In vitro development of donated frozen-thawed human embryos in a prototype static microfluidic device: a randomized controlled trial

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Objective: To compare the development of human embryos in microfluidic devices with culture in standard microdrop dishes, both under static conditions.
Design: Prospective randomized controlled trial.
Setting: In vitro fertilization laboratory.
Patient(s): One hundred eighteen donated frozen-thawed human day-4 embryos.
Intervention(s): Random allocation of embryos that fulfilled the inclusion criteria to single-embryo culture in a microfluidics device (n = 58) or standard microdrop dish (n = 60).
Main Outcome Measure(s): Blastocyst formation rate and quality after 24, 28, 48, and 72 hours of culture.
Result(s): The percentage of frozen-thawed day-4 embryos that developed to the blastocyst stage did not differ significantly in the standard microdrop dishes and microfluidic devices after 28 hours of culture (53.3% vs. 58.6%) or at any of the other time points. The proportion of embryos that would have been suitable for embryo transfer was comparable after 28 hours of culture in the control dishes and microfluidic devices (90.0% vs. 93.1%). Furthermore, blastocyst quality was similar in the two study groups.
Conclusion(s): This study shows that a microfluidic device can successfully support human blastocyst development in vitro under static culture conditions. Future studies need to clarify whether earlier stage embryos will benefit from the culture in microfluidic devices more than the tested day-4 embryos because many important steps in the development of human embryos already take place before day 4. Further improvements of the microfluidic device will include parallel culture of single embryos, application of medium refreshment, and built-in sensors.
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Key Words: Blastocyst, embryo culture techniques, human, in vitro fertilization, microfluidics

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The static culture format currently used for human assisted reproduction differs profoundly from the dynamic microenvironment found in the female reproductive system (1). An embryo encounters physically and chemically changing conditions during its journey through the fallopian tubes and crypts in the lumen of the uterus (2, 3). Microfluidics may revolutionize in vitro embryo culture by mimicking these in vivo conditions more closely. First, microfluidic devices can be designed with a smaller culture volume than conventional culture dishes. Improved culture conditions may arise from the accumulation of autocrine factors in the vicinity of each embryo without dilution by a large culture volume (4) or a reduction

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in localized oxygen tension (5). A smaller culture volume also allows an embryo to regulate its own microenvironment better during single-embryo culture (6). Second, a reduced culture volume can facilitate a comprehensive analysis of culture medium because embryo activity would cause measurable changes in the local environment of each embryo. The integration of sensors in microfluidic culture chambers and the combination with in situ time-lapse microscopy (7) represent unique opportunities for the identification of the embryo with the highest chance to implant. Third, microfluidic technologies offer more sophisticated approaches for the refreshment of culture medium (8).

Several research groups have published encouraging findings on the microfluidic culture of embryos from a number of animal species (9–14). Promising results were notably reported by Heo et al. (8), who cultured groups of mouse embryos in a semimicrofluidic device consisting of a microfunnel with a volume of 10 μL and a microfluidic network for medium perfusion. Blastocyst development in the microfunnel and conventional droplets was comparable. However, the proportion of mouse zygotes developing to hatching or hatched blastocysts was significantly enhanced in the dynamic setting compared with the static microfunnel control. Furthermore, culture in the microfunnel led to improved implantation and ongoing pregnancy rates of mouse embryos.

However, all of the aforementioned microfluidic studies have been limited to the culture of embryos in a group, but a single-embryo culture approach is essential for embryo selection in human assisted reproduction and online monitoring of embryo development. Studies on the culture of human embryos in microfluidic culture devices are still scarce. Mizuno et al. (15) reported comparable blastocyst development using a coculture microfluidic system and a microdrop control, with slightly higher quality embryos in the microfluidic system compared with the control group. However, the sample size was limited. Alegretti et al. (16) cultured human zygotes either in a microfluidic dynamic device or in a traditional static system, yielding higher embryo quality on day 3 using microfluidics.

Recently, a microfluidic embryo culture device has been developed to mimic the in vivo environment in the female reproductive system (17). Microfluidic culture chambers of two different volumes (30 nL and 270 nL) were shown to support blastocyst development of mouse preimplantation embryos (strain B6C3F1). Furthermore, single-embryo culture in microfluidic chambers yielded blastocyst rates of more than 90% at 4.5 days after fertilization compared with significantly lower rates in 5-μL microdrops. Additionally, blastocysts could be retrieved from the microfluidic device and transferred to pseudo-pregnant mice, resulting in viable full-term development. Based on the promising findings with mouse embryos by Esteves et al. (17), we hypothesized that human embryos could benefit from the confined culture conditions offered by such microfluidic devices as well, with the idea that the decreased culture volume would enable each embryo to generate its own microenvironment and accumulate factors in its close proximity. Clearly, new technologies have to be validated with great care before an introduction into the clinical in vitro fertilization (IVF) setting is possible. Initial work on animal models should be followed by well-designed studies on donated human embryos and clinical randomized controlled trials (18). The promising results from the study with mouse embryos (17) allowed us to take the next step by culturing human day-4 embryos in microfluidic culture chambers.

