ORIGINAL ARTICLE: ASSISTED REPRODUCTION

Sequential versus Monophasic Media Impact Trial (SuMMIT): a paired randomized controlled trial comparing a sequential media system to a monophasic medium

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Objective: To determine whether sequential or monophasic media is the more optimal formulation for blastocyst development and sustained implantation rates (SIR) in IVF.
Design: Paired randomized controlled trials.
Setting: Academic.
Patient(s): Infertile couples (N = 192) with female partner ≤42 years old and normal ovarian reserve.
Intervention(s): Fertilized zygotes from each patient were randomly divided into two groups: [1] cultured in sequential media and [2] cultured in monophasic medium. Sequential media consisted of Quinn's Advantage Cleavage Medium (SAGE) followed by Blast Assist (Origio). The monophasic medium used was Continuous Single Culture (Irvine Scientific). Paired ETs were accomplished by transferring the best euploid blastocyst from each media group. DNA fingerprinting was used to link outcomes.
Main Outcome Measure(s): The primary outcome measure was the proportion of blastocysts suitable for clinical use. Secondary outcome measures included timing of blastulation, aneuploidy rates, and SIR. Sustained implantation rate is defined as the number fetal heart beats at 8–9 weeks of gestation, divided by the number of embryos transferred.
Result(s): A total of 192 patients had their 2PN embryos (N = 2,257) randomized to each culture system. Sequential media had higher blastulation rate than monophasic medium (55.2% vs. 46.9%). No differences were found in the day of blastulation or aneuploidy rate. Of the 168 patients who had euploid blastocysts suitable for transfer, 126 completed a paired ET. Among the double ETs, there was no difference in implantation between groups.
Conclusion(s): This is the first randomized controlled trial to examine paired euploid transfers of sibling zygotes cultured in sequential versus monophasic media. This study demonstrates that the usable blastocyst rate is greatest after culture in the sequential media tested in comparison with the monophasic formulation selected for study. However, no difference exists in timing of blastulation, aneuploidy, or SIR. Whether these observations are generalizable to other media systems remains to be determined.
Clinical Trial Registration Number: NCT01917240. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Embryo culture, media for IVF, embryo development, in vitro fertilization, culture media

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Two widely used but distinct approaches of culture media have emerged to support embryonic growth to the blastocyst stage of development: sequential and monophasic media. Sequential media, otherwise known as the "back to nature," approach is a two-step formulation designed to accommodate changes in embryo metabolism before and after the compaction stage of development (1, 2). In contrast, monophasic formulations are one step and capable of supporting embryonic growth through all stages of development. Functionally, monophasic formulations "let the embryo choose" which nutrients are required whereas sequential formulations may mimic in vivo conditions more closely (3, 4). Both culture systems have demonstrated excellent clinical outcomes. However, at present, there is no consensus among clinical programs as to which approach is optimal as evidenced that both systems remain in widespread clinical use.

The first attempts at blastocyst culture used monophasic formulations, as referenced, but these were replaced by sequential culture systems, which purported better implantation rates (5–7). Sequential culture systems reflected an increased understanding of the physiologic requirements of the growing embryo in vivo (8–11). Step 1 primarily consists of nonessential amino acids, ethylenediaminetetraacetic acid (EDTA), pyruvate, and a reduced concentration of glucose to support before compaction development. In a temporally correct manner, step 2 removes EDTA, adds essential amino acids, decreases the concentration of pyruvate and increases the concentration of glucose to support after compaction development. Most of the pioneering work in support for sequential media was performed using animal models that demonstrated improved fetal development and increased implantation rates (12–14).

A continued strong interest remained in optimizing monophasic formulations, as they are simpler to use and less expensive in practice. Monophasic medium incorporates all the necessary ingredients for culture to blastocyst stage including nonessential and essential amino acids, EDTA, glucose, pyruvate, and lactate in a constant concentration. Consequently, monophasic medium requires less manipulation of the embryo and fewer resources to support development, especially if medium is not renewed on day 3 of development. The improvement in monophasic culture systems was rigorously developed in animal model using simplex optimization (4, 15).

