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Individual demands of human embryos on IVF culture medium: influence on blastocyst development and pregnancy outcome

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Abstract The elucidation of the metabolic requirements of human embryos *in vivo* or *in vitro* remains, despite being intensively investigated, a work in progress. The adoption of extended embryo culture to the blastocyst stage during the last decade has entailed new challenges. With the increased attention to culture media formulations, more evidence on the sensitivity of embryos to their early environmental conditions is accumulating which might affect phenotype and developmental potential. A retrospective study was conducted that comprised 286 IVF cycles to evaluate the effect of two different culture media on blastocyst development and pregnancy outcome. Embryos were either cultured in a one step or a sequential medium. Higher fertilization rates and augmented blastocyst rates as well as higher implantation rates were observed when embryos were cultured in one step medium ($P < 0.05$). Interestingly, the transfer of two embryos where one embryo was cultured in either medium resulted in a significantly higher rate of twin pregnancies. Although multiple pregnancies should be avoided in assisted reproduction treatment to reduce risks for offspring and mother, this higher frequency of twin pregnancies resulting from the transfer of embryos derived from different culture media suggests that each embryo makes individual demands on its early environment.

KEYWORDS: blastocyst, culture medium, embryo, implantation, live birth rate, pregnancy

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Introduction

In the last three decades, vast efforts have been undertaken in the area of IVF to improve the service as well as the possibilities and success rates of assisted reproduction technologies for couples seeking infertility treatments. One crucial aspect of these efforts comprises improvements to the in-vitro culture of embryos. Although understanding of the metabolic requirements of human oocytes and embryos during the first 5 days of in-vitro culture is essential in this respect, there are still many open questions about the best culture media for the various stages of embryo development. Numerous studies have been published on different culture media formulations and their effects on embryo development (Biggers and Summers, 2008; Gardner et al., 2002; Pool, 2005).

Since the beginnings of IVF, the majority of embryo transfers have been conducted between days 1–3, at either the pronuclear or the cleavage stage. The primary reason for this is the inability of past culture systems to support embryo development at acceptable rates for longer than 3 days. The use of extended embryo culture to the blastocyst stage in assisted reproduction treatment has brought with it new requirements for culture media. The main advantage of embryo culture to day 5–6 is that it allows us to identify those embryos with limited or no development potential (Gardner, 1998; Papanikolaou et al., 2008; Zech et al., 2007). Extended embryo culture can therefore be considered as a quality control of laboratory techniques. Furthermore, with transfer of embryos at the blastocyst stage there is also a more physiological synchronization of the naturally occurring time of embryo implantation and the receptivity of the endometrium for the embryo (implantation window).

A number of publications have stressed the importance of choosing 'the right' culture medium based on the findings that culture environments *per se* or changes to the culture environment result in quantitative differences in the level of mRNA for a variety of genes. Microarray analysis in mouse embryos has revealed that, of many thousands of genes expressed within the blastocyst-stage embryo, in-vitro culture causes a small but significant change in the transcriptome. Those culture conditions more closely resembling the physiological situation were shown to produce more good-quality embryos, which were better aligned with in-vivo-derived embryos (Johnson, 2005; Lonergan et al., 2006; Rinaudo and Schultz, 2004; Thompson et al., 2007). Additionally, fewer deviations in the transcriptome and proteome were seen compared with embryos derived from culture under reportedly more stressful conditions.

Among the long list of different media commercially available for human blastocyst culture, generally two concepts of culture are applied. The first concept is the single medium culture, where the same medium formulation – usually changed on day 3 – is used during the 5 days of in-vitro culture. Second, knowing that there is a shift in the metabolic milieu of embryos from the early (days 1–2) to the later (day 3–5) stages of development, sequential media were established. In sequential culture systems, the medium used for culture for days 1–2 differ in composition and concentration of components, such as glucose, amino acids, pyruvate and others, as compared with the one for days 3–5. Sequential media were expected to more closely mimic the physiological conditions in the female reproductive tract and thereby better support blastocyst development (Lane and Gardner, 2007). However, the optimal environmental conditions *in vivo* have still not been determined as the measurement of fluids in the Fallopian duct and the uterus are difficult due to the small volumes of probes and the complexity of cell-to-cell interactions.