Our proof of concept study had the primary objective of assessing whether microfluidic culture chambers could support the development of preimplantation human embryos under static conditions or could even provide enhanced culture conditions. For this purpose, donated frozen-thawed human day-4 embryos were cultured individually in submicroliter microfluidic chambers or in standard microdrop dishes, both under static conditions. The morphologic characteristics of the developing embryos and their blastocyst formation rate were assessed at four different time points.

MATERIALS AND METHODS

Study Design

This study was designed as a prospective randomized controlled trial. The institutional review board and Central Committee on Research Involving Human Subjects approved of the study (no. NL38300.000.11). The randomized controlled trial was conducted at the IVF center of an academic hospital in the Netherlands (VU University Medical Center Amsterdam) and was registered at the Dutch Trial Register as NTR3867. Embryos were included from August 2012 to August 2013.

Origin of Embryos

Cryopreserved embryos donated for scientific research were thawed and included in this study. The stimulation protocol and routine laboratory procedures at VU University Medical Center Amsterdam were described earlier by Vergouw et al. (19). In short, IVF or intracytoplasmic sperm injection (ICSI) was performed after oocyte retrieval on day 0, and embryo transfer took place on day 3. Supernumerary, good-quality embryos (eight cells or more with <20% fragmentation) were assessed in the morning on day 4 and cryopreserved with a standard slow freezing protocol approximately 2 hours later. Embryos were exposed to increasing levels of the cryoprotectant dimethyl sulfoxide (ReagentPlus; Sigma-Aldrich) and were frozen in high-security straws (CBS; Cryobiosystems) in a controlled-rate freezer (Planer; Cryobiosystems). After that, the embryo straws were stored in nitrogen vapor tanks or in liquid nitrogen.

Because cryopreservation on day 4 can be seen as an unconventional approach outside of the Netherlands, we provide basic data on our clinical success. The numbers below refer to the year 2012, which was when we started our study. The average maternal age of our patients in 2012 was 35.4 years, and we cryopreserved embryos in 72.2% of all ovum pickups. The implantation rate of our frozen-thawed embryo transfers was 17.4% compared with the national average of 14.9%.

Culture Dish Preparation at VU University Medical Center Amsterdam

Standard culture dishes (Falcon Easy Grip; Becton Dickinson) were prepared according to routine laboratory procedures at

least 18 hours in advance to allow the culture medium to equilibrate. Standard dishes were filled with eight 25-μL microdrops of Sage blastocyst culture medium (Quinn's Advantage Protein Plus blastocyst medium; Cooper Surgical) and were covered with paraffin mineral oil (Repromed; IM Services). Microfluidic devices contained a culture chamber with a volume of 640 nL measuring 1,500 μm in diameter and were fabricated as described in the *Supplemental Material and Methods* and Supplemental Figure 1 (available online) and were prepared approximately 20 hours before embryo thawing.

First, the microfluidic systems were sterilized with 75% ethanol. Next, the devices were immersed in phosphate-buffered saline, and the channels were flushed thoroughly with phosphate-buffered saline before placing them in center-well organ culture dishes (Falcon; Becton Dickinson). The outer and inner rings of the center-well dishes were filled with medium to counter evaporation: IVF Basics HTF (Gynotec) with human albumin (Albuman; Sanquin). The channels then were flushed with Sage blastocyst medium, using the passive pumping technique described by Walker and Beebe (20). Six passive pumping cycles were needed (using 2-μL droplets of medium) to replace the culture medium in the device about three times. Subsequently, the devices were placed in the incubator to allow the medium to equilibrate overnight. The next morning, the channels were flushed again with pre-equilibrated Sage blastocyst medium. After 1 more hour of incubation, the devices were ready to use. The same incubator (NU-4950-E; NuAire) at 36.8°C, 5% CO_2, and atmospheric O_2 concentration was used for both culture groups.

Inclusion Criteria and Randomization

Donated human embryos that had been cryopreserved on day 4 were thawed according to routine laboratory protocol in decreasing levels of dimethyl sulfoxide. All embryos were graded morphologically after thawing by the experimenter. Only embryos of sufficient morphologic quality after thawing were included. Embryos had to meet the following inclusion criteria: a minimum of eight blastomeres, less than 20% fragmentation, and less than 25% degenerated cells. A randomization table was generated using Random Allocation Software version 1.0.0 (http://mahmoodsaghaei.tripod.com/Softwares/randalloc.html) before the start of the study. The availability of multiple embryos from one patient demanded randomization in blocks of four to make sure that embryos from one patient were randomized across the two study arms.