Several sequential and monophasic media formulations are US Food and Drug Administration approved and commercially available. Ready availability of these high quality culture media has likely contributed to increased utilization of blastocyst culture in clinical settings. Some studies suggest equivalent outcomes in embryological parameters when tested among sibling zygotes, but are underpowered to assess differences in implantation and have not yet assessed aneuploidy risk (16–18). At present, there has not been an adequately powered randomized rigorously controlled trial to evaluate blastocyst culture in contemporary IVF practice. The goal of the present analysis is to perform a paired, prospective randomized controlled trial to assess usable blastulation rates, aneuploidy risk, and sustained implantation rates among sibling zygotes cultured in monophasic compared with sequential media. For the purposes of this analysis the sequential media tested consisted of Quinn's Advantage Cleavage Medium (SAGE) followed by Blast Assist (Origio). The monophasic media used was Continuous Single Culture (Irvine Scientific).

MATERIALS AND METHODS

Patient Population

Infertile couples attempting conception through IVF at a single center from August 2013 to March 2015 were evaluated to determine study eligibility. Given the paired design where each patient served as her own control, it was critical to recruit patients who met the American Society for Reproductive Medicine guidelines for having two (or more) embryos transferred (19). All study participants had also elected to use comprehensive chromosomal screening to assess for embryonic aneuploidy.

Inclusion criteria reflected the paired nature of the study design and targeted patients with normal ovarian reserve, therefore each patient would serve as her own control. Patients $\leq$42 years old at the time of IVF cycle were eligible for inclusion if the FSH level was $<$12 IU/L, AMH $\geq$1.2 ng/mL, and basal antral follicle count $\geq$12. To exclude confounding variables, patients with the following characteristics were not offered enrollment [1] chronic endometrial insufficiency, [2] use of oocyte donation or gestational carriers, [3] medical contraindication to double embryo transfer or IVF, [4] severe male factor infertility requiring surgical sperm extraction, or [5] single gene disorders.

The protocol was Institutional Review Board approved and registered with clinicaltrials.gov (NCT01917240). All patients that met inclusion criteria and expressed the desire to participate in the study were consented before ovarian stimulation.

IVF Cycle Management

There were no restrictions on stimulation protocols as the study intervention began after fertilization was confirmed. Due to the paired nature of the study design every patient had their zygotes randomized to sequential and monophasic media. This design allowed for a comparison of zygotes exposed to identical endocrine milieus during follicular stimulation, thereby eliminating confounding variables associated with follicular stimulation.

Standard regimens for controlled ovarian hyperstimulation (COH) were employed using purified urinary FSH or recombinant FSH and LH activity in the form of low-dose hCG or hMG along with GnRH agonist (long down-regulation or microdose flare) or GnRH antagonist to prevent a premature LH surge. Monitoring of IVF cycles were per practice routine. Oocyte maturation was induced with recombinant hCG, purified urinary hCG, or with GnRH agonist. This was performed when at least two follicles reached 17–18 mm or when the follicular cohort was deemed to be mature by the patient's primary physician. Transvaginal oocyte aspiration was performed approximately 36 hours later. Cumulus

stripping occurred after retrieval. Insemination of mature oocytes was performed by intracytoplasmic sperm injection (ICSI).

