Prediction of live birth – selection of embryos using morphokinetic parameters

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Backround. The goal of assisted reproduction is for a couple treated with IVF techniques to end the treatment by giving birth to a healthy baby. A neccessary presumption for success is the identification of the best embryo with high implantation and developmental potential. One option is to select an euploid embryo by invasive preimplantaion genetic testing for aneuploidy (PGT-A) or it is possible to select the best embryo by non-invasive time-lapse monitoring (TLM), specifically based on morphokinetic parameters and morphological markers that are able to identify an embryo with high developmental potential.

Materials and Methods. The study involved a total of 1060 embryos (585 euploid and 475 aneuploid embryos after PGT-A) with good morphology from 329 patients in the period 01/2016-10/2021. All embryos were cultured in a time-lapse incubator, trophectoderm (TE) cells biopsies for PGT-A examination were performed on day 5 (D5) or day 6 (D6) of culture. During the study period, 225 frozen embryo transfers (FET) of one euploid embryo were performed. Based on the treatment outcome, the embryos were divided into 2 groups – euploid embryos, which led to the birth of a healthy child, and euploid embryos that did not show fetal heartbeat (FHB) after FET.

Results. Based on the statistical analysis of the embryos without implantation and the embryos with live birth, it is clear that the morphokinetic parameters t5 (time of division into 5 cells) and tSB (time of start of blastulation) are significantly different.

Conclusion. The results suggest that of the morphokinetic parameters tSB and t5 are predictive indicators for selecting an embryo with high developmental potential and with a high probability of achieving the birth of a healthy child.

Key words: embryo, time-lapse, morphokinetics, implantation, live birth

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INTRODUCTION

The basic goal of assisted reproduction is to select a high-quality embryo that, after transfer to the uterus, will result in the birth of one healthy child. Single embryo transfer (SET) reduces the incidence of multiple pregnancies after IVF (ref.[1,2]). The key to successful IVF treatment is selection of embryos with high developmental potential[3]. Because more than half of IVF embryos are aneuploid[4,5], techniques have been developed that are able to detect chromosomal abnormalities in human embryos – preimplantation genetic testing for aneuploidies (PGT-A). PGT-A is an invasive techniques where a biopsy of trophectoderm cells is required for self-genetic testing[6]. Modern non-invasive techniques finding a quality embryo for ET include time-lapse monitoring[7], metabolomics[8] and proteomics[9].

At present, it is not realistic for many reasons to test all developing embryos with PGT-A for all IVF patients. Morphological evaluation has long been used to assess embryo quality. Unfortunately, this assessment is highly subjective[10]. Static evaluations of embryos are performed only a few times during culture, and it is not possible to notice what happens when embryos are uncontrolled[11,12]. Embryo morphology can vary considerably over a period of several hours, resulting in high variability in embryo evaluation[13]. In the last 15 years, time-lapse monitoring has been gradually introduced as a non-invasive method for the selection of quality embryos. This technology, which combines frequent imaging of developing embryos[2,11] with undisturbed culture conditions[7,14], allows for more accurate embryo morphological assessment combined with morphokinetic evaluation[15,16]. Time-lapse culture systems have been verified as safe for human embryo culture[12] and certainly have the potential to create a culture environment leading to higher blastocyst formation[17], higher number of implanted embryos and higher clinical pregnancy rate[11,12,18]. By using the cultivation of embryos in time-lapse incubators, it is possible to obtain important information both on embryo development, specifically embryo morphokinetics, but also on other developmental signs such as fragmentation and multinucleation of blastomeres[19].

Numerous studies have investigated the relationships between morphokinetic parameters and embryonic competence[2,11,12,20] and between morphokinetics and embryonic ploidy[21-23].

Based on evaluation of the relationships between various morphokinetic parameters and the developmental competence of the observed embryo, various selection hierarchical models were created – the first hierarchical model was created in 2011 by Meseguer[11]. A recent meta-analysis confirmed the importance of morphokinetic selection of embryos, as it showed an increased number of pregnancies, a higher birth rate, and a lower loss of early pregnancies[24].

Despite the large number of published time-lapse studies, different and even conflicting data are presented. There is still significant controversy about which parameters are useful for predicting blastocyst stage, implantation potential, or embryonic ploidy[13,25-27].

