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# Timing of Embryo Cleavage

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## Abstract

Time-lapse system can provide a culture environment to observe the development of embryos continuously. There are many morphokinetic markers to help us to find out the best quality of embryos. We review the studies to clarify the relationship of markers between implantation potential and embryo chromosome status. Surprisingly, most of markers are controversial or no significant effect on implantation potential and pregnancy rate. We suppose that some uncertain factors may influence embryonic implantation and pregnancy. Here we provide a new method for selecting optimal quality of embryos by many morphokinetic markers in the time-lapse system. Therefore, we can expect that the time-lapse system helps us to choose the good quality embryos for subsequent embryos transfer to improve implantation potential, euploid chromosome and pregnancy rate. Furthermore, studies need to understand the other maternal physical conditions correlation with embryos implantation.

**Keywords:** time-lapse, cleavage embryo, morphokinetic markers

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## 1. Introduction

The morphology of embryo is the most widespread method to select the embryo with high implantation potential in assisted reproductive technology (ART). Conventionally, embryo development was daily observed after insemination, which could assist the embryologists to select the optimal embryo to transfer for elevating live birth rate eventually. However, the daily observation is considered as a disadvantage for embryo development because of the frequent transfer between incubator and atmospheric environment. Thus, a new and powerful tool, time-lapse monitor (TLM), was developed to estimate the morphokinetic markers of embryos. Currently, TLM can be used to evaluate the embryo growing status from the time of insemination to blastocyst formation. The sequential assessment of pronuclear, cleavage stage, and blastocyst morphology can continuously evaluate the morphology of embryos

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through automatically obtaining images in every 5–20 min. Besides, TLM offers a steady culture condition due to bypassing the daily observation. Here, we discuss the timing of embryo cleavage and the following effects of implantation potential in this chapter.

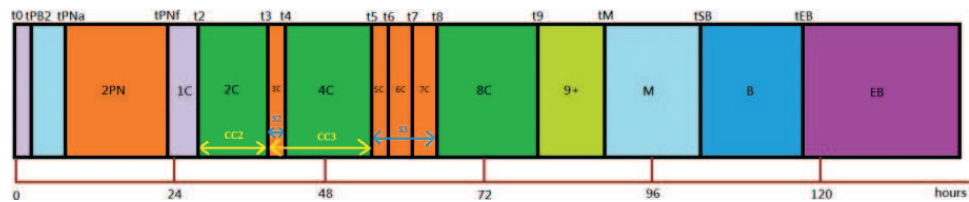
2. Morphokinetic markers

Generally, there are many milestones (Figure 1), including pronucleus appearance, pronucleus breakdown, first division, second division and blastulation, during the period of fertilization to blastocyst formation. The TLM fails to obtain the pictures at every minute since the capturing period was limited. Although the limitation of the time lapse is obvious, currently, it is still the most practical manner to evaluate the timing of embryo development rather than daily observation. Here, we listed the morphokinetic markers and discussed the timing of different time point during the development of embryos and the effect of clinical outcomes.

(1) The timing of second polar body extrusion (tPB2): the time of the second polar body extrusion is  $2.9 \pm 0.1$  h after Intra-cytoplasmic sperm injection (ICSI). The range of extrusion time is around 0.7–10.15 h. If the oocytes from female age >38 years old, the timing of second polar body extrusion was significantly delayed but no other effects were observed in further embryo development [1]. The mean time of tPB2 is 3.9 h in euploid and 4.0 h in aneuploid embryos, respectively. The chromosome integrity of embryos is irrelevant to the timing of second body extrusion [2].

(2) The timing of pronuclear appearance (tPNa): the time of pronuclear appearance is  $8.4 \pm 2.4$  h in the implantation group and  $8.2 \pm 1.9$  h in the non-implantation group [3]. In euploid embryos, the mean time of tPNa is 10.2 h and 10.1 h in aneuploid embryos [2]. Therefore, the timing of pronuclear appearance has no significant effect on implantation potential and chromosome status.

(3) The timing of pronuclear fading (tPNf): longer time taken in pronucleus (PN) breakdown might be beneficial for live birth. Azzarello et al. [4] claimed that the timing of tPNf was longer in live birth group ( $24.9 \pm 0.6$  vs.  $23.3 \pm 0.4$  h), and there was no live birth if the timing of PN breakdown was less than 20 h. The timing of PN breakdown was equal between implanted and non-implanted embryos [3, 5]. The mean time of tPNf is 24.4 h in euploid embryos and 24.8 h in aneuploid embryos [2]. The timing of pronuclear fading has no significant difference in embryo implantation and chromosome status but no live birth when tPNf is less than 20 h.