Randomization and Blastocyst Selection

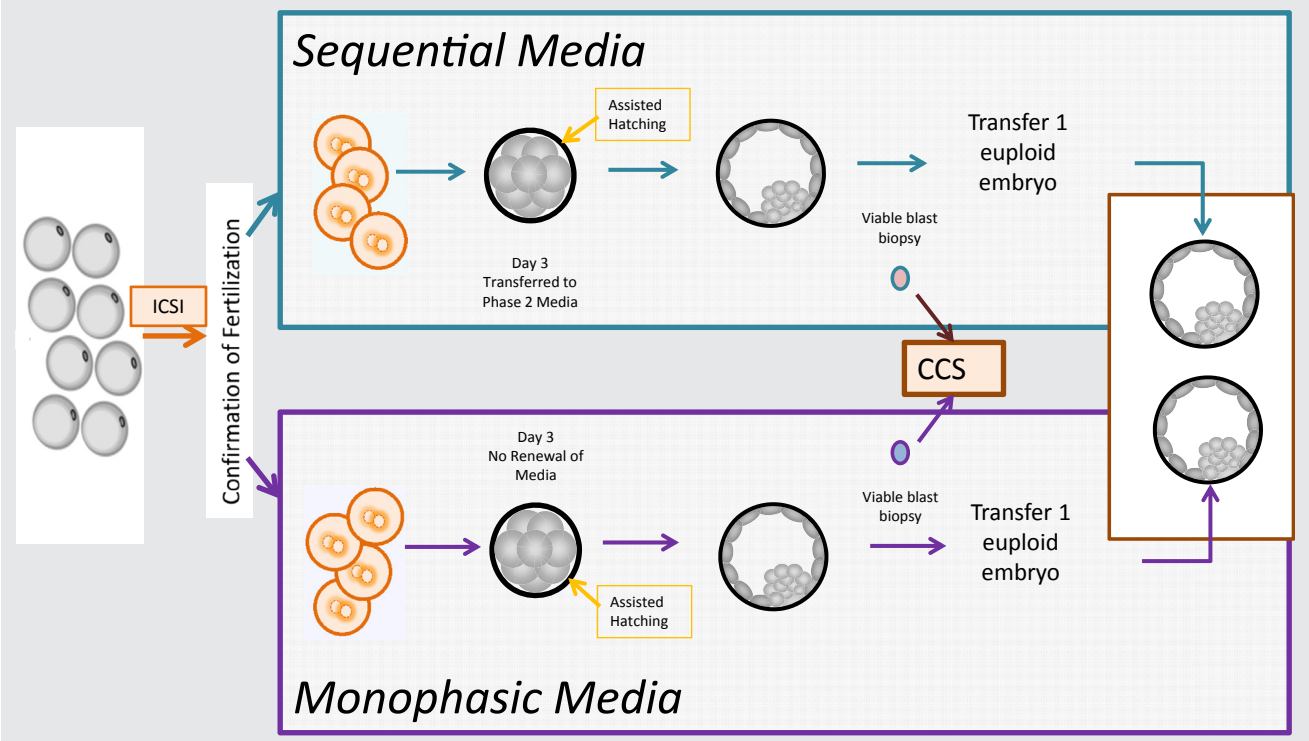
The study was designed to provide a paired comparison of two embryonic culture systems on in vitro embryo development. To achieve this paired design, each patient's cohort of fertilized zygotes was split into two dishes of equivalent number and labeled "A" or "B." The embryologist then opened an envelope designating which media system group A and group B was to be cultured in (Fig. 1). This experimental design has been used successfully in multiple prior studies involving the clinical assisted reproductive technology (ART) laboratories (20–23). Allocation concealment was achieved using sequentially numbered, opaque, sealed envelopes. Dishes were placed into their respective incubators immediately after confirmation of fertilization.

On day 5 of in vitro culture, embryos from both groups were assessed. Given that the dishes were coming from incubators with different CO_2 concentrations, it was not possible to blind the embryologist grading and selecting embryos for transfer as to which culture system had been used for a given group of embryos. For any given case, one embryologist evaluated the entire cohort of embryos for that single patient, including embryos grown in sequential and monophasic media. Morphological selection was a based on a modified Gardner criteria that examined the appearance of the inner cell mass (ICM) and trophectoderm. All embryos underwent assisted hatching on day 3 of development, which in the experience of the laboratory team allows hatching at the time of expansion and typically earlier than would be seen in unhatched embryos. Assisted hatching was performed in <2 minutes for each culture dish, to minimize any fluctuations in pH or temperature.