All embryos that fulfilled the inclusion criteria were randomly placed in culture medium droplets by the experimenter and allocated to the microdrop control or microfluidic group by a computerized randomization table using sealed envelopes. The embryos in the control group were placed in standard microdrop dishes overlaid with paraffin oil. For the microfluidic culture, embryos were introduced in the inlet reservoir of the microfluidic device with a conventional glass pipette and moved into the microchamber by creating a gentle flow in the channels by passive pumping (20). All embryos were cultured in a static environment without medium refreshment and were not retrieved from the microfluidic devices before disposal at the end of the 72-hour culture period.

Embryo Grading and End Points

Each embryo was graded morphologically at four different time points during culture: 24, 28, 48, and 72 hours after thawing. The number of blastomeres, the percentage of fragmentation, and the degree of degeneration were recorded. Blastocyst quality was scored based on the Gardner and Schoolcraft blastocyst grading system, which includes a detailed assessment of the expansion, inner cell mass, and trophectoderm of each blastocyst (21) with an adaptation using numeric scores for inner cell mass and trophectoderm instead of letters, as recommended by the Istanbul consensus meeting (22). In short, the degree of blastocyst expansion was graded from 1 to 6. Blastocysts with an expansion score from 3 to 6 also received numerical scores from 1 to 3 for both their inner cell mass and trophectoderm.

Our main study end point was the blastocyst formation rate in both the control dishes and microfluidic devices 28 hours after thawing. This moment was chosen because this is the time point when an embryo is usually transferred in a thawing cycle at our IVF center. The morphologic features of each embryo at the different time points were analyzed as secondary study parameters. Also the number of embryos that would have been suitable for embryo transfer 1 day after thawing according to standard laboratory criteria was recorded. Suitability for embryo transfer after overnight culture was determined by the following criteria: the presence of at least seven blastomeres and less than 50% degenerated cells.

For the purpose of comparing blastocyst quality between the two experimental groups, embryos were categorized as high-quality or low-quality blastocysts according to their expansion status, inner cell mass, and trophectoderm morphology (Table 1). The number of high-quality blastocysts, low-quality blastocysts, or no blastocysts was analyzed at the four different time points in the control dishes and microfluidic culture chambers.

Sample Size Calculation and Statistics

According to 2,500 frozen-thawed embryo transfers that were performed at our IVF center in past years, the blastocyst formation rate for human embryos 1 day after thawing was 60% in standard culture dishes. To detect a 25% increase in blastocyst formation rate, with a power of 83% at the 5% level of statistical significance, at least 50 embryos were needed in each experimental arm. Statistical analysis was conducted

TABLE 1

Blastocyst quality categories.

Blastocyst quality	Expansion	ICM/TE
High	3, 4, 5, 6	11, 12, 21, 22
Low	1, 2, 3, 4, 5, 6	23, 32, 33
No formation	Cleavage-stage embryos, morulas (>10 cells), and compaction-stage embryos	

Note: Blastocysts were assessed and categorized based on their expansion stage, inner cell mass (ICM), and trophectoderm (TE) quality using the Gardner and Schoolcraft (21) scoring system in combination with numbers instead of letters for ICM and TE, as recommended by the Istanbul consensus meeting (22).

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using IBM SPSS statistics version 20.0.0 and Microsoft Excel 2010. Data on the development of each embryo in the control and experimental conditions was recorded by experienced laboratory personal. Chi-square and Fisher's exact tests were used to analyze the data.

RESULTS

A total of 293 embryos were thawed for this study, and 41% of these embryos met our inclusion criteria. We included 120 embryos from 44 patients in the study, and randomized them in one of the experimental groups. We performed experiments on nine separate days and included an average of 13 embryos per day. Two embryos had to be excluded from the microfluidics group due to embryo loading issues. The problems were caused by an initially too narrow inlet opening, which was addressed by adjusting the inlet diameter. Therefore, 60 embryos were cultured in standard microdrop dishes (control) and 58 embryos in microfluidic devices.

We compared the distribution of morphologic scores in both the experimental groups (embryo baseline characteristics) directly after thawing and randomization. In the control group, 41.7% of all embryos were cleavage-stage embryos, 33.3% were compaction-stage embryos, and 25% were morulas. In the microfluidic group, 41.4% were cleavage-stage embryos, 39.7% were compaction-stage embryos, and 19% were morulas. The proportion of cleavage-stage embryos, compaction-stage embryos, and morulas was not statistically significantly different between the two groups before the beginning of the culture period (chi-square test, $P=.646$).