The aim of this study was to compare two commercially available embryo culture media systems. A modified simplex medium containing amino acids (single medium; Global; LifeGlobal, Ontario, Canada) and a sequential medium system (G1/G2; Vitrolife, Gothenburg, Sweden) were chosen and evaluated in terms of blastocyst development, implantation (IR), ongoing pregnancy (PR) and live birth rate.

Materials and methods

A retrospective study was conducted comprising a total of 269 patients undergoing IVF treatment at the study centre between April 2005 and May 2006. Patients with no embryo transfer or with embryo transfer other than day 5 were excluded from this study. Application of the long protocol for stimulation was an inclusion criterion. Stimulation protocols, embryo culture procedures and embryo transfer techniques were similar for all patients during this period. No differences in the past medical history of patients between the groups were found. The percentage of patients with primary infertility was 74.7% (75.0% in the single medium group, 73.2% in the sequential medium group and 75.8% in the sibling group). The mean duration of treatment for infertility from the time of diagnosis to the presented IVF cycle was 2.6 years with 61.2% patients having prior IVF cycles in other centres. Thereby, a mean of 2.6 years of treatment was calculated for the single and sequential medium groups and 2.5 years was calculated for the sibling group. A mean of 61.2% of the patients experienced prior IVF cycles in the single medium group, 61.1% in the sequential medium group and 61.3% in the sibling group. The cause of infertility was male factor (32.3%), female factor (24.1%) and combined infertility (43.6%), with no differences in the distribution between the groups. In the single medium group, there were 33.4% patients with male factor, 24.0% with female factor and 42.6% with combined infertility and the distribution in the sequential medium group was 31.2%, 23.9% and 44.9%, respectively. In the sibling group, the percentages were 32.4% for male factor, 24.3% for female factor and 43.3% for combined infertility.

Oocytes were obtained from 269 patients (mean age 35.2 ± 4.3, range 23–47 years) in 286 stimulation cycles using the long-protocol (Zech et al., 2007). Oocyte retrieval was performed 36–38 h after the administration of human chorionic gonadotrophin (HCG), after which the cumulus–oocyte–complexes were cultured in human tubal fluid media (LifeGlobal) for 2–4 h. Oocytes were then fertilized using either standard insemination or intracytoplasmic sperm injection. Conventional IVF was performed in about 8% of the IVF cycles, equally distributed between the groups. The study design is summarized in **Figure 1**. The patients were first randomized to either one medium