All fair and good quality expanded blastocysts underwent trophectoderm biopsy and comprehensive chromosome screening (24). Aneuploidy screening results were available the following morning (day 6 of development). At that time the euploid blastocyst with the best morphological development was selected from each group. These two embryos were then transferred. All patients and physicians were blinded to the results.

All patients met American Society for Reproductive Medicine criteria for a double blastocyst transfer and had already designated their preference for a double-ET before study participation. As such, no patients had additional risk for multiple gestation based on their participation in the study. In spite of this, all patients were thoroughly counseled before study enrollment about the risk of multiple gestation.

FIGURE 1



Experimental design. Retrieved zygotes for each patient were split into two groups and cultured in sequential or monophasic media. Embryological developmental outcomes and rates of implantation resulting in a live birth were compared. CCS = comprehensive chromosome screening; ICSI = intracytoplasmic sperm injection.

Werner. Two-step medium yields more blastocysts. Fertil Steril 2016.

Patients at risk for ovarian hyperstimulation syndrome (OHSS) or who did not have expanded blastocysts until day 6 of development underwent a frozen double-ET of one euploid blastocyst from each media group. Patients with euploid embryos from only one culture condition received a single-ET. Pregnancies were observed until delivery, after which a DNA fingerprinting assay was done to determine the culture group from which the delivery originated (Fig. 1).

Laboratory Intervention

Tri-gas incubators (Panasonic model MCO-5M-PA) were used for this study. An oxygen tension of 5% was used in all incubators. A group of incubators were dedicated to the study, half of which were calibrated for culture in sequential media and the other half for monophasic medium. The CO_2 and temperature of each incubator was measured and recorded every morning at the same time. The pH was measured in each incubator on a weekly basis, and the CO_2 gas level was adjusted to maintain the pH within the range found to be optimal for blastulation in the laboratory and this value differed for the two types of media. The optimal pH for monophasic medium was 7.31 and for sequential media, it was slightly more acidic for precompaction development (7.25–7.28) and slightly more alkaline (7.35–7.38) for postcompaction development. Sequential media consisted of Quinn's Advantage Cleavage Medium (SAGE) followed by Blast Assist (Origio). The monophasic media used was Continuous Single Culture (Irvine Scientific).

Laboratory management of each case was broken into three phases. The first phase extended from isolation of the oocyte-cumulus complexes from the aspirated follicular fluid (FF) to the completion of ICSI and confirmation of fertilization. These procedures were performed per routine laboratory protocol and were identical for both groups.

The second phase represented the experimental phase. After confirmation of fertilization, the zygotes were transferred to dishes that had been pre-equilibrated with either sequential or monophasic media. Embryos grown in sequential media were moved on day 3 from phase I to phase II medium as per laboratory routine. Media was not renewed for embryos culture in monophasic medium. The embryos from both groups were hatched on day 3 as per routine in the laboratory for embryos planned to undergo preimplantation genetic screening.

The third phase was embryo development to the expanded blastocyst stage followed by trophectoderm biopsy. All supernumerary blastocysts were vitrified.

Statistical Analysis

The primary outcome was the proportion of zygotes with two pronuclei (2PN) that developed into expanded blastocysts suitable for clinical use (either transfer or cryopreservation, with a modified score 3CC or better). These morphologically normal embryos were termed usable blastocysts. This measure of in vitro development is the end point of choice when comparing laboratory outcomes and is the parameter by which quality assurance and quality control activities are performed in this laboratory.

Secondary outcomes included timing of blastulation and ploidy status. Timing of blastulation was deemed "on time" if an embryo blastulated before the afternoon of day 5 (~120 hours after ICSI) and deemed "late" if the embryo did not become an expanded blastocyst until the morning of day 6. Finally, sustained implantation, as defined by the presence of a fetal heart beat after 9 weeks gestation, was compared between groups.