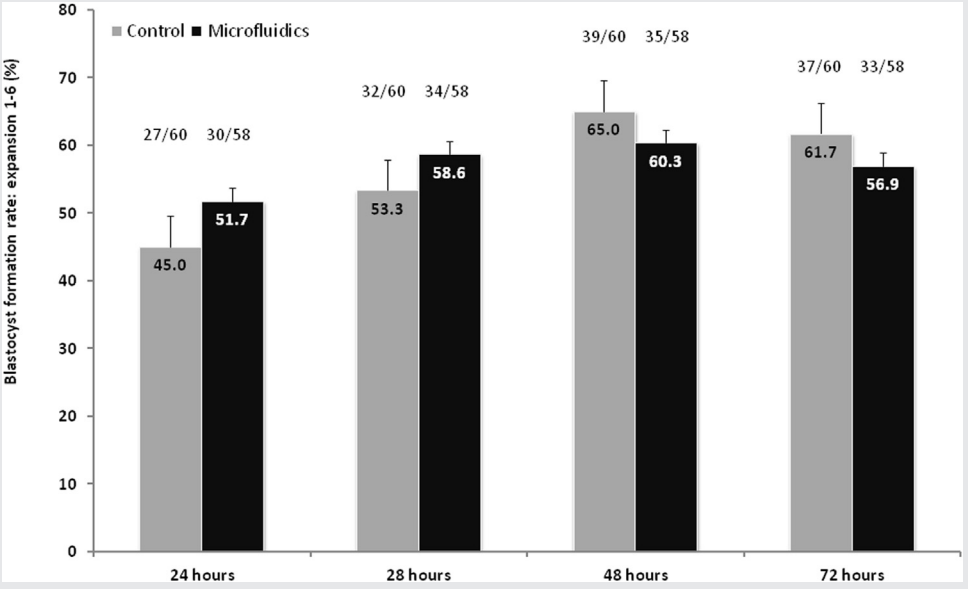
The blastocyst-formation rates for the two study groups are shown in Figure 1. The proportion of frozen-thawed day-4 embryos that developed to the blastocyst stage after 28 hours of culture (primary end point) did not statistically significantly differ in the microdrop dishes (control) and microfluidic devices (53.3% vs. 58.6%, respectively; Fisher's exact test, $P=.583$). The proportion of blastocysts after 24, 48, and 72 hours did not statistically significantly differ between the study groups either. We observed the highest blastocyst formation rate after 48 hours of culture as well as a small decrease after 72 hours at the end of the culture period in both groups.

The percentage of embryos that would have been suitable for embryo transfer did not statistically significantly differ after 28 hours of culture in the microdrop control dishes and the microfluidic devices (90.0% vs. 93.1%, respectively; Fisher's exact test, $P=.743$). These results are comparable to the mean proportion of embryo thawing procedures that resulted in an embryo transfer in our IVF center in the past 3 years (91.2%).

The analysis of the proportion of embryos that developed to an advanced blastocyst stage (expanded, hatching or fully hatched blastocysts) after 48 hours and 72 hours did also not reveal a statistically significantly difference between the standard microdrop dishes (control) and the microfluidic devices (48 hours: 46.7% vs. 48.3%, $P=1.000$; 72 hours: 56.7% vs. 55.2%, $P=1.000$).

Table 2 shows the percentage of high-quality and low-quality blastocysts as well as the embryos with no blastocyst formation after 24, 28, 48, and 72 hours of culture. Blastocyst quality was comparable in the microdrop control dishes and microfluidic culture chambers at all time points.

FIGURE 1



Blastocyst formation rate after 24, 28, 48, and 72 hours of culture in the standard microdrop dishes (control) or microfluidic devices. Values within the bars indicate the blastocyst formation rate; numbers above the bars display the fraction of all embryos that formed a blastocyst in each group. No statistically significant differences were found regarding the number of blastocysts in the control and microfluidic culture devices at any of the four time points.

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TABLE 2

Blastocyst quality after culture in standard microdrop dishes (control) and microfluidic devices at the four time points (24, 28, 48, and 72 h).

	24 h		28 h		48 h		72 h	
Blastocyst quality	**Control**	**Microfluidics**	**Control**	**Microfluidics**	**Control**	**Microfluidics**	**Control**	**Microfluidics**
High	18.3	25.9	28.3	34.5	43.3	48.3	51.7	50.0
Low	26.7	25.9	25.0	24.1	21.7	12.1	10.0	6.9
None	55.0	48.3	46.7	41.4	35.0	39.7	38.3	43.1
P value	.593		.794		.405		.822	

Note: Data are expressed as the percentage (%) of blastocysts with respect to the total number of embryos in each group. None = no blastocyst formation.

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DISCUSSION

This study demonstrates the successful in vitro development of donated frozen-thawed human embryos in microfluidic culture chambers with a volume of 640 nL. Embryo culture in static microfluidic devices resulted in blastocyst rates that were not statistically significantly different from the culture in microdrop control dishes. The proportion of embryos that would have been suitable for embryo transfer was comparable after 28 hours of culture in the microfluidic devices and control dishes. Also the proportion of expanded, hatching, and hatched blastocysts did not differ between the two culture devices after 48 and 72 hours of culture. The observation of a small decrease of the blastocyst rate in both groups after 72 hours of culture was most likely related to the extended culture period.