The aim of this study was to follow up and confirm the results of our previous study[28], where it was found that morphokinetic predictive parameters for achieving clinical pregnancy with a high probability are t5 and tSB; and also that the presence of multinucleations in 2-cell and 4-cell embryos is associated with a higher probability of embryonic aneuploidy. This study, unlike the one mentioned above, used patients who gave birth to a healthy child after FET of one time-lapse cultured euploid embryo.

MATERIALS AND METHODS

This retrospective cohort single-center study is based on data from the database of the Clinic of Reproductive Medicine and Gynecology Zlín. The study involved patients who completed IVF treatment at the clinic with PGT-A and culture of embryos in a time-lapse system in the period 01/2016 to 10/2021. A total of 329 patients mean age of 33.1 ± 4.4 years were included. The indication for PGT-A was mainly older age of the patient, miscarriage, or implant failure in previous IVF cycles.

All laboratory procedures and materials were used the same as in the previous study[28], to which this current work follows.

Ovarian stimulation and oocyte collection were performed according to standard treatment protocols. Ovarian stimulation was performed using the GnRH antagonist protocol, Decapeptyl/Dipherelin was used to induce ovulation, and oocyte collection was under ultrasound control 36 h after trigger application.

All oocytes were denuded and ICSI was performed on mature oocytes (MII) (4 h after oocyte collection). All embryos were cultured individually in an EmbryoSlide culture dish (Vitrolife) with one-step GTL medium (Vitrolife), the slide was overlaid with 1.5 mL of mineral oil (Ovoil, Vitrolife). Embryos were cultured in a time-lapse system (EmbryoScope, Vitrolife) at 37 °C, 6% CO_2, 5% O_2 to blastocyst or expanded blastocyst stage (D5, D6). Laser-assisted hatching (OCTAX) was performed on the D3 to open the zona pellucida and allow the trophectoderm to hernia out.

A total of 1060 morphologically high-quality embryos from 329 patients were genetically tested. Finally there were 585 euploid and 475 aneuploid embryos after PGT-A. During the study, 225 frozen embryotransfers (FET) of 1 euploid embryo were performed. The actual biopsy of the trophectoderm was performed on all quality embryos on D5 or D6, the required quality of the embryos was 4AA, 4AB, 4BA, 5AA, 5AB, 5BA, 6AA, 6AB, 6BA according to Gardner and Schollcraft[29] as soon as the embryo was sufficiently hernia from the zona pellucida (minimum 10 cells) using a laser (OCTAX). TE cells were washed according to standard protocol in PVP buffer (1% polyvinylpyrrolidone), placed in a PCR microtube with 2 µL PBS buffer, frozen at –20 °C and transferred to an accredited molecular genetic laboratory, where SurePlex DNA isolation and whole genome amplification (SurePlex) was performed. DNA Amplification system, Illumina). The obtained DNA sample with a final concentration of about 50 ng/µL was examined by array-CGH (DNA labeling, on-chip hybridization – 24sure +, BlueGnome, Illumina, analysis using BlueFuse Multi software) or the chromosomal profile of the samples was determined by NGS (VeriSeq PGS Kit, Illumina). The principle of the method is the preparation of a sequencing library, where individual samples are marked with specific indices, which is then amplified and sequenced by SBS (MiSeq, Illumina). Biopsied embryos were vitrified 1 h after TE biopsy (Rapid VitBlast, Vitrolife).

For all embryos where PGT-A was performed, the image from the EmbryoScope (image every 10 min, 7 focal planes) was analyzed and all known morphokinetic and morphological data were read using EmbryoViewer software. The parameters used for the study were: duration of the second cell cycle (cc2), duration of the third mitosis – time between division of the 3-cell embryo into 4-cell embryo (s2), time to division of the embryo into 5-cell embryo (t5), time until the start of blastulation (tSB). The time of cell division is the moment when a complete septum is formed from the cytoplasmic membrane and the two new blastomers are completely separated. The blastulation initiation time (tSB) is when the first blastocoel cavity forms between embryonic cells. The incidence of multinucleation (MN) in the 2-cell and 4-cell stages was further evaluated, with each embryo with multinucleations in at least one blastomere being considered an embryo with multinucleations. The annotation was performed all the time by the one experienced embryologist. Following the menstrual cycle, the patient was prepared for frozen embryotransfer of one euploid embryo using an HRT-substituted cycle or a native cycle.