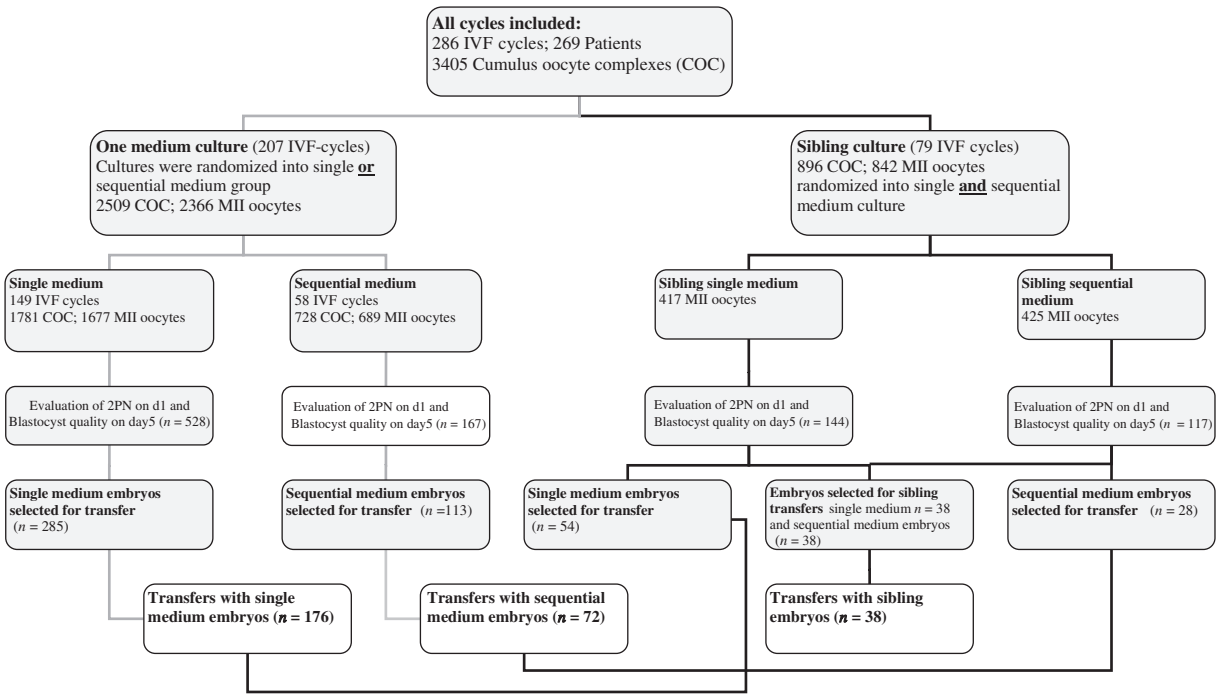

Figure 1 Flow diagram of the conducted study comparing single versus sequential medium culture. In the ''One medium culture'', all oocytes of a patient were cultured in the same medium whereas in the ''Sibling culture'' the oocytes of each patient were randomly split and maintained in both tested culture media. COC = cumulus—oocyte—complex; PN = pronuclei; MII = metaphase II.

culture, i.e. all the oocytes from one patient were cultured in the same medium or sibling culture, i.e. an individual patient's oocytes were randomized to single and sequential medium culture. The patients allocated to one medium culture were randomly assigned into the single medium or sequential medium group. Therefore oocytes were either cultured in a single medium (149 cycles) or in a sequential medium (58 cycles), with both media being supplemented with 7.5% human serum albumin (LifeGlobal). Randomization was performed by allocating the patients to the different media (single or sequential medium) according to their day of birth (pair or impair). Thereby a larger number of patients were involved in the single medium group.

In a total of 79 cycles, the oocytes were allocated to sibling cultures. Patients with a minimum of 10 oocytes and pick-up on Mondays were allocated to this group to facilitate the laboratory procedures. Oocytes for sibling cultures were randomly grouped and half of the fertilized oocytes were exposed to single medium whilst the other half was cultured in sequential medium for a direct comparison between the two culture systems. The only inclusion criteria for sibling cultures was that at least 10 mature oocytes were retrieved at the pick-up.

Fertilization was assessed 16—20 h after insemination. The fertilization rate was calculated by dividing the number of 2 pronuclei (2PN) embryos obtained by the number of mature oocytes retrieved. Embryos were maintained in group cultures in 0.75 ml culture medium in four-well dishes (Nunc, Roskilde, Denmark) until day 3. Embryos were then washed and transferred to new dishes with fresh media, either single medium or sequential medium (G2 medium) for another 48 h. All embryos were cultured under a humidified atmosphere of 5% CO_2 and air at 37.0°C. On day 5, embryo quality was evaluated and the best blastocysts were chosen for embryo transfer. Blastocyst quality was assessed according to the degree of blastocoele expansion and the quality of both the inner cell mass and the trophoectoderm as described elsewhere (Gardner et al., 2000). Blastocysts with A-grading for inner cell mass and trophoectoderm or a combination of A- and B-grading were classified as 'top-blastocysts'.

The IR was calculated by the number of fetal heart beats observed by ultrasound 8 weeks after embryo transfer divided by the number of embryos transferred. The birth rate was estimated by dividing the number of babies born by the number of transfers. Differences in frequencies of fertilization, cleavage, implantation, pregnancies and live births were evaluated with Pearson's chi-squared test. A two-tailed *t*-test was used to test for differences in blastocyst quality and growth. Differences between the groups were considered statistically significant when the *P*-value was <0.05. Statistical analysis was performed using the Statistical Package for Social Sciences version 17.0 for Windows (SPSS, USA).

Results

Embryo development

In a total of 149 IVF cycles, all 1677 MII oocytes collected were cultured in single medium after insemination. In 58 cycles, all 689 inseminated MII oocytes were kept in sequential medium until transfer. Sibling cultures were performed in 79 IVF cycles. Here the injected oocytes were randomly divided in two groups, one half (417 oocytes) cultured in

Table 1 Fertilization rate and blastocyst development in single versus sequential culture medium.