Morphologic assessments of blastocysts can be used to evaluate culture systems according to Matsuura et al. (23), who have demonstrated a positive correlation between blastocyst morphologic characteristics and cell numbers. We found that the quality of the developing blastocysts did not statistically significantly differ after culture in microfluidic devices and standard microdrop dishes.

The prototype of our microfluidic device was initially validated on mouse embryos. Esteves et al. (17) found that single and group culture of mouse embryos in these microfluidic devices yielded blastocyst rates above 90%. Furthermore, single mouse embryo culture in microfluidic devices resulted in viable full-term development, which is extraordinary because mouse embryos generally develop better in groups (24).

Several explanations for the inability of our study to demonstrate higher developmental rates for human embryos cultured in microfluidic devices compared with standard dishes are conceivable. One explanation could be that the design or material of the microfluidic device may not have been optimized enough to obtain advanced culture conditions.

The literature has highlighted the potential adverse effects of culturing cells in polydimethylsiloxane devices (25, 26). The absorption of components of the culture medium in the porous polydimethylsiloxane matrix can change solution concentrations (27). Furthermore, evaporation can alter medium osmolarity during incubation (26), which was prevented in our study by adding medium around the device in the center-well dishes. Regehr et al. (28) reported that uncured monomers in the polydimethylsiloxane porous matrix could diffuse in the microfluidic structures to contaminate the culture medium.

In contrast to the promising findings of the murine study, we experienced difficulties replicating these results with human embryos in our pilot experiments. In fact, we found poor developmental rates with the microfluidic device at first (data not shown). Eventually, we discovered the importance of a highly critical step during the preparation of the microfluidic systems. The devices used for the mouse experiments were flushed with culture medium again shortly before the introduction of embryos in the culture chambers. Adding this flushing step after overnight equilibration of the devices on the morning of embryo thawing significantly improved the development of the human embryos. As a consequence, we excluded the initial negative results of our pilot experiments from the analysis. To alleviate such issues in the future, a different material may be considered for the fabrication of the microfluidic devices.

Embryonic genome activation takes place in human embryos between the four- and eight-cell stages (29). Although many important steps in the development of an embryo have already occurred by day 4, our pilot experiments showed that these embryos are still highly responsive to different culture conditions. Cavitation and differentiation into inner cell mass and trophectoderm are crucial processes that only occur under favorable culture conditions, and our pilot experiments have indicated that a suboptimal culture environment can impair the development of day-4 embryos.

Two earlier studies compared human embryo quality after culture in microfluidic devices and control dishes. It is noteworthy that both studies started embryo culture at an earlier embryonic stage than we did in our current study. Alegretti et al. (16) transferred embryos in their IVF Chip on day 1, and Mizuno et al. (15) started the culture in microfluidic devices on day 2. In contrast with our results, these studies found superior embryo quality after culturing human embryos in microfluidic devices compared with their control dishes (15, 16). However, these studies were only published as conference papers and did not state whether the embryos were cultured in groups or individually. The exposure of embryos to a more optimal culture environment from an earlier developmental stage onward may have led to superior embryo quality in those studies. In contrast, we randomized more advanced human embryos that were frozen on day 4 of their development. Therefore, the

donated day-4 embryos may not have benefited as optimally from the culture in microfluidic culture chambers as earlier-stage embryos would have. However, the presented randomized controlled trial with day-4 embryos was a prerequisite to permit future research with earlier-stage human embryos.

In our laboratory, embryos are routinely cryopreserved on day 4 and may be donated to scientific research after the completion of the patient's IVF treatment. Therefore, both legally and ethically, these human day-4 embryos were the only available option to conduct a follow-up study of the research with the precursor of the microfluidic device on mouse embryos by Esteves et al. (17). Future studies will have to show whether earlier-stage human embryos will benefit from the culture in microfluidic devices more than the frozen-thawed day-4 embryos.

Aside from the use of mature embryos, several other limitations of the present study should be considered. Embryos were not retrieved from the microfluidic device at the end of the culture period, which would have allowed additional assessments of embryo quality. Furthermore, the use of mineral oil in the control dishes but not in the microfluidic devices represents a potential confounding factor; however, mineral oil has been shown to be toxic in some cases (30, 31) and therefore its exculsion could also be seen as a chance to reform human embryo culture. Further tests will need to establish whether microfluidic embryo culture without oil works in different types of incubators (17).

It would be interesting to study the effect of medium refreshment on the development of human embryos in the future. However, we decided to test the microfluidic devices in a static configuration first because we wanted to study the effect of a confined culture volume before assessing the influence of medium refreshment on human embryo development. Heo et al. (8) demonstrated that the developmental rate of mouse embryos cultured in a microfluidic device was proportional to the duration of dynamic culture but not the developmental stage. A microfluidic circuit, driven by a Braille pin actuation sequence that pumps fresh medium to a microfunnel, accelerated mouse embryo development to the blastocyst stage and increased pregnancy rates (8). While continuous but low flow rates impaired mouse embryo development (9), pulsatile delivery of medium has been shown to improve embryo development in mice (8). Esteves et al. (17) compared the development of groups of five mouse embryos cultured under static conditions with dynamic conditions in the precursor of our microfluidic device. A single event of medium refreshment in the microfluidic device significantly improved mouse embryo birth rates.

