REVIEW

Should we forget about embryos till day 5?

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Purpose of review
To find the way of having more and better blastocyst is essential. How to culture embryos up to blastocyst stage remains critical.

Recent findings
Several studies show how a blastocyst score can predict the implantation potential. If that score is enough to choose the best blastocyst, as culture conditions would not be affected in these days, we would not need to check early cleavage embryos, even it could be better for the embryo development.

Summary
The item that should be discussed is if it is better to evaluate or not embryos at early cleavage stages. If we do not check embryos on days 2 and 3, we should change our way to work and how to culture those embryos. First step would be to perform all embryo transfers on day 5 or 6. If we let embryos grow to blastocyst without any morphology evaluation, we should adapt several steps in our laboratory, for example we should move to a single-step culture medium or we should not do assisted hatching on day 3 embryos.

Keywords
ammonium, blastocyst, culture conditions, morphology, score

INTRODUCTION

On the basis of bibliography of the last years, there is a trend toward blastocyst transfer rather than cleavage embryo transfer. There are several studies that point out that blastocyst transfer could achieves better results [1▪]. Culture media is one of the most important factors in in-vitro fertilization (IVF); it can affect live birth rate, pregnancy rate, implantation rate, fertilization rate and the number of good quality embryos. Embryos cultured *in vitro* may be exposed to constant stress. Suboptimal culture conditions force the embryo to undergo adaptations, and thus cause lower pregnancy and higher miscarriage rates. Embryo culture can be performed sequentially with change to a medium with a different composition proposed to follow the embryo's physiological needs, or single-step media with refreshment, without change of the medium composition; or even, single-step media without any refreshment for uninterrupted embryo culture, especially for applications like time-lapse microscopy [2] or even limiting the embryo evaluation only to fertilization and blastocyst stage. If blastocyst score can predict implantation likelihood better than cleavage embryo score, we could avoid disturbing the embryos by checking their morphology on days 2 and 3 and keep stable the culture conditions up to day 5. Then, we could pick the best blastocyst according to their morphology. However, there are still some questions that should be discussed, the first one could be: should we transfer embryos always at blastocyst stage? And if we do that, we should change how to work and how to culture those embryos. There are some more questions that remain important and should be answered: should we let embryos grow to blastocyst without any morphology evaluation? If yes, how should we do it? Should we move all to a single-step culture medium? Should we do assisted hatching on day 3 embryos that will be helpful in biopsy on blastocyst stage?

SHOULD ALL THE EMBRYO TRANSFERS BE PERFORMED ON BLASTOCYST STAGE?

The last review performed by *The Cochrane* database where timing of embryo transfer has been studied concludes that live birth rate and clinical pregnancy rate in a fresh blastocyst transfer are higher than in a fresh cleavage stage embryo transfer [1▪]. According

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KEY POINTS

- Clinical outcome in blastocyst stage seems to be higher than in cleavage stage embryo.
- Checking embryos on day 3 helps us to choose between two similar blastocysts; however, when embryos have been evaluated on day 3, there is less agreement between embryologists in order to choose embryos for transfer.
- Checking embryos on day 3 is mandatory when assisted hatching is performed.
- Single-step media allow us culture up blastocyst stage; however, ammonia concentration could be higher.

to this information, we should consider blastocyst stage transfer like the best choice to get pregnant. In this review, 27 parallel-design randomized control trials were included (4031 participants), four more than in the last review [3]. Several parameters have been included in the review: live birth rate, cumulative pregnancy rate, multiple pregnancy rates, miscarriage rate and failure rate to transfer embryos. After the analysis of all articles, the review concludes that blastocyst stage transfer achieves higher clinical outcome; however, there is low-quality evidence for live birth and moderate-quality evidence for clinical pregnancy that blastocyst stage transfer is associated with higher rates than cleavage stage transfer. We have to take into consideration that three of the studies were published more than 10 years ago, and there have been important changes over this time.