Based on the result of the blood pregnancy test (level of hCG, human choriogonadotropin) and the further development of the pregnancy and its termination by the birth of a healthy child, 2 groups were created in this study: euploid embryos that led to the birth of a healthy child (n=126) and euploid embryos that did not lead to ultrasound confirmed fetal heartbeat – FHB (included embryos with negative hCG test, no visible gestational sac, gestational sac withoout FHB) (n=99).

The main data were continuous variables (cc2, s2, t5 and tSB). The distributions of calculated embryo timings

Table 1. Comparison of morphokinetic parameters in groups of PGT-A normal embryos that led to the birth of a healthy child after FET and PGT-A normal embryos where FHB was not achieved after FET (continuous variable analysis, two-sample t-test).

Parameter	Embryos – live birth n=126/mean	Embryos – no FHB n=99/mean	*P*
cc2/h	11.579	11.749	0.294
s2/h	0.671	0.608	0.483
t5/hpi	48.338	50.148	0.052
tSB/hpi	93.438	95.287	0.014

h, hours; hpi, hours post insemination; cc2, duration of the second cell cycle; s2, time between second and third mitosis; t5, time to reach 5-cell embryo; tSB, time to start of blastulation; FHB, fetal heartbeat.

from a sample of genetically normal embryos that led to the birth of a healthy child and genetically normal embryos that did not lead to implantation was tested using the Shapiro-Wilks test.

The means of samples with normal distribution and of sufficient size were compared by a two-sample t-test. In the case of non-normal distribution, the medians were compared by Man Whitney non-parametric test.

Categorical variables including MN in 2-cell and 4-cell embryos were interpreted as counts and percentages and analyzed using the Fisher Exact test. To assess the predictive values of the variables, all combinations of variables were subjected to multivariate logistic regression analysis. The many resulting combinations were tested in automated loops, so that all possible combinations from 1 to 6 concurrent parameters were explored. The only variables kept in the different trials, were these where the null hypothesis that the coefficients were insignificant had a probability below 0.05.

Results were considered significant at $P<0.05$.

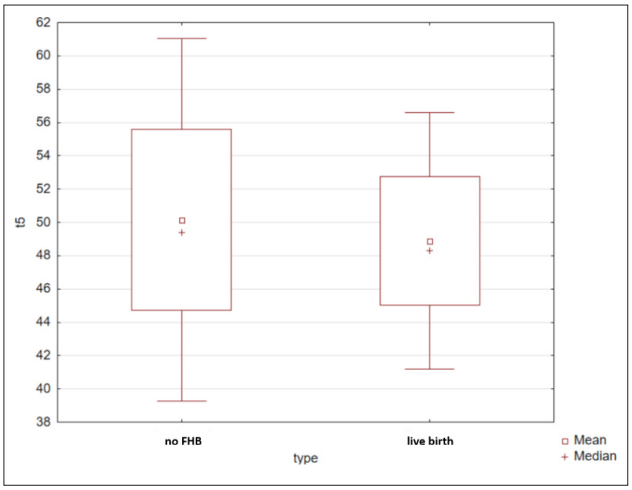

Fig. 1. t5 – graph of continuous variables – euploid embryos without FHB and euploid embryos with live birth.
FHB, fetal heartbeat.

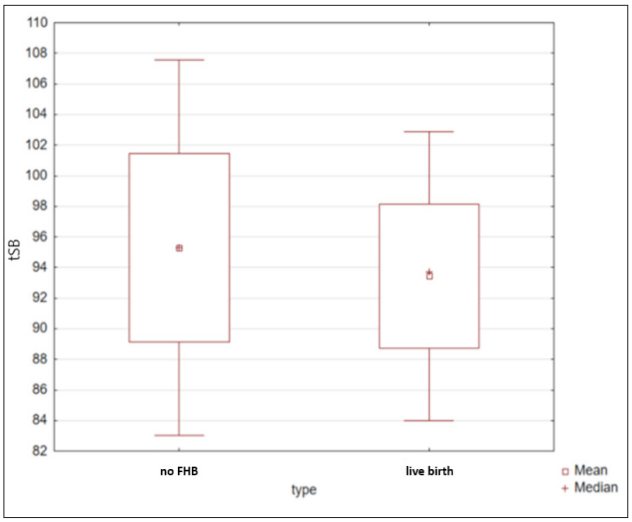

Fig. 2. tSB – graph of continuous variables – euploid embryos without FHB and euploid embryos with live birth.
FHB, fetal heartbeat.