The refreshment of medium in microfluidic devices could offer several advantages for human embryo culture, such as the delivery of fresh nutrients to the embryos, the removal of waste products, and mechanical stimulation of the embryos (8, 32). Moreover, because conventional pipetting can be harmful to embryos due to shear stress (33), a drop in temperature, or even the loss of embryos (34), the replacement of pipetting with passive pumping (20) may be advantageous for embryo development. Medium refreshment in a microfluidic device can also be employed for the delivery of sequential culture media. Finally, microfluidic medium refreshment can be used for the exchange of cryopreservation solutions during the vitrification of embryos and oocytes (8, 35).

In the future, further work must be dedicated to improving the prototype microfluidic device tested in this first step. An automated, technician-friendly interface will be essential for medium refreshment as well as loading and retrieval of embryos out of the device (30, 31). The current design of the microfluidic platform must be adjusted and multiplexed for the parallel culture of multiple individual embryos in one culture dish. Furthermore, sensors could be integrated to conduct a range of measurements in the microenvironment of each embryo, such as oxygen and metabolite concentration, pH, reactive oxygen species, temperature, and medium composition (36–38). In this fashion, the new microfluidic platform may serve as an advanced tool for combined embryo culture and characterization by providing valuable data on embryo metabolism and development. Additionally, a combination with time-lapse imaging (7) would allow the parallel analysis of multiple culture parameters and morphologic data.

In conclusion, our study presents the first evidence that a microfluidic device supports the culture of human embryos in a nanoliter culture volume under static culture conditions. Further research is needed to determine whether an enhanced design, the use of earlier stage embryos, or medium refreshment in the microfluidic device can improve blastocyst formation rates or blastocyst quality. Embryo selection using multiple parameters recorded by built-in sensors will hopefully encourage single-embryo transfer policies on a larger scale in the future.

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REFERENCES

1. Da Rocha AM, Smith GD. Culture systems: fluid dynamic embryo culture systems (microfluidics). Methods Mol Biol 2012;912:355–65.
2. Fauci LJ, Dillon R. Biofluidmechanics of reproduction. Annu Rev Fluid Mech 2006;38:371–94.
3. Leese HJ, Tay JI, Reischl J, Downing SJ. Formation of fallopian tubal fluid: role of a neglected epithelium. Reproduction 2001;121:339–46.
4. Swain JE, Smith GD. Advances in embryo culture platforms: novel approaches to improve preimplantation embryo development through modifications of the microenvironment. Hum Reprod Update 2011;17:541–57.
5. Swain JE, Pool TB. Microfluidics in ART: time to go with the flow? IVF Online 2008;9:15–20.
6. Wheeler MB, Walters EM, Beebe DJ. Toward culture of single gametes: the development of microfluidic platforms for assisted reproduction. Theriogenology 2007;68(Suppl):S178–89.
7. Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat Biotechnol 2010;28:1115–21.
8. Heo YS, Cabrera LM, Bormann CL, Shah CT, Takayama S, Smith GD. Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates. Hum Reprod 2010;25:613–22.
9. Hickman DL, Beebe DJ, Rodriguez-Zas SL, Wheeler MB. Comparison of static and dynamic medium environments for culturing of pre-implantation mouse embryos. Comp Med 2002;52:122–6.

