not find any deviations of early morphokinetics after fertilization with class I or class II/III spermatozoa. These observations confirm previous findings suggesting that the grade of sperm head vacuolization has no impact on the embryo outcome on day 3 [22, 28, 33, 55]. Thus, the value of embryo selection prior to EGA remains questionable as the real developmental potency of the embryo cannot be properly estimated. In regard to the increased blastocyst rates, when class I sperm was injected and all three MKc were met, this might give some predictive value of (top) blastocyst formation. However, TLC-based embryo selection on day 3 with the here-applied selection model reveals an unacceptable error rate with a potentially high risk of embryo wastage. In consequence, the application of IMSI and embryo selection at the blastocyst stage seems still to be superior.

Conflict of interest The authors declare that they have no competing interests.

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