Continuous uninterrupted single medium culture without medium renewal versus sequential media culture: a sibling embryo study

Eight hundred ninety-three sibling embryos from 80 IVF cycles were cultured side by side in either: 1) a single medium continuously, without medium renewal on day 3; or 2) sequential media. There were no significant differences between the two culture media systems regarding embryo quality or the proportion of embryos selected for transfer on day 3 from either media; however, for day 5 embryo transfer, a greater number of blastocysts were available, and were selected for transfer, from the continuous single medium culture compared with sequential media culture. (Fertil Steril® 2009;■:■–■. ©2009 by American Society for Reproductive Medicine.)

Media for culturing human embryos in vitro have evolved away from somatic cell culture formulations toward stage-specific sequential formulations to facilitate presentation of different mediu, components to the embryos during pre- and postgenomic activation. There are many commercial sequential media formulations for stage-specific use and, more recently, two formulations that are designed, as a single medium, to present all components to the embryos during all stages of postfertilization in in vitro development (1–6).

The movement of embryos to new dishes, or refreshing the culture medium at intervals has been suggested as a technique to avoid exposure of embryos to the potential buildup of ammonium from the breakdown of amino acids or volatile atmospheric compounds. Single media (sometimes referred to as monoculture media) are designed to provide all components to embryos at all times; a modern single-culture medium, designed to limit the buildup of ammonium by replacement of glutamine with a more stable form, could be used for continuous uninterrupted culture of human embryos, as has been suggested and demonstrated by others (6–9).

The purpose of the present study was to compare the sequential media protocol currently in use to a continuous uninterrupted single-medium protocol as well as to challenge the concept that embryos must be moved to new dishes with fresh media at an interim time point during culture and that sequential culture is required to mimic the movement of embryos from the oviduct to the uterus.

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Global medium for embryo culture was supplied free of charge by IVFonline, Guelph, Ontario, Canada.
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In this study, standard assisted reproductive technology (ART) laboratory procedures, commercial embryo culture media, and commercial media supplements were used; therefore, Institutional Review Board approval was not required. Global medium (IVFonline; Guelph, Ontario, Canada) was provided free of charge for this study.

All patients undergoing in vitro fertilization between June 2008 and December 2008 were considered to reduce selection bias toward any one diagnosis or age grouping. Patients were excluded from the study if there were fewer than two fertilized or cleaving ova, if the cycle included biopsy for preimplantation genetic diagnosis/screening, or if the cycle was destined for cryopreserved embryo banking.

Ninety-five oocyte retrievals occurred during the study period. Seventy patient oocyte and ten donor oocyte cycles were included; 15 cycles were excluded according to the criteria stated above. Patients were assigned to day 3 or day 5 transfer according to established protocol, based primarily upon patient age and the number of fertilized ova. Thirty-six and 34 patients were assigned to day 3 and day 5 transfers, respectively, and all ten donor oocyte recipients were assigned to day 5 transfer.

For each case, a clean Pyrex desiccator jar (2.2 L volume) was prepared the day before oocyte retrieval: approximately 50 mL sterile Water for Assisted Reproductive Technologies (Irvine Scientific, Santa Ana, CA) was added to the bottom of the jar. The ground glass surface of the lid was lightly coated with Dow Corning high-vacuum grease (Dow Corning Corporation, Midland, MI) to facilitate a gas-tight seal, and the jar, with the lid slightly ajar, was placed into a 37°C gas (6% CO_2/air)- and humidity-equilibrated incubator for warming overnight. One jar was used for each patient. The Dow Corning brand silicone grease has passed, and continues to pass, the mouse embryo toxicity assay used in this laboratory.

Oocytes exposed to sperm via conventional fertilization or intractyoplasmic sperm injection (ICSI), or embryos

after fertilization check, were placed into culture dishes that had been equilibrated overnight at 37°C with 6% CO_2/air. Dishes with oocytes exposed to sperm, or embryos after fertilization check, were then placed into a prepared glass jar and charged with certified medical grade 6% CO_2, 5% O_2, 89% N_2 (filtered through an activated carbon bed, followed by passage through a water gas washing bottle, and finally a 0.2 μm inline filter) at 10–15 psi for 3 minutes, sealed, and returned to the incubator.