10. Raty S, Walters EM, Davis J, Zeringue H, Beebe DJ, Rodriguez-Zas SL, et al. Embryonic development in the mouse is enhanced via microchannel culture. Lab Chip 2004;4:186–90.
11. Melin J, Lee A, Foygel K, Leong DE, Quake SR, Yao MWM. In vitro embryo culture in defined, sub-microliter volumes. Dev Dyn 2009;238: 950–5.
12. Kim MS, Bae CY, Wee G, Han YM, Park JK. A microfluidic in vitro cultivation system for mechanical stimulation of bovine embryos. Electrophoresis 2009; 30:3276–82.
13. Han C, Zhang QF, Ma R, Xie L, Qiu TA, Wang L, et al. Integration of single oocyte trapping, in vitro fertilization and embryo culture in a microwell-structured microfluidic device. Lab Chip 2010;10:2848–54.
14. Ma R, Xie L, Han C, Su K, Qiu TA, Wang L, et al. In vitro fertilization on a single-oocyte positioning system integrated with motile sperm selection and early embryo development. Anal Chem 2011;83:2964–70.
15. Mizuno J, Ostrovidov S, Sakai Y, Fujii T, Nakamura H, Inui H. Human art on chip: improved human blastocyst development and quality with IVF-Chip. Fertil Steril 2007;88:S101.
16. Alegretti JR, Rocha AM, Barros BC, Serafini P, Motta ELA, Smith GD. Microfluidic dynamic embryo culture increases the production of top quality human embryos through reduction in embryo fragmentation. Fertil Steril 2011;96(Suppl):S58–9.
17. Esteves TC, van Rossem F, Nordhoff V, Schlatt S, Boiani M, Le Gac S. A microfluidic system supports single mouse embryo culture leading to full-term development. RSC Adv 2013;3:26451–8.
18. Harper J, Magli MC, Lundin K, Barratt CLR, Brison D. When and how should new technology be introduced into the IVF laboratory? Hum Reprod 2012; 27:303–13.
19. Vergouw CG, Kieslinger DC, Kostelijk EH, Botros LL, Schats R, Hompes PG, et al. Day 3 embryo selection by metabolomic profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: a randomized controlled trial. Hum Reprod 2012;27:2304–11.
20. Walker GM, Beebe DJ. A passive pumping method for microfluidic devices. Lab Chip 2002;2:131–4.
21. Gardner D, Schoolcraft W. In vitro culture of human blastocysts. In: Jansen R, Mortimer D, editors. Toward reproductive certainty: fertility and genetics beyond 1999. London: Parthenon; 1999:378–88.
22. Balaban B, Brison D, Calderon G, Catt J, Conaghan J, Cowan L, et al. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Reprod Biomed Online 2011;22:632–46.
23. Matsuura K, Hayashi N, Takiue C, Hirata R, Habara T, Naruse K. Blastocyst quality scoring based on morphologic grading correlates with cell number. Fertil Steril 2010;94:1135–7.
24. Lane M, Gardner DK. Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. Hum Reprod 1992;7: 558–62.
25. Berthier E, Young EWK, Beebe D. Engineers are from PDMS-land, biologists are from Polystyrenia. Lab Chip 2012;12:1224–37.
26. Heo YS, Cabrera LM, Song JW, Futai N, Tung YC, Smith GD, et al. Characterization and resolution of evaporation-mediated osmolality shifts that constrain microfluidic cell culture in poly(dimethylsiloxane) devices. Anal Chem 2007;79:1126–34.
27. Toepke MW, Beebe DJ. PDMS absorption of small molecules and consequences in microfluidic applications. Lab Chip 2006;6:1484–6.
28. Regehr KJ, Domenech M, Koepsel JT, Carver KC, Ellison-Zelski SJ, Murphy WL, et al. Biological implications of polydimethylsiloxane-based microfluidic cell culture. Lab Chip 2009;9:2132–9.
29. Braude P, Bolton V, Moore S. Human-gene expression first occurs between the four-cell and eight-cell stages of preimplantation development. Nature 1988;332:459–61.
30. Otsuki J, Nagai Y, Chiba K. Damage of embryo development caused by peroxidized mineral oil and its association with albumin in culture. Fertil Steril 2009;91:1745–9.
31. Morbeck DE, Khan Z, Barnidge DR, Walker DL. Washing mineral oil reduces contaminants and embryotoxicity. Fertil Steril 2010;94:2747–52.
32. Smith GD, Takayama S, Swain JE. Rethinking in vitro embryo culture: new developments in culture platforms and potential to improve assisted reproductive technologies. Biol Reprod 2012;86:62.
33. Xie Y, Wang F, Puscheck EE, Rappolee DA. Pipetting causes shear stress and elevation of phosphorylated stress-activated protein kinase/jun kinase in preimplantation embryos. Mol Reprod Dev 2007;74:1287–94.
34. Smith GD, Swain JE, Bormann CL. Microfluidics for gametes, embryos, and embryonic stem cells. Semin Reprod Med 2011;29:5–14.
35. Meng L, Huezo X, Stone BA, Baek K, Ringler G, Marrs RP. Development of a microfluidic device for automated vitrification human embryo. Fertil Steril 2011;96(Suppl):S207.
36. Heo YS, Cabrera LM, Bormann CL, Smith GD, Takayama S. Real time culture and analysis of embryo metabolism using a microfluidic device with deformation based actuation. Lab Chip 2012;12:2240–6.
37. Urbanski JP, Johnson MT, Craig DD, Potter DL, Gardner DK, Thorsen T. Noninvasive metabolic profiling using microfluidics for analysis of single preimplantation embryos. Anal Chem 2008;80:6500–7.
38. Wu CC, Saito T, Yasukawa T, Shiku H, Abe H, Hoshi H, et al. Microfluidic chip integrated with amperometric detector array for in situ estimating oxygen consumption characteristics of single bovine embryos. Sens Actuators B Chem 2007;125:680–7.