Obstetric and perinatal outcomes are also important factors that have to be taken into account. As far as we know, the most recent published study (at this time), which includes 318 live births, shows no increased risk of obstetric or perinatal complication in pregnancies resulting from single blastocyst transfers compared with single cleavage embryo transfers [4]. In the same year in a previous review, which involves 12 observational studies with 195,325 singleton pregnancies, the authors conclude that as there is a low quality of available evidence, more large well-conducted studies are needed to clarify the potential risks and benefits of blastocyst transfer [5]. Regarding perinatal outcome, there is an important parameter to consider, oxygen concentration; most of the perinatal outcome studies when blastocyst is transferred have been done in clinics with oxygen atmospheric concentration (≈20%) and it has been documented that it can predispose embryos to be cellular stressed [6].

Taken into account all this information, we could think that if we have all the requirements that are needed, we can achieve higher outcome rates by transferring blastocyst stage embryos. However, there is another nonanswered question: what are the real epigenetic effects in an extended culture? Is it possible that all necessary steps in assisted reproductive technology (ART) as part of the treatment of infertility can influence the epigenetic programming during early development [7]. We still need more deeper studies to understand it.

TO CHECK OR NOT TO CHECK EMBRYOS ON DAY 3

There is a lack of randomized controlled trials comparing clinical outcomes of embryos that have been evaluated during all culture days versus embryos without any evaluation up to blastocyst stage. With no data, it is difficult to make a decision, but if we decide that culture up to blastocyst stage is the best option now, then we have to find the best way to do it. Checking embryo morphology at days 2 and 3 means open the incubator and disturb culture conditions. If we believe that the best morphological blastocyst can achieve the highest implantation potential, then we should avoid any factor that can modify the environment where the embryo is growing. However, this is not so easy.

It is quite often to find two similar blastocysts, in terms of morphological quality, and the decision of which one has the highest likelihood to implant it is not clear. We can find more information of the embryo that can help us by looking back on embryos development on days 2 and 3, and depending on the morphology on those days we can choose the one that had better cleavage. Indeed, by adding information from other days, we could choose between two similar blastocysts. This means that it is necessary to open the incubator and how does it affect the embryo is difficult to know. However, in a recent study, authors found that when we evaluated embryos on day 3 for blastocyst embryo transfer, there seems to be less agreement between embryologists in order to choose embryos for transfer on blastocyst stage, although it is unclear whether this disagreement affects clinical outcomes, and in what sense [8].

Even if we have blastocyst with different morphologies and we think that the choice is easy, we have to take into account a recent study where authors found that morphology does not add or change their relationship model with the implantation potential [9]. In this study, time lapse system was used to evaluate morphokinetic parameters, with these data the authors found that the prediction of blastocyst formation, using a predictive model based on early morphokinetic parameters,

does not correlate with implantation rate and they built a new predictive model for implantation potential, using later kinetic parameters (t8–t5 and time for expansion blastocyst). Taken into consideration this result, if we decide to check early cleavage embryos, we should consider that we are focused on some parameters that do not have the best relationship with implantation rate; and probably, we should change the important time points to evaluate in order to identify the embryo with the highest implantation potential.

Group culture has been proposed like an option to let embryos reach blastocyst stage and then evaluate each one. Group culture of embryos in a small volume could improve development, potentially through secretion of autocrine/paracrine factors. Embryos can communicate through paracrine biomolecules that have been suggested to affect embryo homeostasis and growth. In 2010, Ebner *et al*. [10] published results from a prospective randomized comparison of single embryo culture and group culture on blastulation, implantation and pregnancy rates. Group culture was shown to be superior in terms of compaction and blastulation rates and blastocyst quality as compared to individual culture. A tendency toward a higher cumulative clinical pregnancy and live birth rates was also observed. Although some authors did not observe any differences in IVF outcomes in patients having their embryos either cultivated individually or in groups [11], others demonstrated a significant increase in blastocyst developmental rate when embryos were grouped on day 3 based on the quality [12], although the embryo culture may be performed more advisable at the single embryo level to avoid the negative influence of degenerating embryos in contact with viable embryos.