RESULTS

The results show that PGT-A normal embryos that led to the birth of a healthy child after FET had statistically significantly different morphokinetic parameters t5 ($P=0.05$) and tSB ($P=0.01$) compared to the euploid embryos (post PGT-A) which after insertion into the patient's uterus did not lead to implantation or fetal heartbeat (FHB) was not demonstrated. There was no statistically significant difference in morphokinetic parameter cc2 and s2 in the groups of PGT-A normal embryos which resulted in the delivery of a healthy baby after FET and PGT-A normal embryos that did not lead to implantation and fetal heartbeat (FHB). The results are summarized in Table 1, Fig. 1 and 2.

After statistical evaluation, no statistically significant difference was detected in the incidence of multinucleations in 2-cell and 4-cell embryo stage between the groups of euploid embryos (post PGT-A) with live birth and euploid embryos (post PGT-A) embryos without FHB.

The obtained data also show that if multinucleations occur in 2-cell embryo, then there is a 9.55x higher chance of multinucleations occurring in 4-cell embryo as well as embryos without multinucleations in 2-cell stage ($P<0.0001$).

Using the stepwise regression method of the logistic model, it was found that the higher the time of the parameter t5, the greater the chance of MN occurring in 2-cell stage - every hour the chance of MN increases by 7%. The s2 parameter is significant for the occurrence of MN in 4-cell embryos.

The statistical analysis shows that the mean time of t5 and tSB is longer in patients who have failed implantation of the transferred embryo.

It is clear from the results that the parameter tSB has a significant effect on the successful termination of pregnancy by childbirth; however, the higher the tSB value, the lower the chances of delivery.

DISCUSSION

Our study confirmed that the morphokinetic parameters tSB and t5 observed in PGT-A normal preimplantation human embryos have a statistically significant relationship with their developmental potential – achieving the delivery of a healthy child. A statistically significant difference was detected in the values of tSB and t5 parameters in the group of PGT-A normal embryos, which led to the birth of a healthy child after FET, and in the group of PGT-A normal embryos without achieving a fetal heartbeat. This is consistent with the results of our previous study[28], where it was found that the values of morphokinetic parameters cc2, t5 and tSB are significantly shorter in genetically normal embryos than in genetically abnormal embryos; furthermore, tSB and t5 parameters were significantly shorter in the group of genetically normal embryos with a proven clinical pregnancy compared to genetically normal embryos with a negative pregnancy test after FET.

In addition, multinucleations in 2-cell and 4-cell embryo stage were monitored in this study, but no significant difference was found in PGT-A normal embryos that led to the birth of a healthy child and PGT-A normal embryos without FHB. In the previous study, a statistically significant difference was found in the groups of PGT-A normal and PGT-A abnormal embryos in occurrence of multinucleations in 2-cell and 4-cell embryo stage[28].

The main reason for implantation failure and miscarriage in IVF treatment is the incidence of aneuploidy in oocytes and embryos, which can occur in more than 50% of human embryos and which increases with maternal age[4,30]. Embryos for ET are still very often selected based on their morphology on the day of ET, but it has been found that even aneuploid embryos can achieve a high morphology score on the day of ET (ref.[31,32]).

Evaluation of embryos using time-lapse monitoring is currently still developing and the number of embryological laboratories using this evaluation method is increasing. Many studies have tried to identify prognostic factors obtained from time-lapse monitoring that would identify a euploid embryo or embryo with high developmental potential, but their results have been different from each other[25].