SUPPLEMENTAL MATERIALS AND METHODS

Microfluidic Device: Design and Fabrication

Our design of the microfluidic device for the culture of preimplantation mouse embryos (1) was adapted for the culture of human embryos. The microfluidic culture chamber is connected to inlet and outlet reservoirs via two 300-μm wide channels, as presented in Supplemental Figure 1. Diffusion-based delivery of nutrients from the reservoirs to the culture chamber occurs in this configuration. At the entry of the chamber, a 165-μm constriction prevents embryos from escaping the chamber during the culture. At the outlet of the chamber, a 180-μm width obstacle is placed in the channel to trap the embryos in the chambers (see Supplemental Fig. 1). Finally, the dimensions of the device have been adjusted to accommodate human embryos, which are larger in size than mouse embryos: the height of the structure is 360 μm, and the diameter of the chamber is 1,500 μm, to yield a final volume of 640 nL for the culture chamber.

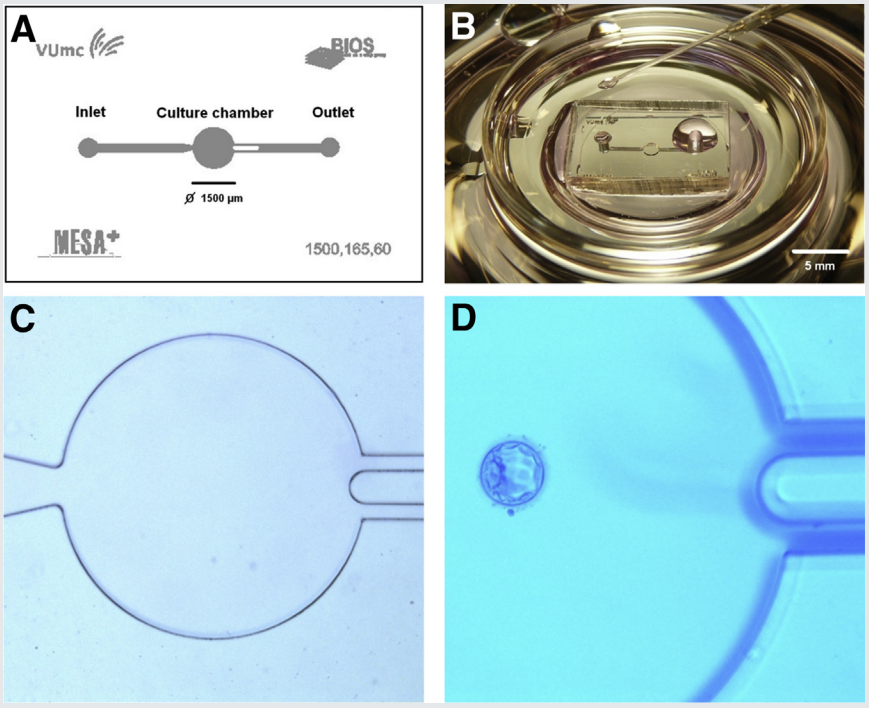
Polydimethylsiloxane (PDMS) is a frequently used material for the production of microfluidic culture devices because it is transparent, gas permeable, and biocompatible. Devices are fabricated from PDMS using a soft-lithography technique, as previously described elsewhere (2).

Briefly, an SU-8 on silicon mold is produced in a cleanroom using photolithography techniques based on a design previously drawn with Clewin (WieWeb software; Hengelo). The height of the SU-8 structures is 360 μm. Before use, the mold is coated with perfluorodecyltrichlorosilane (FDTS; ABCR GmbH, Germany) for easy removal of the PDMS layer from the mold after curing. A 10:1 mixture of prepolymer:curing agent (Sylgrad 184; Dow Corning GmBH), previously thoroughly degassed, is poured on the mold, and after a second degassing step to remove any remaining bubbles, the mold covered with PDMS is placed in the oven for curing overnight at 60°C. After curing, the PDMS layer is gently removed from the mold, cut into individual devices using a sharp knife, and reservoirs are punched manually using sharp needles. Punching PDMS creates perfectly vertical reservoirs for easy loading and possible removal of the embryos. Finally, glass cover slips and the resulting PDMS devices are activated using gas plasma treatment before they are assembled together to yield the microfluidic systems.

REFERENCES

1. Esteves TC, van Rossem F, Nordhoff V, Schlatt S, Boiani M, Le Gac S. A microfluidic system supports single mouse embryo culture leading to full-term development. RSC Adv 2013;3:26451–8.
2. Zhao XM, Xia YN, Whitesides GM. Soft lithographic methods for nanofabrication. J Mater Chem 1997;7:1069–74.

SUPPLEMENTAL FIGURE 1



Microfluidic device for the culture of human embryos. (**A**) Design of the device as drawn using Clewin software. (**B**) Introduction of an embryo in the microfluidic device by passive pumping. (**C**) Microscopy picture of the culture chamber showing the trapping structures implemented at the inlet and outlet of the culture chamber. (**D**) Blastocyst in a microfluidic culture chamber.

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