**Figure 1.** The milestones of embryo development. tPB2: the timing of second polar body extrusion, tPNa: the timing of pronuclear appearance, tPNf: the timing of pronuclear fading, t2, 3, 4, 5, 6, 7, 8, 9: time from insemination to the 2, 3, 4, 5, 6, 7, 8, 9 cell stages, tM: time from insemination to morula, tSB: time from insemination to starting blastulation, tEB: time from insemination to expanded blastulation, cc2:t3-t2, cc3:t5-t3, s2:t4-t3, s3:t8-t5.

(4) Time from insemination to the 2-cell stage ( $t_2$ ): it is still controversial in the period. Meseguer et al. [6] presented that the  $t_2$  of implanted embryos group was shorter than non-implanted embryos ( $25.6 \pm 2.2$  vs.  $26.7 \pm 3.8$  h). Chamayou et al. [3] showed no significant difference in implanted and non-implanted embryos ( $26.9 \pm 3.2$  vs.  $27.0 \pm 4.0$  h). Kirkegaard et al. [5] claimed that  $t_2$  was similar in the pregnancy and non-pregnant groups. Curiously,  $t_2$  is shorter when embryo was incubated in single culture medium than sequential culture medium ( $27.36 \pm 4.12$  vs.  $29.09 \pm 4.86$  h) [7]. The mean time of  $t_2$  is no significant between euploid (28 h) and aneuploid embryos (28.4 h) [2]. The development of the 2-cell stage may be faster in implanted embryos but no significant in chromosome status.

(5) Time from insemination to the 3, 4, 5 cells ( $t_3$ ,  $t_4$ ,  $t_5$ ): some studies have shown that the enhanced implantation potential has been observed in shorter  $t_3$ ,  $t_4$  and  $t_5$ . The time periods of  $t_3$ ,  $t_4$  and  $t_5$  were significantly shorter in implanted embryos than non-implanted embryos. The times of  $t_3$  ( $37.4 \pm 2.8$  h),  $t_4$  ( $38.2 \pm 3.0$  h) and  $t_5$  ( $52.3 \pm 4.2$  h) are significant difference in implanted embryos compared with the times of  $t_3$  ( $38.4 \pm 5.2$  h),  $t_4$  ( $40.0 \pm 5.4$  h) and  $t_5$  ( $52.6 \pm 6.8$  h) in non-implanted embryos [6]. However, Chamayou et al. [3] and Kirkegaard et al. [5] demonstrated that there was no difference in embryo implantation and pregnancy rate. The embryo development is faster in single culture medium than in sequential culture medium ( $t_3$ ,  $37.75 \pm 6.64$  vs.  $39.53 \pm 6.15$  h;  $t_4$ ,  $40.07 \pm 5.98$  vs.  $41.45 \pm 6.07$  h;  $t_5$ ,  $48.77 \pm 9.49$  vs.  $52.22 \pm 9.34$  h) [7]. The mean time of  $t_3$  (37.4 vs. 37.2 h) and  $t_5$  (50.4 vs. 50.6 h) is no significant difference between euploid and aneuploid embryos, but the mean time of  $t_4$  (40 h) is significant difference between the euploid (40 h) and aneuploidy (41.1 h) blastocysts [2]. Consequently, faster embryo development of  $t_3$ , 4, 5 is beneficial for implantation, but only  $t_4$  might influence the euploid rate of blastocysts.

(6) Time from insemination to the 6, 7, 8, 9 cells ( $t_6$ ,  $t_7$ ,  $t_8$ ,  $t_9$ ): according to the previous report, although the time from insemination to the 8 cells exhibited faster in implanted embryos ( $54.9 \pm 5.2$  vs.  $58.0 \pm 7.2$  h) [8], the other report showed that there are no statistical difference between the implanted and nonimplanted embryos at  $t_6$  ( $54.3 \pm 5.8$  vs.  $54.5 \pm 8.2$  h),  $t_7$  ( $57.4 \pm 8.6$  vs.  $57.6 \pm 9.8$  h),  $t_8$  ( $61.0 \pm 10.8$  vs.  $60.8 \pm 11.5$  h) and  $t_9$  ( $77 \pm 8.5$  vs.  $76 \pm 11.3$  h) [3]. In addition, Kirkegaard et al. [5] also proved that the pregnant rate was irrelevant to the period. In euploid embryos, the  $t_6$  (53.9 h),  $t_7$  (57.8 h),  $t_8$  (61.9 h) and  $t_9$  (76.1 h) are similar to the time in aneuploid embryos [2]. Statistically, the  $t_6$ ,  $t_7$ ,  $t_8$  and  $t_9$  have no significant difference between the implanted and non-implanted embryos and between the euploid and aneuploid embryos.