Paired analyses were performed. The Wilcoxon signed-rank test was used to compare paired rates of embryological development. McNemar's χ^2 for concurrence was used to compare sustained implantation rates for those patients undergoing paired ET. Pearson's χ^2 was used to compare sustained implantation rates for all transferred embryos.

Parameters of in vitro development were reported first as an overall effect on all embryos in aggregate, and then secondly in a paired fashion. Table 1 reports the overall mean of these rates.

RESULTS

One hundred ninety eight patients were enrolled and the population reflected inclusion criteria with an average age 34.3 years old, average day 3 FSH level of 6.6 IU/L, AMH value of 4.2 ng/mL, and 12 ± 5.4 fertilized zygotes. Six patients withdrew from study protocol after consent, five for personal reasons not related to study design and one for media that failed to pre-equilibrate. This resulted in 192 patients whose zygotes underwent randomization for growth in either sequential or monophasic media. After ICSI, 2,257 of the 2,543 metaphase II were fertilized (88.8% fertilization rate). Patients (192) completed the study protocol through blastocysts development; however, an additional six patients withdrew from the study at this point and did not complete an ET, with explanations ranging from natural conception (1 patient), marital issues (1), desire for sex selection (2), or embryo banking (2 patients). Eleven patients had no ET due to complete embryonic arrest, and seven patients had no ET as all embryos were aneuploid. This resulted in 168 patients who completed the study protocol, including ET. No patients were lost to follow-up and all pregnancy outcomes were available. Throughout the study, the culture systems performed as planned, passing regular quality assurance with pH optimized as described.

Overall In Vitro Development

A total of 2,257 zygotes were randomly assigned to each culture system—1,127 to monophasic medium and 1,130 to sequential media. One thousand one hundred fifty zygotes formed usable blastocysts, yielding an overall blastocyst conversion rate of 51.0% (monophasic medium 46.9% [528/1,127] and sequential media, 55.2% [622/1,130]) (Table 1). These proportions are consistent with results typically attained in this embryology laboratory.

Comprehensive chromosomal screening was performed for all usable blastocysts and 802 were deemed euploid. Of usable blastocysts, 69.1% (365/528) resulted from monophasic culture and 70.3% (437/622) from sequential media. Of the remaining embryos, 9 resulted as nonconcurrent where the

TABLE 1

In vitro development: sequential media yielded more usable blastocysts per patient.

Parameter	Sequential	Monophasic	*P* value
2PNs	1,130	1,127	
No. of usable blastocycts (rate)	622 (55.2%)	528 (46.9%)	.0001
No. of euploid (rate, expressed per no. of usable blastocysts)	437 (70.3%)	365 (69.1%)	.5518
No. of usable euploid blastocysts (transferred and cryopreserved) (rate expressed per no. of 2PNs)	437 (38.7%)	365 (32.4%)	.0018

Note: PN = pronuclei.

Werner. Two-step medium yields more blastocysts. Fertil Steril 2016.

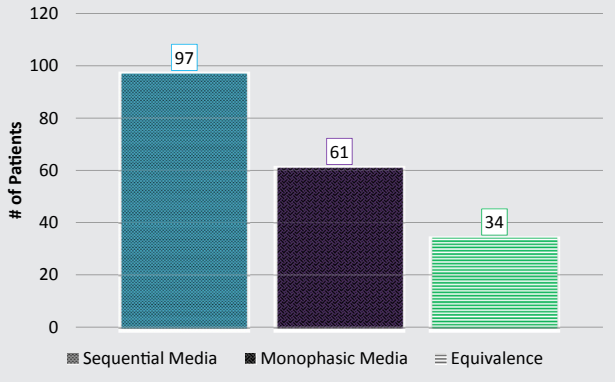
consistency of data within each of the 24 chromosomes was insufficient to determine ploidy status, 1 sample failed to amplify, and the rest of the embryos were aneuploid.