A special case would be the patients who undergo preimplantation genetic diagnosis or chromosomal comprehensive screening. If the biopsy will be done on blastocyst stage and only euploid blastocyst will be transferred, then we should not need to check cleavage embryos and we will just need to culture up to blastocyst stage and evaluate what embryos can be biopsied. However, most of the clinics that perform blastocyst biopsy usually do an artificial shrinkage of the Zona Pellucida, to do that is mandatory to take the embryos out the incubator on day 3, so if we open the incubator and going to disturb that embryo, we can evaluate the embryo without spending too much time.

HOW MUCH IMPORTANT TOXICS CONCENTRATION IS?

An optimal embryo culture medium is important for embryonic development and subsequently the success of ART treatment. There has been much controversy about the most appropriate embryo culture medium or culture system, although a single-step medium does not mimic the in-vivo changes an embryo would experience as it moves through the oviduct to the uterus, any metabolic stress is potentially balanced by the absence of disruptions to the in-vitro environment and elimination of physical manipulation [13]. Sequential media were developed to mimic the changing environment that the embryo experiences and also its changing metabolic requirements [14]. Therefore, to use sequential media, it is necessary to remove the culture dish containing the embryos from the incubator and physically move the embryos into the second-phase culture medium. A current alternative strategy is to culture the embryo in a single medium that has been optimized to cover the metabolic needs of the embryo during culture. The advantages and disadvantages of sequential media and single-step media have been discussed [14,15], and there is a long debate about how to choose the best culture conditions and medium [2,13,16,15,17▪▪]. However, there is no clear evidence of which type of media will support embryo development in a better way, and which culture conditions are the most appropriate. One of the disadvantages of those continuous single-step media might be the accumulation of toxins, including ammonium [16]. Especially during the cleavage stage, embryos are vulnerable to ammonium in culture media, and can also negatively affect blastocyst formation. The main sources of ammonium are unstable glutamine and the protein supplement, it is known that amino acids, especially free L-glutamine, spontaneously break-down into ammonium during incubation at 37°C and result in the altered expression of close to 400 genes involved in metabolism, cell growth and/or maintenance, transcription, cell communication, transport, development and regulation of transcription. The break-down of these components into ammonium is more pronounced during incubation at 37°C; however, it is not negligible during storage at 2–8°C. This results in an increase in ammonium levels in these media over time [18]. Apart from amino acids, other factors, such as the protein supplementation, might contribute to the ammonium accumulation during culture [19]. There could be big differences in ammonium accumulation among different media, and it seems that it could be lower in single-step media [17▪▪,20]. In fact, it has been observed an increased rate of blastocyst formation following culture in single medium, particularly if the medium is used in a two-step fashion with renewal on day 3 [21]. However, a recent study

performed with a time lapse system [22] found the same clinical outcomes (clinical pregnancy rate, implantation rate and live birth rate) when it compared embryo culture with or without renewal of the medium on day 3.

WHAT IF WE COMBINE SEVERAL TECHNIQUES?

Currently, there are a lot of devices and technologies that are available in the market and can be used. In the last years, time lapse systems have been used in IVF laboratories to identify the optimal cleavage behavior of the embryos and to find a relationship between these morphokinetic parameters and the implantation potential. Time lapse systems allow us to study the embryos without opening the incubator. One of the main advantages of the uninterrupted culture is that it minimizes changes of the in-vitro culture environment by eliminating the need to remove the embryos from the incubator. In fact, reducing the number of incubator door openings and increasing the stability of the culture environment are likely to improve embryo development in itself. The benefits of a successful continuous single-medium protocol in a time lapse incubator could include additional practical advantages offered by this protocol, including to avoid movement between culture drops, pipetting, which can cause an increase in stress factors in embryos, in addition each manipulation also increases the chance of accidents, contaminations and embryo loss. Also, decreased embryonic stress, including temperature changes and pH fluctuations or even a reduction in the cost of materials used than when a two-step protocol is used [22]. However, the uninterrupted protocol may be more susceptible to the volatile organic compound levels and to the environmental culture conditions, so its application should be limited to certain IVF laboratories. We could find one key by using this system; we could let the embryo up to blastocyst stage and have the information of their cleavage behavior so that we could choose better. Recently, it has been shown that combining morphological examination through time lapse imaging and oxygen consumption measurements allowed knowledge on the developmental competence of the embryo to be gained, eventually identifying more complete selection criteria for embryo selection before transfer [23], giving relevance to morphokinetic parameters which take place in the early stages of embryo development, which is faced with the slope of not evaluating the embryos until blastocyst stage. Moreover, good quality blastocyst could be aneuploid embryos and end in an implant failure or in a miscarriage. Time lapse technology could help us to identify the blastocyst with the highest likelihood of being euploid embryo [24,25].