(7) Time from insemination to morula ( $t_M$ ): morula is defined as all cells fused together. There is no difference that the  $t_M$  is  $86 \pm 9.1$  and  $84.4 \pm 11.4$  h in implanted and non-implanted embryos, respectively [3]. The  $t_M$  of euploid (94.4 h) and aneuploid (95.3 h) are insignificant [2]. Therefore, statistically, the  $t_M$  does not involve in the implantation potential and chromosome status.

(8) Time from insemination to starting blastulation ( $t_{SB}$ ): the initiation of blastulation means the time point of the blastocoel cavity observation. There is no significant difference in the mean time of  $t_{SB}$  in implantation and pregnancy [3, 5]. Therefore, the time from insemination to starting blastulation does not affect embryo implantation potential and pregnancy rate. However, the mean time of  $t_{SB}$  (103.4 h) in euploid embryos is significant shorter than

aneuploid embryos (103.4 h,  $p = 0.007$ ) [2]. Furthermore, the shorter tSB refers to more chance of euploid embryos for embryo transfer.

(9) Time period from insemination to expanded blastulation (tEB): expanded blastulation means the diameter of blastocyst had increased by more than 30%, the expanding results in a thin zona pellucida [9]. There is no *statistical significance* between implanted embryos and non-implanted embryos (111.7 vs. 110.5 h) [3]. Kirkegaard et al. [5] also indicated that there is no significant difference in pregnancy and non-pregnancy groups (104 h). However, the mean time of tEB is significant shorter in euploid embryos than that in aneuploid embryos (118.7 vs. 122.1 h) [2]. In addition, the shorter time of embryos achieved expanded blastulation is more likely to be euploid embryo. The faster embryos of expanded blastulation have more euploid embryos but the mean time of tEB has no difference between implantation potential and pregnancy.

(10) Time period between 2-cell and 3-cell stage (t3-t2, cc2): cleavage cycle 2, time of the second cycle is also known as the time between 2-cell and 3-cell stage. The mean of cc2 is 11.4 h in implanted embryos and 11.8 h in non-implanted embryos [3]. Meseguer et al. [6] found the same cc2 (11.8 h) in implanted and non-implanted embryos. The mean of cc2 (11 h) is also no *statistical significance* between pregnancy and non-pregnancy group [5]. There is no difference in the mean of cc2 in euploid and aneuploid embryos (10.5 vs. 10.4 h) [2]. Therefore, cc2 cannot predict the implantation potential, pregnancy rate and chromosome status.

(11) Time period between 5-cell and 3-cell stages (t5-t3, cc3): it is also defined as cleavage cycle 3 by Chamayou et al. [3]. They presented that the median of cc3 was significant longer in implanted embryos than nonimplanted embryos (14.4 and 13.0 h, respectively). As a result, longer cc3 may be beneficial for embryo development.

(12) Time of synchrony of the second cell cycle (s2, t4-t3): time between 4-cell and 3-cell stages or 3-cell stage also means s2. The mean of s2 is 2 h in implanted embryos and 1.7 h in non-implanted embryos [3]. It also has no significant difference between pregnancy and non-pregnancy groups [5]. However, the mean of s2 is significant smaller in euploid embryos than aneuploid embryos (2.6 vs. 4.2 h) [2]. Therefore, the mean of s2 might be used for predicting the chromosome status of embryos.

(13) Time of synchrony of the third cell cycle (s3, t8-t5): S3 also signifies the time between 8-cell and 5-cell stages. It includes the sum of 5-cell, 6-cell and 7-cell stages. There is no difference in the mean of s3 between implanted embryos and non-implanted embryos (8.0 vs. 8.1 h) [3]. Kirkegaard et al. [5] also found no difference between pregnancy and non-pregnancy groups. There are no data compared with the mean of s3 in aneuploid and non-aneuploid embryos. Hence, the effect of s3 on implanted potential and pregnancy rate remains no significantly.