Controlled ovarian stimulation was achieved with antagonist or agonist administration, combined with a mixed FSH/LH protocol, tailored to each patient. Oocyte harvest occurred approximately 35 hours after administration of 10,000 U hCG. Oocyte cumulus complexes were isolated from follicular aspirates, rinsed twice in Hepes-modified human tubal fluid (HTF) with 5 mg/mL albumin (Quinn's Sperm Washing Medium; Sage In-Vitro Fertilization, Trumball, CT), and then held in equilibrated HTF medium supplemented with 10 mg/mL human serum albumin (Irvine Scientific, Irvine, CA) in a 37°C humidified 5% CO_2/air atmosphere until processing for conventional insemination or ICSI. The overnight culture medium for inseminated oocytes (conventional fertilization or sperm-injected ova) was GIVF medium supplemented with 10 mg/mL albumin (Vitrolife, Denver, CO), where dishes were prepared with 100-μL drops under 12 mL Oil for Embryo Culture (light mineral oil; Irvine Scientific, Santa Ana, CA). Approximately 18 hours after insemination or ICSI, oocytes were examined for the presence of pronuclei, and one-cell embryos with two pronuclei were placed randomly into the two culture media treatments, so that, as much as possible, equal numbers of sibling embryos within each patient were placed into each treatment.

Two commercial formulations were used for embryo culture: the G5 series sequential media already in use at this laboratory (G1.2v5, G2.2v5; Vitrolife) and Global medium (IVFonline, Guelph, ON). The pH of both culture media (without protein) was found to be similar, approximately pH 7.2 to 7.25 after overnight equilibration under 6% CO_2. In an earlier report (10), ammonium concentrations were monitored in several media, over time, and were found to linearly increase in formulations with glutamine, and, because exposure of mouse embryos to culture medium containing high concentrations of ammonium, e.g. 300 μg/mL as ammonium chloride, was concluded to be detrimental (11, 12), the current formulation of Global medium, containing a stable form of glutamine (with protein) was assayed; ammonia did not exceed 40 μg/mL after 7 days, nor did the pH significantly change. The stability of glutamine alternatives has been demonstrated elsewhere (13–15).

Culture dishes for postfertilization embryos were prepared the day before use and equilibrated overnight in a standard humidified cell culture incubator at 37°C with 6% CO_2/air. For the sequential media arm of the study, days 1–3 (day 0 is day of retrieval), a dish was prepared with 50-μL drops (seven drops total) of G1.2v5 supplemented with 10% v/v Synthetic Serum Substitute (SSS; Irvine Scientific, Irvine, CA), under 12 mL mineral oil; for days 3–6, a dish was prepared with 50-μL drops (seven drops total) of G2.2v5 supplemented with 10% v/v SSS under 12 mL mineral oil. For the continuous uninterrupted single medium arm of the study, a single dish was prepared for days 1–6, with 50-μL drops (seven drops total) of Global medium supplemented with 10% v/v SSS under 12 mL mineral oil. The authors recognize that equilibration of oxygen with the medium would require time, perhaps several hours, after dishes are moved from room air conditions to sealed jars with a reduced oxygen atmosphere.

In as much as possible, pronuclear stage embryos were placed into the drops in groups, typically 2–3 embryos per 50-μL drop. Both treatment dishes were placed side by side in the same prewarmed humidified glass desiccator jar, after which the jar was gassed for 3 minutes at 10–15 psi with triple mix gas, sealed, and placed back into a 37°C incubator. On day 3 after retrieval, embryos from the first sequential medium were moved to the new dish with the second of the sequential media. The embryos in the dish with Global medium were not moved to a new dish, nor were the microdrops "renewed" with fresh equilibrated medium.

For day 3 transfer, embryos were scored for cell number and morphology (score Q1–5, 1 being best). Embryos in sequential medium were then moved to the second sequential medium. Embryos in the single medium remained in the original dish, without renewal of the microdrops. Selection of embryos for transfer was based on cell number and quality score, regardless of the culture media treatment. Immediately before transfer, selected embryos were moved to approximately 3 mL 36°C Quinn's Sperm Washing Medium. The rationale for using this medium for embryo transfer is the pH stability provided by the Hepes buffer in case the embryo transfer took longer than anticipated. The catheter was rinsed with a small volume of medium, and the embryos were aspirated into the catheter tip in approximately 10–15 μL volume, located between two small air bubbles. The embryo transfer was assisted using ultrasound guidance. Dishes with embryos that were not transfered were returned to the glass jar, which was then gassed as described and returned to the incubator for culture to day 5 and/or 6 for evaluation for cryopreservation (expanded blastocysts with obvious inner cell mass).