Another approach could be the use of microfluidic technology. Embryos are still mostly cultured in groups, in large amounts of medium and mostly under static conditions. In terms of dynamic culture, medium with a constant composition has been perfused continuously or in bursts, whereas some in-vitro culture protocols require a single change in the medium composition on day 3 [26], with the consequent opening of the incubator and the handing of the embryos. This change of culture medium and embryo handing can be used to evaluate the embryos on day 3 without an extra opening of the incubator. Interestingly, using microfluidic technology, this change could be accomplished gradually including, if necessary, a pH adaption due to medium requirements, as happens *in vivo*, to closely follow the developmental stages of the embryo without incubator openings and maintaining immutable the embryo culture conditions. This could mean the end of the embryo evaluation on days 2 and 3, if not for the incorporation of time-lapse technology as a complement of microfluidic technology. Although for the moment, the main niche for microfluidic technology in the field of ART in the next coming years is for basic research [27▪].

CONCLUSION

Published data suggest that blastocyst stage has the highest clinical outcomes. If culture up to blastocyst stage is the chosen decision, not to check cleavage embryos seems to be beneficial for the embryos; however, it is not possible in all cases. There is insufficient evidence to recommend either sequential or single-step media as being superior for the culture of embryos to blastocyst stage, and if one clinic achieves better results with a sequential medium, to take embryos out the incubator is mandatory. Assisted hatching on day 3 implies to work out of the incubator too. Currently, one of the best approaches to not disturb culture conditions and culture embryos up to blastocyst stage could be the use of time lapse systems, and we can keep follow the whole development process without taking embryos out the incubator, and even identify the one with highest implantation potential between two similar morphological blastocysts. This way, incubators that include time-lapse system represent a very good tool in order to not disturb the embryos during the culture.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ■ of special interest
- ■■ of outstanding interest

1. Glujovsky D, Farquhar C, Quinteiro Retamar AM, *et al.* Cleavage stage versus
■ blastocyst stage embryo transfer in assisted reproductive technology. Cochrane Database Syst Rev 2016; 1–103; CD002118.

This is the last review of the *Cochrane Database* that studies whether blastocyst stage (days 5–6) embryo transfers improve clinical outcome compared with cleavage stage embryo transfers.

2. Swain JE, Carrell D, Cobo A, *et al.* Optimizing the culture environment and embryo manipulation to help maintain embryo developmental potential. Fertil Steril 2016; 105:571–587.
3. Glujovsky D, Blake D, Farquhar C, Bardach A. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. Cochrane Database Syst Rev 2012; 244–250; CD002118.
4. Oron G, Nayot D, Son WY, *et al.* Obstetric and perinatal outcome from single cleavage transfer and single blastocyst transfer: a matched case-control study. Gynecol Endocrinol 2015; 31:469–472.
5. Martins WP, Nastri CO, Rienzi L, *et al.* Obstetrical and perinatal outcomes following blastocyst transfer compared to cleavage transfer: a systematic review and meta-analysis. Hum Reprod 2016; 31:2561–2569.
6. Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human, IVF. Reprod Biomed Online 2016; 32:137–141.
7. Mantikou E, Jonker MJ, Wong KM, *et al.* Factors affecting the gene expression of in vitro cultured human preimplantation embryos. Hum Reprod 2016; 31:298–311.
8. Storr A, Venetis CA, Cooke S, *et al.* Inter-observer and intra-observer agreement between embryologists during selection of a single day 5 embryo for transfer: a multicenter study. Hum Reprod 2016. [Epub ahead of print]
9. Motato Y, de los Santos MJ, Escriba MJ, *et al.* Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. Fertil Steril 2016; 105:376–384.
10. Ebner T, Shebl O, Moser M, *et al.* Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. Reprod Biomed Online 2010; 21:762–768.
11. Spyropoulou I, Karamalegos C, Bolton VN. A prospective randomized study comparing the outcome of in-vitro fertilization and embryo transfer following culture of human embryos individually or in groups before embryo transfer on day 2. Hum Reprod 1999; 14:76–79.
12. Tao T, Robichaud A, Mercier J, Ouellette R. Influence of group embryo culture strategies on the blastocyst development and pregnancy outcome. J Assist Reprod Genet 2013; 30:63–68.
13. Youssef MM, Mantikou E, van Wely M, *et al.* Culture media for human preimplantation embryos in assisted reproductive technology cycles. Cochrane Database Syst Rev 2015; 1–97.
14. Gardner DK, Lane M. Mammalian preimplantation embryo culture. Methods Mol Biol 2014; 1092:167–182.
15. Machtinger R, Racowsky C. Culture systems: single step. Methods Mol Biol 2012; 912:199–209.
16. Biggers JD, Summers MC. Choosing a culture medium: making informed choices. Fertil Steril 2008; 90:473–483.
17. Hardarson T, Bungum M, Conaghan J, *et al.* Noninferiority, randomized,
■■ controlled trial comparing embryo development using media developed for sequential or undisturbed culture in a time-lapse setup. Fertil Steril 2015; 104:1452–1459.