### 3. Special markers in time-lapse system

Some morphokinetic markers are only revealed in the time-lapse system because the continuously and frequently recording system. Traditional observation has difficulty in observing these transitory phenomena. Following this, we listed these morphokinetic markers and conclude the effect of embryos.



**Direct cleavage** ( $\leq 5$  h from 2 to 3 cells): generally, the time from 2 to 3 cells is around 10–11 h [2, 3, 5, 6]. Rubio et al. [10] found that embryos with direct cleavage ( $\leq 5$  h) have lower implantation rate than embryos with normal cleavage pattern (1.2 vs. 20%). The incidence rate of direct cleavage is 14%. What is the reason causing direct cleavage is still obscure. Based on the announcement of Rubio et al. [10], the centrioles introduced by the sperm control the first mitotic divisions of the oocytes. Therefore, the impairment of sperm neck, the location of centrioles, during ICSI procedure may alter the timing of first embryos cleavage. Rejection of direct cleavage embryos for transfer could enhance the implantation rate.

**Direct unequal cleavage (DUC)**: actually, direct cleavage could occur at any cleavage cycle. Zhan et al. [11] defined as the abrupt cleavage of one blastomere into three daughter blastomeres or an interval of cell cycles less than 5 h. Therefore, they describe direct unequal cleavage at first cleavage as DUC-1, at second cleavage as DUC-2, at third cleavage as DUC-3 and embryos exhibiting multiple DUCs as DUC-Plus. They found that the embryos fertilized with the sperm from epididymis, and testicles have significant higher DUC-1 percentage (13.6 vs. 11.4%). However, the incidence of DUC-1 is 9.1% in embryos fertilized with sperm from ejaculation. Besides, the embryos with multinucleation blastomere (MNB) have 2–3 times of incidence compared to non-MNB embryos. They conclude that blastocyst rate, implantation potential and euploid rate are significantly lower in DUC embryos. Non-DUC embryos should be the first choice for embryos transfer.

**Reverse cleavage**: reverse cleavage can be divided into two types. Reverse cleavage type 1 (complete): blastomeres rejoin after completely separating. Reverse cleavage type 2 (incomplete): zygote or blastomere fails to separate (type I, Supplemental Video 1; type 2, Supplemental Videos 2 are available online at [www.fertstert.org](http://www.fertstert.org)). It could occur up to three times in 27.4% of embryos during the first three cleavage cycles [12]. They found GnRH antagonist protocol and ICSI procedure had higher incidence of reverse cleavage compared with GnRH agonist protocol and IVF procedure. Embryos fertilizing with poor sperm motility ( $<21\%$ ) also have higher rate of reverse cleavage. Besides, embryos with reverse cleavage are associated with poor grade embryos and lower implantation potential. Therefore, reverse cleavage is a negative factor for embryos selection.

## 4. Conclusion

The continuously morphokinetic change of embryo development is the main characteristic of time-lapse system. We can observe many milestones of embryos development and calculate the time intervals to understand the relationship of implantation potential, chromosome status and pregnancy rate. Unfortunately, all the morphokinetic markers could not predict implantation potential, chromosome status and pregnancy rate exactly. Most of markers are controversial or no significant effect. Conventionally, embryos with quicker development would be recommended for transfer to raise the pregnancy rate. However, after reviewing all the data, not all markers can support this principle.

The reason of controversial descriptions of the markers is very incomprehensive. We suppose that some factors might influence embryos implantation and pregnancy. Obviously, maternal

and physical conditions, such as endometrial receptivity, endometrial polyps, endometrial or endocervical infection, hydrosalpinx, immune disorder, subclinical hypothyroidism etc., can also impede the embryos implantation and the following pregnancy. We also know that aneuploid embryos show poor implantation rate or result in spontaneous abortion. Although some markers correlate with higher rate of euploid embryos, it still cannot be used for predicting euploid embryos precisely. If people want to know the chromosome status of embryos, pre-implantation genetic screening (PGS) is still the first choice.

Therefore, the time-lapse system can help us to evaluate the quality of embryos. We can use more precise morphokinetic markers to distinguish the embryos quality. The embryos with good quality have higher rate of implantation potential and normal chromosome. Currently, PGS is the optimal manner to find out the euploid embryos. However, the good quality of euploid embryo is not a guarantee of embryo implantation and pregnancy. It is the basic condition for better embryo implantation. We have to consider many other maternal and physical situations which greatly affect embryo implantation to promote the implantation and pregnancy rate. It also needs further studies to clarify the mystery of implantation process.

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