	Only single medium	*Only sequential medium*	*Sibling group*		*All*	
			Single medium	*Sequential medium*	*Single medium*	*Sequential medium*
IVF cycles	149	58	79	79	228	137
MII oocytes per culture	1677 (11.3 ± 6.3)	689 (11.9 ± 7.8)	417 (5.3 ± 3.0)	425 (5.4 ± 3.0)	2094 (9.2 ± 6.1)	1114 (8.1 ± 6.4)
Zygotes per culture	1274 (8.6 ± 4.5)	487 (8.4 ± 5.6)	328 (4.2 ± 2.2)	309 (3.9 ± 2.5)	1602 (7.1 ± 4.4)[b]	796 (5.8 ± 4.6)[b]
Fertilization rate (%)	76.0[c]	70.7[c]	78.7[c]	72.7[c]	76.5[b]	71.5[b]
Blastocysts per culture	528 (3.5 ± 2.7)	167 (2.9 ± 2.4)	144 (1.8 ± 1.6)	117 (1.5 ± 1.5)	672 (2.9 ± 2.5)[a]	284 (2.1 ± 2.1)[a]
Blastocyst rate (%)	41.4%	34.3	43.9%	37.9%	42.0[c]	35.7[c]
Top-quality blastocysts per culture	298 (2.0 ± 2.1)[c]	81 (1.4 ± 1.7)[c]	94 (1.2 ± 1.2)[c]	69 (0.9 ± 1.2)[c]	392 (1.7 ± 1.9)[a]	150 (1.1 ± 1.4)[a]
Top-quality blastocyst rate (%)	23.4	16.6	28.7[c]	22.3[c]	24.5[c]	18.8[c]

Values are number (mean ± SD) unless otherwise stated.
[a,b,c]Differences between the groups were calculated using the Pearson's chi-square test. [a]*P*-value <0.001; [b]*P*-value <0.01; [c]*P*-value < 0.05.

single medium, the other half (425 oocytes) in sequential medium (**Figure 1**). As shown in **Table 1**, no significant difference in the number of MII oocytes cultured in the different media was observed. Significantly more 2PN embryos were detected in single medium (mean of 7.1 ± 4.4 zygotes per culture) as compared with sequential medium (5.8 ± 4.6 zygotes per culture; $P < 0.01$). The fertilization rate was significantly higher in single medium cultures in all groups (in one medium group and sibling culture group, both $P < 0.05$; overall $P < 0.01$; **Table 1**).

The quality and number of blastocysts was evaluated after 5 days in culture. A significantly higher number of blastocysts developed in single medium: average of 2.9 blastocysts per culture compared with 2.1 blastocysts in sequential medium ($P < 0.001$). In line with this observation, a significantly higher blastocyst per zygote rate was found with 42.0% in single medium and 35.7% in sequential medium ($P < 0.05$). The culture conditions affected both the number of embryos developing into blastocysts as well as the quality of those blastocysts. Embryos cultured in single medium were more likely to develop into top-quality blastocysts with an average of 1.7 top-quality blastocysts per culture as compared with 1.1 in sequential medium. The better quality of blastocysts in single medium as compared with sequential medium was significant in both the groups (both $P < 0.05$). The top-quality blastocyst rate per zygote was significantly higher in single medium cultures: 24.5% compared with 18.8% in sequential medium cultures ($P < 0.001$).

Pregnancy rates after embryo transfer

After 5 days of in-vitro culture, the best blastocysts (one or two) were chosen for embryo transfer according to their morphological appearance. A total of 176 patients received a transfer of embryos all cultured in single medium and 72 patients had an embryo transfer with embryos cultured in sequential medium. Thirty-eight women had an embryo transfer from sibling cultures where one embryo was cultured in single medium and the other one in sequential medium (**Figure 1**). There were no differences in the number of embryos transferred per patient and in the mean age of patients in each of these three groups (**Table 2**). There were also no significant differences in the quality of embryos transferred as determined by the number of top-quality blastocysts or blastocysts and morulae or compactions in each of these three groups.

Two weeks after transfer, urinary βHCG concentrations were tested as an early indicator of pregnancy. The highest rate of patients testing positive was found in the sibling group where two embryos from either medium was transferred (60.5%) followed by the group of patients who received embryos cultured in single medium (49.4%) and was lowest among those patients who received embryos cultured in sequential medium (38.9%). Although the differences are not statistically significant, they do, in fact, suggest a higher probability of pregnancy if one of the transferred embryos is cultured in single medium and the other was maintained in sequential medium. The transfer of embryos derived only from single medium culture still seems more likely to result in a pregnancy compared with the transfer of embryos from sequential medium culture.