This work is the largest prospective randomized sibling embryo study to date that has compared a single-step medium and sequential media (from the same manufacturer) using time-lapse, showing that the two media systems are equivalent in relation to blastocyst quality and rates.

18. Mantikou E, Jonker MJ, Wong KM, *et al.* Factors affecting the gene expression of in vitro cultured human preimplantation embryos. Hum Reprod 2016; 31:298–311.
19. Meintjes M. Media composition: macromolecules and embryo growth. Methods Mol Biol 2012; 912:107–127; Review.
20. Kleijkers SH, van Montfoort AP, Bekers O, *et al.* Ammonium accumulation in commercially available embryo culture media and protein supplements during storage at 2-8°C and during incubation at 37°C. Hum Reprod 2016; 31:1192–1199.
21. Sfontouris IA, Martins WP, Nastri CO, *et al.* Blastocyst culture using single versus sequential media in clinical IVF: a systematic review and meta-analysis of randomized controlled trials. J Asist Reprod Genet 2016; 33:1261–1272.
22. Costa-Borges N, Bellés M, Meseguer M, *et al.* Blastocyst development in single medium with or without renewal on day 3: a prospective cohort study on sibling donor oocytes in a time-lapse incubator. Fertil Steril 2016; 105:707–713.
23. Tejera A, Castelló D, de Los Santos JM, *et al.* Combination of metabolism measurement and a time-lapse system provides an embryo selection method based on oxygen uptake and chronology of cytokinesis timing. Fertil Steril 2016; 106:119–126.
24. Basile N, Nogales M, Bronet F, *et al.* Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. Fertil Steril 2014; 101:699–704.
25. Del Carmen Nogales M, Bronet F, Basile N, *et al.* Type of chromosome abnormality affects embryo morphology dynamics. Fertil Steril 2017; 107:229–235.
26. Schulte K, Ehmcke J, Schlatt S, *et al.* Lower total cell numbers in mouse preimplantation embryos cultured in human assisted reproductive technique (ART) media are not induced by apoptosis. Theriogenology 2015; 84:1620–1630.
27. Le Gac S, Nordhoff V. Microfluidics for mammalian embryo culture and
■ selection: where do we stand now? Mol Hum Reprod 2016; First published online September 27, 2016. [Epub ahead of print]

This article shows us how the miniaturized platforms combining different capabilities for embryo culture and characterization, also without any automated interface. For instance, platforms allowing real-time and multiparametric monitoring of embryos would be instrumental in screening various physical or chemical culture parameters and optimizing the medium composition. Furthermore, such integrated platforms are very likely to provide more fundamental knowledge on embryo development, which could in turn benefit clinical treatments, and help in identifying the best embryos leading to more successful and healthy pregnancies.