For day 5 transfer, embryos were briefly viewed on the morning of day 3, but were not assigned quality scores. Embryos in sequential medium were moved to the second sequential medium. Embryos in the single medium treatment remained in the original dish, without renewal of the microdrops. The dishes were returned to the glass jar, gassed as described, and returned to the incubator for continued culture to day 5. On day 5, embryos in both dishes were scored as to developmental stage and subjective morphology

TABLE 1

Cycle characteristics according to transfer day and oocyte source, and sibling embryo developmental data using two culture media systems, according to transfer day and oocyte source.

		Culture medium day 3 transfer			Culture medium day 5 transfer			Culture medium day 5 transfer	
	Patient oocytes day 3 transfer	Sequential culture	Continuous culture	Patient oocytes day 5 transfer	Sequential culture	Continuous culture	Donor oocytes day 5 transfer	Sequential culture	Continuous culture
No. of retrievals	36			34			10		
No. of transfers	36			34			10		
Mean (SD) patient age, yrs	35.8 (3.9)			34.2 (4.2)			42.1 (4.9)		
Total no. of fertilized ova	232	116	116	480	238	242	181	91	90
Mean (SD) no. of fertilized ova	6.4 (2.6)	3.2 (1.3)	3.2 (1.3)	14.1 (4.5)	7.0 (2.3)	7.1 (2.4)	18.1 (6.8)	9.1 (3.1)	9.0 (3.8)
Mean (SD) no. of Q1/Q2 $\geq$6-cells on day 3[1]		1.0 (1.0)	1.1 (0.9)						
Mean (SD) no. of blastocysts on day 5					2.5 (2.0)[a]	3.4 (2.5)[b]		4.0(3.3)[a]	4.7 (4.2)[b]
Mean (SD) no. of embryos replaced	2.8 (0.7)	1.3 (0.7)	1.6 (0.7)	2.1 (0.3)	0.7 (0.6)[c]	1.4 (0.6)[d]	2.0 (0.0)	0.8 (0.8)[c]	1.2 (0.8)[d]
Mean (SD) quality score for embryos replaced[2]		2.5 (0.8)	2.4 (0.7)		2.5 (1.4)	2.7 (1.3)		1.4 (0.5)	1.8 (1.0)
Mean (SD) total no. of blastocysts frozen on days 5 and 6		0.2 (0.5)	0.4 (0.6)		3.1 (2.2)	4.0 (2.3)		5.1 (3.3)	4.9 (2.6)
Clinical pregnancies/ retrievals	21/36 (58.3%)			27/34 (79.4%)			10/10 (100.0%)		

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TABLE 1

Continued.

	Patient oocytes day 3 transfer	Culture medium day 3 transfer: Sequential culture	Culture medium day 3 transfer: Continuous culture	Patient oocytes day 5 transfer	Culture medium day 5 transfer: Sequential culture	Culture medium day 5 transfer: Continuous culture	Donor oocytes day 5 transfer	Culture medium day 5 transfer: Sequential culture	Culture medium day 5 transfer: Continuous culture
Clinical pregnancies/ transfers	21/36 (58.3%)			27/34 (79.4%)			10/10 (100.0%)		
Ongoing pregnancies/ retrievals	17/36 (47.2%)			26/34 (76.5%)			9/10 (90.0%)		
Ongoing pregnancies/ transfers	17/36 (47.2%)			26/34 (76.5%)			9/10 (90.0%)		
Implantations/ embryos	26/101 (25.7%)			41/70 (58.6%)			18/20 (90.0%)		

Note: Data for sibling embryos within culture media treatments were analyzed using the paired *t* test and Wilcoxon rank test where appropriate. Day 3 subjective embryo quality scores rank Q1–5, where 1 is best and 5 is worst. Day 5 subjective embryo quality scores rank Q1–4, where 1 is best and 4 is early blastocyst stage. Clinical pregnancy is the presence of a gestational sac at approximately 7 weeks gestation. Ongoing pregnancy is the presence of fetal cardiac activity at approximately 7 weeks' gestation. Implantation rate is calculated by the number of gestational sacs divided by the number of embryos transfered. Q1 = quality score 1; Q2 = quality score 2.