Implantation rates

The implantation rate was calculated on the basis of positive heart beat detected by ultrasound per embryo transferred. Embryos from sibling cultures, where one sibling was maintained in single medium and the other in

Table 2 Pregnancy and implantation rates after transfer of embryos cultured in single medium or sequential medium.

	Only embryos from single medium culture	*Only embryos from sequential medium culture*	*Sibling embryos from single and sequential medium culture*
No. of transfers	176	72	38
Age of patients (years) (mean ± SD)	35.4 ± 4.3	35.7 ± 4.3	34.5 ± 4.5
No. of embryos transferred	339	141	76
Top-quality blastocysts	181 (53.4)	60 (42.6)	42 (55.3)
Blastocysts	129 (38.0)	59 (41.8)	26 (34.2)
Morulae and compactions	29 (8.6)	22 (15.6)	8 (10.5)
βHCG positive	87 (49.4)	28 (38.9)	23 (60.5)
Implantation rate	113 (33.3)[a]	31 (22.0)	33 (41.3)

Values are number (%) unless otherwise stated. Et = embryo transfer.
[a]Implantation rate is significantly higher in the single medium culture group ($P < 0.05$).

sequential medium, were most likely to implant (IR 41.3%). Transfers with embryos where both were cultured in single medium showed an IR of 33.3%. The lowest IR (22.0%) was found in patients where the embryos were cultured in sequential medium ($P < 0.05$).

Live birth rate, miscarriage rate and multiple births

As summarized in **Table 3**, the rate of miscarriages did not differ between the groups tested (13.6% in the single medium group, 9.7% in the sequential medium group and 13.2% in the sibling group). The live birth rate was highest in the sibling group (47.4%), followed by the single medium group (35.8%) and the sequential medium group (29.2%). Although a trend towards more live births after transfer of sibling embryos can be observed, the groups showed no statistically significant differences. Interestingly, a statistically significant difference in the rate of twin births was observed between the different cultures. The highest percentage of twins born was found in the sibling group (26.3%), followed by those patients who had received embryos from a single medium culture (12.5%). Only two out of 21 patients (2.8%) gave birth to twins in the sequential medium group, although the number of embryos transferred was equal in all groups.

Discussion

In this study, single medium was demonstrated to have better abilities than sequential medium to support early embryo development. The percentage of zygotes on day 1 as well as the number and quality of blastocysts on day 5 were significantly higher when embryos were cultured in single medium rather than sequential medium. The appearance of 2PN 16–20 h after insemination or injection of spermatozoa reflects the proper development of the early embryo and depends not only on the oocyte and sperm quality but also shows that culture conditions are well adjusted to the embryo's needs. The possible influence of the culture conditions is underlined by the observation that the number of MII oocytes per cumulus–oocyte–complex as well as the sperm parameters did not differ between the groups. Therefore, it is hypothesized that, in single medium, the fertilized oocyte finds better conditions to develop even in the very early stages.

Consequently, this study also observed a higher IR for embryos cultured in single medium. However, when sibling embryos were transferred, where one was derived from single medium and the other from sequential medium, a significantly higher IR was found in patients as compared with embryos all maintained in single or sequential medium. This

Table 3 Birth rate, percentage of singletons or twins and miscarriage rate after transfer of embryos cultured in single medium or sequential medium.

	Single medium embryos (n ET = 176)	*Sequential medium embryos (n ET = 72)*	*Sibling embryos from single and sequential medium (n ET = 38)*
Miscarriages	24 (13.6)	7 (9.7)	5 (13.2)
Births	63 (35.8)	21 (29.2)	18 (47.4)
Singletons	41 (23.3)	19 (26.4)	8 (21.1)
Twins	22 (12.5)	2 (2.8)	10 (26.3)[a]

Values are number (%).ET = embryo transfer.
[a]The rate of twin births was statistically significantly different between the three groups: $P < 0.01$.

corresponds with the observation of a trend towards a higher live-birth rate in sibling embryos transferred. Interestingly, the rate of twins was also found to be significantly higher in the sibling group as compared with other groups.