In our work, embryos were cultured in a time-lapse system for 5 to 6 days (D5, D6), i.e. usually to the expanded blastocyst stage. Cultivation in the time-lapse incubator (EmbryoScope®) is uninterrupted if one-step medium is used and evaluation is performed with EmbryoViewer® software using images from multiple focal planes. The camera takes pictures at a low intensity of red LED lighting (635 nm); the total exposure dose is much lower compared to standard evaluation under a laboratory microscope[33]. Continuous culture improves culture medium stability, which is an essential factor for blastocyst formation, and has the potential to increase the number of quality embryos in a patient's IVF cycle[17]. A disadvantage is that this system does not allow embryo rotation, which can negatively affect the visual assessment of the embryo, especially when the blastomeres overlap or if the embryo has a high degree of cytoplasmic fragmentation[34].

Several studies have evaluated the relationship between morphokinetic parameters derived from time-lapse monitoring (TLM) and IVF / ICSI results. Faster fertilization kinetics were associated with better embryo development[35], while faster cleavage kinetics, in addition to better embryo development[36-38], has also been associated with higher implantation[11,21,39] and higher pregnancy rate[40-42]. Recently, it has been shown that faster blastulation kinetics is associated with higher birth rate[43]. This was also confirmed in our work, namely that the parameter tSB has a significant effect on the successful completion of pregnancy.

Meseguer et al.[11] were among the first to publish the results of their retrospective study of morphokinetic parameters, blastomere size and multinucleation in embryos and found that embryos with t5 = 48.8 to 56.6 h had a higher chance of developing blastocysts of good morphology and have a higher implantation potential. We chose for the study the morphokinetic parameters cc2, s2, t5, tSB, the occurrence of multinucleations in the 2-cell stage and the 4-cell stage. The morphokinetic parameters and morphological markers focused on in our work have already been evaluated in the following studies: t5 (ref.[21]), t5 and cc2 (ref.[11]), s2 (ref.[11,30]), cc2 and s2 (ref.[30,44]), tSB (ref.[30,45,46]) and were significantly shorter in genetically normal embryos than in genetically abnormal embryos. Fishel et al.[43] reported that the morphokinetic parameter tSB and the duration of blastulation, dB = (tB – tSB), strongly correlate with a higher probability of live births after ET. Goodman et al.[47] identified tSB as the only predictive factor for implantation in their study. Campbell et al.[45] found that in embryos with a low risk of aneuploidy, the start of blastulation (tSB) should occur within 96.2 hpi.

A recent large-scale study evaluating 1810 transferred embryos suggested that the blastulation kinetics assessed from the start of blastulation and to blastocyst development was a better predictor of live births than classical morphology assessments[48]. This is in contrast to a previous smaller study involving 235 patients where the use of blastulation kinetics and early morphokinetic information did not improve the number of implanted embryos or clinical pregnancies compared with evaluation based on classical morphology[47].

Minasi et al.[30] in their study of predictive markers at the level of blastocysts found that the morphokinetic parameters tSB – start of blastulation, tB – time of formation of a complete blastocyst, tEB – time of formation of an expanded blastocyst, tHB – time to start of hatching, were remarkably in euploid embryos shorter compared to aneuploid embryos. These results are consistent with previously published data from Campbell et al.[22,45], where in euploid embryos compared to aneuploid embryos, a delay in the start of compactation (tSC), the start of tSB blastulation and the reaching of the full blastocyst phase (tB) were observed.

As already mentioned, even aneuploid embryos are

able to achieve a high morphological score[31,32,49]. The findings of the Del Carmen Nogales et al.[50] that morphokinetic parameters can connected with the kind of chromosomal abnormality, i.e., embryos with a high degree of chromosomal abnormalities develop differently than euploid embryos, are very interesting. Surprisingly, trisomy embryos have very similar morphokinetic features to euploid embryos. The results of the study by Fragouli et al.[32] show that in the cleavage stage (up to stage 4–8 cells) embryo ploidy does not affect embryo morphology, on the other hand, at the blastocyst stage, a higher probability of euploidy was found in blastocysts with good morphological score and in blastocysts with faster growth progression[31,32]. A possible explanation is that the activation of the embryo genome begins at the stadium of 4 to 8 cells[51]. As a result, it is only during the third day of development that the embryo starts to express its own genes and the genetic abnormalities present begin to affect embryonic development[31,32]. It is neccessary to mention that attempting to choose an euploid blastocyst based on its morphology alone can be highly hazardous, as many aneuploid blastocysts are capable of achieving the highest quality[30].

