ORIGINAL PAPER

Prospective-randomized study comparing clinical outcomes of IVF treatments where embryos were cultured individually or in a microwell group culture dish

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Abstract

Culturing embryos together in a microdrop of media may improve embryo quality, based on the results of animal studies, however individual identification of the embryos in such a system is not possible. The microwell group culture dish contains 9 or 16 microwells with a minimal well-to-well distance and a specific well morphology that facilitates paracrine and autocrine effects. The microwell group culture dish enables individual identification of the embryos while providing the environment that comes with similar benefits as group culture. Our aim was to investigate whether embryo culture in the microwell group culture dish (Primo Vision Dish, Vitrolife) improves IVF outcomes compared to individual culture in human IVF treatment. Five hundred thirty-two IVF-ET cycles were enrolled in this prospective randomized study in a university hospital. IVF cycles were randomized into microwell group culture and individual culture groups. Primary outcome measure was clinical pregnancy rate and secondary outcome measures were embryo quality, fertilization, implantation, delivery and embryo utilization rates. Fertilization rate in ICSI cycles was significantly higher in the microwell group culture group (70.6% vs. 64.9%, $P = 0.001$). Clinical pregnancy rate was 50.8% in the group culture and 40.6% in the individual culture ($P = 0.022$). Live birth rate was 41.5% in microwell and 32.9% in individual culture ($P = 0.0496$). Embryo utilization rate was higher in microwell group culture than in individual culture (80.6% vs. 75.0%; $P < 0.001$). Microwell group culture has a beneficial effect on IVF outcome and it also allows following up individual embryo development.
ClinicalTrials.gov: NCT01774006.

Introduction

Embryos are cultured either in groups or individually during in vitro fertilization (IVF) treatment. In group culture, more embryos are cultured together in the same medium. Single or individual culture means that each embryo is cultured alone in a separate droplet of medium. A higher rate of murine (Lane and Gardner 1992; O'Neill 1997; Kelley and Gardner 2019), bovine (Ieda et al. 2018) and human (Ebner et al. 2010) embryos developed to blastocyst in group culture. The higher cell number was also observed following group culture in murine (Lane and Gardner 1992; O'Neill

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1997) and porcine (Stokes et al. 2005) blastocyst. Higher pregnancy rate was achieved following the embryo transfer of murine (Lane and Gardner 1992) or human (Ebner et al. 2010) embryos when they are cultured in groups. Moreover a higher rate of embryo utilization (Rebollar-Lazaro and Matson 2010) have been shown when embryos are cultured in groups rather than individually. On the other hand, some studies showed no clinical benefit of group culture compared to single culture (Spyropoulou et al. 1999). However, none of the studies showed any negative effect of group culture.

The main reason behind single embryo culture in human IVF is to provide a possibility for the individual embryo monitoring. The advantages of group culture may be the effect of the secretion of different substances on the neighboring embryos and on the embryo itself. Paracrine (the effects of one embryo on the other) and autocrine (the effects of the embryo on itself) effects may account for the improved laboratory outcome in group culture (Matsuura 2013).

The microwell culture allows individual identification in group culture and it has been used first for culturing zona-free embryos (Vajta et al. 2000, 2010). Later, the method became commonly used to culture mammalian embryos as well (Vajta et al. 2010). To harvest the alleged benefit of the paracrine effects of group culture, embryos/microwells need to be within a certain distance from each other (Stokes et al 2005; Gopichandran and Leese 2006). Well morphology is also an important feature and an optimally designed, narrow microwell may improve embryo quality (Matsuura 2013; Kelley and Gardner 2017) and blastocyst rates (Vajta et al. 2000, 2008; Sugimura et al. 2010) compared to single culture.

The aim of the present study was to compare the effect of culture conditions on human embryos in individual culture in a conventional culture dish with that of group culture in a microwell group culture dish. For this purpose, we compared the IVF outcome following embryo transfer between embryos cultured under the two conditions.

Materials and methods

Overall study design

This was a prospective, randomized, controlled trial with two arms, performed at the Division of Assisted Reproduction, Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Hungary.

The study compared two routinely used embryo culture practices in a university clinic setting (SE-NOI1-ARO-MF05/1-6). The trial was registered in the ClinicalTrials.gov database (NCT01774006).

We prospectively randomized patients to two groups: group culture in microwells (Primo Vision Dish 3 × 3 wells;

Vitrolife, Sweden) or individual culture [60 mm Falcon 3004 (BD, Erembodegem, Belgium)].

The primary end point was clinical pregnancy rate. The secondary endpoints were fertilization rate, embryo quality, implantation rate, live birth rate and embryo utilization rate.

Recruitment and randomization

Infertile couples undergo IVF-ET treatment were recruited. Altogether, 532 patients with ≥ 1 oocytes collected were enrolled in the study (264 cycles in the microwell group culture and 268 in the individual culture group).

Randomization was performed by the embryologist after oocyte collection using a computer generated randomization script. Patients and gynecologists performing the IVF treatment were blinded.