Paired Analysis of In Vitro Development

A paired analysis was then performed within each patient (N = 192) to determine whether the type of culture media impacted embryo development. Ninety seven (50.5%) patients were more likely to have embryos form usable blastocysts in sequential media. This is in comparison with the 61 (31.8%) patients who had more usable blastocysts in monophasic and 34 (17.7%) patients where culture media made no difference (Fig. 2). For the study population, sequential media was statistically more likely to result in more usable blastocysts in comparison to monophasic medium (*P*=.0001). It follows that the number of usable euploid blastocysts was also higher in embryos cultured in the chosen sequential media in comparison with the selected monophasic medium (*P*=.0018) (Table 1).

This type of analysis was then performed in patients who formed at least one usable blastocyst in each group (N = 154) to determine whether embryos grown in sequential media were more likely to blastulate "on time" in comparison with embryos grown in monophasic medium. There was no statistical difference per patient in the time to blastulation regardless of culture media (*P*=.4063). Similarly this analysis was performed to determine the euploid rate of usable blastocysts per patient and no statistical difference was noted regardless of culture media (*P*=.5518).

FIGURE 2



Paired analysis of usable blastulation rate favors sequential media. Of 192 patients, 97 were likely to have more embryos develop into usable blastocysts when cultured in sequential media. *P*=.0001.

Werner. Two-step medium yields more blastocysts. Fertil Steril 2016.

Paired Analysis of Reproductive Competence among Transferred Euploid Blastocysts

Pregnancy rates (PR) of the transferred blastocysts in the group of patients undergoing planned double-ET (one from each of the culture groups, N = 126) were analyzed. From this group, 60 patients sustained implantation of both embryos resulting in twins, 36 patients had implantation of only one embryo and had a singleton gestation, and 30 patients did not become pregnant.

All double-ETs that resulted in singleton gestations underwent DNA fingerprinting to determine whether the embryo from sequential or monophasic culture media sustained implantation. Of these no statistical difference was noted in sustained implantation as 19 resulted from culture in sequential media and 17 resulted from culture in monophasic medium (*P*=.868) (Table 2).

An analysis was then performed of patients undergoing only a single ET (n = 42). Single ETs were only performed if a patient only had one usable euploid blastocyst from one culture group. Twenty-one single ETs resulted in sustained implantation, 12 from sequential media and 9 from transfer in monophasic culture. The remaining single ETs did not result in pregnancy, 12 from sequential and 9 from monophasic culture. There was no statistical difference in implantation when comparing media to support blastocyst development in patients completing a single ET (*P*=1.0). This study had an 80% power to detect a 15% difference in implantation rates.

DISCUSSION

This study systematically demonstrates that culture in the monophasic medium selected for study (Continuous Single Culture, Irvine Scientific) results in a lower usable blastocyst formation rate when compared with the tested sequential media ((Quinn's Advantage Cleavage Medium, SAGE) followed by Blast Assist (Origio)). There were no significant differences identified in the timing of blastulation or ploidy rates. This suggests that patients will have more usable euploid blastocysts when grown in the chosen sequential media likely allowing each patient a greater cumulative opportunity for ET

TABLE 2

Euploid ET(s): sustained implantation rates were similar between the two culture groups.

Characteristic	Total no. of patients	Total no. of embryos	Sustained implantation rate in sequential media	Sustained implantation rate in monophasic media	*P* value
ET completed	168				
Paired	126	252	19/36	17/36	.868
Single ET	42	42	12/24	9/18	1.0

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and pregnancy. However, the type of media did not impact the reproductive potential of those embryos that did blastulate. Once a euploid blastocyst was of sufficient quality to be selected transfer, sustained implantation rates were equivalent for sequential and monophasic media. This is the first paired randomized controlled trial to assess differences in blastulation rates between sequential and monophasic media.