As embryo cultures in sequential medium were performed using a previous culture medium formulation of the medium employed, some cultures were also prepared with a later version of the sequential medium by Vitrolife (G1.3/G2.3). Although here the blastocyst quality on day 5 was overall better, the IR was still below the one obtained from the single medium cultures (data not shown). A recent study comparing another improved formulation of the same medium (G1.5/G2.5) with single medium (Global) reports enhanced blastocyst development in the single medium culture (Reed et al., 2009); however, in this study, no medium change was performed in the single medium cultures on day 3. These results can therefore not be directly compared with the current study's findings, as the additional impact of medium refreshment on day 3 on embryo development is not considered.

Earlier studies comparing different media for human embryo culture found no benefit of sequential media over single media. A comparative study on sibling zygotes cultured for 5 days in a precursor of the single medium used in the current study ($KSOM^{AA}$) and in the sequential media P-1 and CCM reports no difference in blastocyst development in either medium (Biggers and Racowsky, 2002). Similarly, a prospective, randomized study comparing the development of embryos in Rotterdam medium (single medium) with culture in the sequential G1/G2 medium did not find any differences in blastulation, IR or PR (Macklon et al., 2002). This is corroborated by another publication showing that single medium (Global) is as good as sequential media for the development of human embryos (Angus et al., 2006). However, a better development of blastocysts and a higher IR with single medium as compared with sequential medium was reported in another study (Zech et al., 2006). Similar observations were reported in a recent prospective, randomized study on donor oocytes where the authors excluded the effects of a heterogeneous population of IVF patients (Sepulveda et al., 2009); comparing the in-vitro development, the IR and the PR of embryos cultured in single medium (Global) with culture in a sequential medium (ECM/Multiblast), they were able to demonstrate that the number of embryos developing to blastocysts and IR were significantly higher for embryos cultured in single medium.

One reason why single medium might result in better embryo development is possibly related to the amino acid content. In contrast to the sequential medium used in this study, the single medium used provides a larger spectrum of amino acids for the early embryo until day 3. Amino acid turnover has been shown to be related to blastocyst development and PR (Brison et al., 2004; Houghton et al., 2002). Another explanation for the better outcome in single medium culture might be that embryos are not put under the additional biochemical stress of having to adapt to a new medium composition after 2 days as they have to do in sequential medium culture (Biggers et al., 2000; Ho et al., 1995; Rinaudo and Schultz, 2004).

The current findings that the transfer of embryos cultured in two different media results in such a high percentage of twin pregnancies leads to new speculations about early embryo development. They suggest that the requirements of early embryos differ even between siblings and, that by providing different culture media, the given environment might better match the requirements of more embryos. This means that those embryos can be transferred, are stronger and better equipped and are more likely to implant and develop into healthy babies. Another explanation is that embryos derived from different culture systems still have slightly different requirements during early implantation and therefore do not compete with each other in the uterus as embryos derived from the same culture medium would do. The current findings, that each embryo might have specific demands on its early environment, is supported by the studies of Pomeroy et al. (2009) that reported evidence that splitting embryos between two culture media might increase the implantation rate and by Angle (2006) that suggested an ameliorated pregnancy outcome after transfer of sibling embryos from two different sequential media.

It is known that mammalian preimplantation embryos are sensitive to their environment and that culture conditions can affect both pre- and post-natal growth and development potential. Evidence suggests that the period of post-fertilization embryo culture is the most critical period affecting blastocyst quality, gene expression patterns and the ability to establish a pregnancy (Johnson, 2005; Lonergan et al., 2006; Thompson et al., 2007). Providing optimal culture conditions is therefore crucial for the outcome of assisted reproduction treatment and epigenetic influences might become more important as new insights into this topic are gained.

This present study reports that blastocyst quality differed between the single medium and the sequential medium as expressed by a higher fertilization and blastocyst rate. Accordingly, the IR of embryos cultured in single medium was higher than those cultured in sequential medium. Notably, the transfer of sibling embryos where both originated from different culture media was significantly more likely to result in the birth of twins. Although one of the major goals in assisted reproduction treatment is to avoid multiple pregnancies, the number of multiple pregnancies resulting from the transfer of sibling embryos from different culture media leads to the inference that there is not just one optimal medium for embryo culture. Even sibling embryos seem to have individual needs, even in the very early stages of development, which might be better supported by providing different culture conditions, thus resulting in blastocysts that are more likely to implant.

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