Ovarian stimulation

The gonadotropin-releasing hormone-agonist (GnRH) "long protocol" or multiple dose flexible GnRH-antagonist regimens were used for ovarian stimulation. Transvaginal ultrasound-guided aspiration of follicles was performed at 36 h after hCG administration.

IVF procedure and embryo culture

Laboratory procedures were performed by the same two experienced embryologist using standard protocol (Fancsovits et al. 2015). Oocyte and embryo culture was performed in a culture media product line called "G-series" produced by Vitrolife (Göteborg, Sweden).

Fertilization was performed using conventional IVF or intracytoplasmic sperm injection (ICSI) according to the SOP of the clinic (based on semen quality, patient history and cycle characteristics).

In case of conventional IVF oocytes were placed into the culture dishes after 16–18 h of co-incubation with 3×10^5 progressive motile sperm/ml of culture media.

When ICSI treatment was performed, oocytes were injected following enzymatic removal of cumulus cells and were placed into the relevant culture dish immediately after sperm injection.

Up to 12 embryos were cultured individually in the conventional culture dish and ≤ 9 embryos were cultured together in the microwell group culture dishes in 25 μl medium droplets (Fig. 1).

Embryo development and morphology were assessed 2 and 3 days after fertilization according to standard protocol (Alpha and ESHRE SIG Embryology 2011). Number of blastomeres, and amount of fragmentation was recorded on day 2 and day 3 of embryo culture. Multinucleated embryos (containing blastomeres with more than one nucleus) were

Fig. 1 Culture of embryos in **A** a microwell group culture dish (Primo Vision dish) and in **B** individual culture

A Microwell group culture Vision dish

B Individual culture in a conventional Petri dish
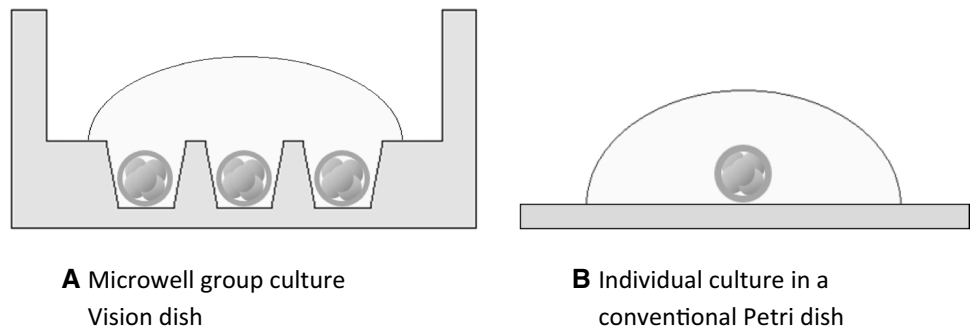
also recorded. The morphology score from 1 to 4 was given to the embryos regarding their quality.

Embryo transfer was performed on day 2 or on day 3 according to patient history and the number of embryos available. Embryo vitrification was performed on day 3 if further normally developed embryos were available.

Outcome measures

Primary endpoint was clinical pregnancy rate, secondary endpoints were fertilization rate, embryo quality, embryo utilization rate, implantation rate and live birth rate. The time needed for the assessment of embryo morphology was also measured.

IVF fertilization rate was calculated as the number of normally fertilized zygotes divided by the total number of oocytes. Fertilization rate following ICSI was determined according to the number of injected oocytes.

Embryo utilization rate was calculated as the number of embryos transferred and cryopreserved, divided by the number of normally fertilized oocytes.

Serum β-hCG levels was measured 11–13 days after ET. Clinical pregnancy was confirmed by the presence of a gestational sac with fetal heart activity 4 weeks after ET. Implantation rate was calculated as the number of gestational sacs with fetal heart activity divided by the number of embryos transferred.

Pregnancies were followed until delivery. The length of pregnancy, the number and birthweight of live newborns and male/female ratio were also recorded. Abortion rates were calculated as the number of clinical pregnancies, while live birth rates were expressed as the number of ETs.

Statistical analysis

Using a two-sided test and Pocock boundaries for estimation, a sample size of 240 patients in each group was calculated (for a total sample size of 480) to achieve a power of 80% at a significance level of 5%. Assuming a 10% dropout rate, a total sample size of 530 (265 in each arm) was needed for the randomized controlled clinical trial.

Student's t test was used to compare mean values, and the Chi^2 test was used to compare proportional values. The time needed for morphology assessment was compared using a paired t-test. Statistical significance was set at $P < 0.05$.

Results

A total of 532 IVF cycles were involved in the study. The number of cycles was 264 in the microwell culture group and 268 in the individual culture group. The cancellation rate (no embryo transfer due to the absence of fertilization or the elevated risk of ovarian hyperstimulation syndrome) did not differ significantly between the two groups (6.8% vs. 7.1%; $\chi^2(1) = 0.02$, $P = 0.902$). Patient characteristics were similar in the two groups (Table 1).