These findings differ from results proposed by earlier studies, which suggested that monophasic media formulations may be better or at least equivalent to sequential media (16, 17, 25). It is possible that different system-specific parameters, including the number of embryos in group culture, the incubators used, and laboratory-specific characteristics, may not be confounding variables given the paired experimental design used in this study (26). Although monophasic media formulations are based on the same principles of development, they may differ widely in the physical concentration of substrates and as such the results presented may not be generalizable to all commercially available formulations.

The "quiet embryo hypothesis" would suggest use of a monophasic culture system to lessen manipulation of the embryo and achieve superior results, however it seems the "back to nature" approach may offer patients a greater opportunity for ET and pregnancy (1, 2). Monophasic formulations have the advantage of using one system over the entirety of embryo development, precluding the need for media renewal on day 3 of development or any assessment that could expose an embryo to fluctuating environmental changes (pH, temperature). In addition, monophasic formulations can be used in conjunction with noninvasive imaging modalities. Furthermore, monophasic formulations are cheaper to use as they require less quality assurance and control testing, fewer resources, and less media (if not renewed on day 3). Sequential formulations tend to be more expensive and do require a media switch on day 3 of development. Although the procedural advantages of monophasic formulations may be advantageous, the results of this study demonstrate that sequential media may afford better clinical outcomes.

The major strengths of the present analysis are the power of the paired zygote design and selection of only euploid embryos for transfer. Whereas meiotic errors should be equivalent in the two groups given that randomization occurred after the completion of meiosis, there was potential to detect a difference in mitotic errors. Mitotic errors contribute meaningfully to aneuploidy in human embryos (27). Although a single biopsy is certainly insufficient to quantitate the absolute level of mosaicism resulting from mitotic errors, it does provide a sampling of errors. Given that the meiotic errors should be equivalent after randomization, any differences in aneuploidy rates would then obligately reflect the proportional difference in the mitotic error rate. The fact that the overall aneuploidy rates were equivalent indicates that the choice of media systems does not meaningfully impact on mitotic error rates during culture. Given the major role that aneuploidy plays in limiting embryonic reproductive competence, this finding is quite reassuring.

Patient inclusion criteria were both a strength and limitation of the study design as only patients with normal ovarian reserve were analyzed. This allowed for the power to assess differences in embryological parameters and assess differences in implantation rates but may prevent applicability to all patients undergoing IVF cycles. Caution should be used in applying these findings to all patient groups and in particular those who are low responders. Although it would need to be demonstrated using a rigorous experimental design, it remains possible that intrinsic differences in the oocytes from low responders might favor a different media formulation than that which is optimal in normal responders.

One of the most significant benefits to monophasic culture is that the embryo remains undisturbed at the cleavage stage, diminishing the need for additional dishes, renewal of media, and dedicated laboratory time and for these reasons the monophasic medium chosen for this study (Continuous Single Culture) was not renewed. Similarly assisted hatching was performed at the cleavage stage on all embryos, as such the effects of the laser on the monophasic culture medium cannot be determined. It should be noted that embryos in sequential culture were both hatched on day 3 and moved into blast medium (phase 2 medium). This second phase of sequential medium would have had less exposure to shifts in temperature and pH then the monophasic formulation.

Although great care was taken in study design, differences still remained in embryo handling between the two study arms that may have resulted in a potential confounding variable. It is unknown whether our findings would be replicated if embryo handing had been identical in both culture systems. Due to the nature of this design it was not possible to blind the embryologists to media as they were responsible for grading and selection. A necessary next step would be to investigate whether renewing media at the cleavage stage may equalize outcomes between groups; however, this may temper the work-flow and economic benefits gained with using a one-step culture system. In conclusion, these data demonstrate that the sequential media formulation tested in this study provided superior efficiency

in supporting production of high quality blastocysts suitable for either transfer or cryopreservation.

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