[a–d] Different superscripts within rows indicate significant difference for sibling embryo data within treatment.

[a,b] $P<.05$.

[c,d] $P<.01$.

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(blastocyst score Q1 to Q3, 1 being best; embryos with early blastocele development without distinct inner cell mass identification were scored as early blastocysts). Embryo selection for transfer was based on the quality score, regardless of the culture medium treatment. The embryo transfer was performed as described above. Embryos not selected for immediate cryopreservation (fully expanded hatching blastocysts with obvious inner cell mass were cryopreserved on day 5) were returned to the glass jar, gassed as described, and returned to the incubator for continued culture to day 6 and evaluation for cryopreservation.

Sibling fertilized embryos within each patient were assigned, as randomly as possible, to each culture media treatment to reduce the experimental variation between patients. The developmental data for sibling embryos were analyzed within each treatment (media system), but not between day of embryo transfer using paired *t* test and Wilcoxon rank test (Statgraphics Plus 6.0; Manugistics, Rockville, MD). Patient and cycle characteristics, pregnancy outcomes, and sibling embryo development data, according to day of embryo transfer within culture media treatments, are presented in Table 1.

For embryo transfer on day 3, the mean number of Q1 + Q2 $\geq$ 6-cell embryos (Q1 is best, Q5 is worst), the mean number of embryos selected for transfer from each media, and the mean quality scores of the embryos transfered were not significantly different between the two culture systems ($P>.05$). Additionally, the number of supernumerary embryos developing to the blastocyst stage for cryopreservation was not significantly different between media ($P>.05$). Embryos were selected for transfer from both media in 30 of 36 transfers (83.3%), 2 of 36 transfers (5.6%) from sequential media only, and 4 of 36 transfers (11.1%) from single medium only.

For embryo transfer on day 5, there was a significantly higher mean number of blastocysts on day 5 in the single medium compared with sequential media ($P<.05$), and a significantly higher number of blastocysts were selected for transfer from the single medium compared with the sequential media, at approximately a 2:1 ratio ($P<.01$), although the mean quality scores of the embryos selected for transfer were similar. A more explicit grading system for blastocysts (16, 17) might provide a more detailed picture of the selection trend observed in this study. Embryos were selected for transfer from both media in 22 of 44 transfers (50.0%), 5 of 44 transfers (11.4%) from sequential media only, and 17 of 44 transfers (38.6%) from single medium only. The significantly higher mean number of blastocysts available on day 5 in the single medium is similar to the findings of Sepulveda et al. (18) and Pomeroy et al. (19), supporting the concept that a single medium can perform as well as sequential media. The results of this study also demonstrate that continuous use of a single medium does not negatively affect embryo development, as has been proposed and demonstrated by others (6–9).

No effort was made in this study to purposefully choose a single embryo from each treatment, rather the best embryos, regardless of treatment, were chosen for replacement; however, for further study, there is the possibility that using two (or more) different culture media in a sibling embryo culture protocol would promote higher implantation rates by allowing embryos within each patient to "choose" which of the two media they prefer, as demonstrated by Angle (20) and Pomeroy et al. (19).

The benefits of a successful continuous single medium protocol could include a reduced potential for embryonic loss and/or introduction of contaminants through handling errors, decreased embryonic stress, including reduced temperature and pH fluctuations (11, 21) or even pipetting of embryos (22), and, though of limited importance, a reduction in the cost of materials used. It should be noted that continuous uninterrupted culture may be less successful if the laboratory or culture environment is less than optimal, because there might be buildup of oil- and/or water-soluble volatile organic compounds over the culture period. The data presented here suggest that with careful control over the culture environment, e.g., using sealed glass jars charged with a specific gas atmosphere, this concern may be minimized.

This study demonstrates that there is no apparent advantage in using sequential media over a single medium and that uninterrupted single medium culture can be successful, regarding embryonic development and clinical outcomes, if laboratory conditions are closely monitored and using precautions against atmospheric fluctuations. It also highlights the need for further carefully designed studies to challenge what is considered to be accepted, or traditional, embryo culture practices.

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