Other published studies report that the morphokinetic parameters of implanted and non-implanted embryos[37,39] and euploid and aneuploid embryos[52] are not different. The study by the team of Barberet et al.[53] shows that there is no difference in morphokinetic parameters for embryos that lead to the birth of a child and for embryos that do not lead to a live birth.

The study by Fishel et al.[54] used morphokinetic data to select embryos for ET; a 19% more live births were found in women under 38 using their own oocytes. The benefit of using morphokinetic data from EmbryoScope for embryo selection was also evident in recipients over 37 years of age, where transfer of one blastocyst had the identical chance of giving birth as two transferred blastocysts after standard treatment, but without the risk of multiple pregnancy.

Some studies have described that morphokinetic parameters of developing embryos can be affect by non-genetic factors: e.g. the quality of the culture media[55], stimulation protocols[56], fertilization method[36], obesity[57], smoking[58] or lower or higher oxygen concentration in the culture environment[59].

Multinucleation, which occurs in the blastomeres of early human embryos, is a common embryonic abnormality[60]. Aneuploidy or mosaicism appears in 50–100 % of genetically screened embryos with multinucleations[49]. However, it is expeted that embryos, based on a "self-correction" mechanism, are capable to repair multinucleated blastomeres, and after the following cell division, two daughter euploid blastomeres with a single nucleus are created. Most embryos with multinucleations in blastomeres are aneuploid or mosaicist, but due to the "self-correction" mechanism, some embryos may eventually develop into euploid[61]. The incidence of multinucleations in 2-cell embryo reduces the rate of embryo implantation after ET as well as the number of clinical pregnancies achieved[19], and advances the number of aneuploidies detected[62]. Interestingly, a higher rate of multinucleations was noted in the 2-cell stage and significantly declined by the changeover of the embryo to the 4-cell stage[63]. Our previous study proposes that a more significant indicator of aneuploidy in the embryo is the incidence of multinucleations in the 4-cell embryo[28].

Objective time-lapse evaluation of embryos is better for embryo selection for its ability to generate embryos for ET that lead to live births than the conventional, subjective blastocyst morphology evaluation system[48].

The use of morphokinetic parameters and morphological markers can facilitate and speed up laboratory procedures, especially in the early exclusion of embryos with abnormal development. However, the same or very similar procedures should be performed during oocyte fertilization and embryo culture to ensure comparable time-lapse results in the long duration. However, it must be acknowledged that not all of these procedures can take into account all the factors influencing the successful outcome of pregnancy, especially in the IVF population cohort; normal placental function[54] and endometrial susceptibility[64,65] are also essential.

Pribenszky et al.[24] confirmed in their meta-analysis that time-lapse embryo culture was correlated with a higher rate of clinical pregnancy (ongoing) and a significant increase in birth rate. Reignier et al.[66] analyzed the success rate and benefit of KIDScore™ day 5 morphokinetic prediction models and showed a significant correlation with the likelihoods of pregnancy and childbirth after blastocyst transfer, but with the finding that there are still opportunities to increase success.

Significant progress in the efficiency of embryo selection is expected in the next decade due to the combination of morphological, metabolic and morphokinetic data using integrative systems using artificial intelligence[67].

Our work suggests that some morphokinetic parameters and morphological markers can identify an embryo that has the potential to develop into a healthy pregnancy and lead to the birth of a healthy child. Therefore, these predictive parameters from TLM should be used to select the best embryo with good developmental potential in patients who cannot undergo PGT-A or who do not wish to have invasive PGT-A examination for any reason.

CONCLUSIONS

By means of time-lapse monitoring using the morphokinetic parameters tSB and t5, which appear to be important indicators of embryo quality, it is possible to select embryos with a high probability for implantation and further for the birth of a healthy child.

Author contributions: KT: conceptualization, analysis and interpretation, data collection, writing the manuscript, manuscript drafting and revision; SB: statistical analysis and interpretation; TR, DR, AMF, FM: manuscript drafting and revision.

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