Semen quality (data not shown), IVF/ICSI ratio and number of Metaphase II oocytes in ICSI cycles were also similar between the two groups (Table 2). For the conventional IVF, the zygotes were placed into culture dishes following 18 h of co-incubation with sperm. Thus, fertilization occurred in the IVF dishes under the same conditions in both groups. On the other hand, for the ICSI cycles, the microinjected oocytes were placed directly into the culture dishes immediately after the sperm injection. Fertilization rates were comparable between the groups when conventional IVF was performed; however, ICSI fertilization rate was significantly higher in the group culture than in the individual culture group (Table 2).

Up to 9 embryos were cultured in the microwell group culture dishes, and the average number of embryos cultured together was 3.4, which resulted in a 7.4 µl/embryo density.

Embryo morphology and the number of good quality embryos were similar between groups; however, the number of blastomeres was significantly higher in the group culture than in the individual culture (Table 3). The number of embryos containing multinucleated blastomeres was slightly lower in group culture than in individual culture dishes.

Clinical pregnancy rate was significantly higher in the microwell culture group than in the individual culture group (50.8% vs 40.6%; $\chi^2(1) = 0.02$, $P = 0.022$); however,

Table 1 Cycle characteristics in the group culture and individual culture groups

	Microwell group culture		Individual culture		Statistics
No. of cycles	264		268		
No. of cycles with ET	246/264	93.2%	249/268	92.9%	$\chi2(1)=0.02, P=0.902$
Patient age[a]	35.7	±4.8	35.9	±4.7	$t(530)=0.40, P=0.689$
FSH (IU/l)[a]	8.0	±3.3	8.0	±3.8	$t(530)=0.22, P=0.823$
BMI (kg/m^2)[a]	23.0	±3.5	23.2	±4.1	$t(530)=0.52, P=0.602$
Cause of infertility					$\chi2(4)=0.49, P=0.241$
Male	123	46.6%	102	38.1%	
Tubal	40	15.2%	38	14.2%	
Other female	22	8.3%	32	11.9%	
Unexplained	67	25.4%	81	30.2%	
Combined (male and female)	12	4.5%	15	5,6%	
Type of stimulation					$\chi2(1)<0.01, P=0.995$
Short protocol	62	23.5%	63	23.5%	
Long protocol	202	76.5%	205	76.5%	
Length of stimulation (days)[a]	11.1	±1.1	11.1	±1.1	$t(530)=0.37, P=0.709$
Gonadotrophin dose (IU)[a]	2773	±1561	2774	±1520	$t(530)<0.01, P=0.995$
No. of oocytes collected[a]	8.7	±5.3	8.7	±5.6	$t(530)=0.04, P=0.966$

BMI Body mass index

[a]Numbers represent averages (±SD)

Table 2 Fertilization results in the group culture and individual culture groups

	Microwell group culture		Individual culture		Statistics
IVF/ICSI ratio	57/207	78.4%[c]	64/204	76.1%[c]	$\chi2(1)<0.40, P=0.529$
No. of Metaphase II oocytes (ICSI only)[a]	6.8	±4.4	6.8	±5.0	$t(409)=0.06, P=0.956$
No. of 2PN zygotes[a]	5.3	±3.9	5.0	±3.8	$t(530)=1.05, P=0.296$
Fertilization rate (IVF only)	421/610	69.0%	439/659	66.6%	$\chi2(1)<0.84, P=0.361$
Fertilization rate (ICSI only)[b]	988/1399	70.6%	898/1384	64.9%	$\chi2(1)<10.49, P=0.001$

[a]Numbers represent averages (±SD)

[b]ICSI fertilization rate was calculated according to the number of injected Metaphase II oocytes

[c]ICSI frequency

Table 3 Embryo morphology in the group culture and individual culture groups

	Microwell group culture		Individual culture		*P* value
Morphology on Day 2					
No. of blastomeres[a]	4.1	±1.4	4.0	±1.3	$t(2747)=2.07, P=0.038$
Morphology score[a]	2.2	±0.8	2.2	±0.8	$t(2747)=0.84, P=0.401$
Fragmentation %[a]	15.2	±9.9	14.8	±10.4	$t(2689)=1.00, P=0.313$
No. of good quality embryos	352/1409	25.0%	357/1337	26.7%	$\chi2(1)=1.06, P=0.304$
Morphology on Day 3					
Number of blastomeres[a]	7.0	±2.2	6.7	±2.3	$t(2476)=2.50, P=0.013$
Morphology score[a]	2.2	±0.7	2.2	±0.8	$t(2476)=0.63, P=0.528$
Fragmentation %[a]	15.3	±10.9	15.2	±11.5	$t(2432)=0.21, P=0.831$
No. of good quality embryos	292/1277	22.9%	280/1196	23.4%	$\chi2(1)=0.10, P=0.748$
No. of multinucleate embryos	160/1409	11.4%	186/1337	13.8%	$\chi2(1)=4.07, P=0.044$

[a]Numbers represent averages (±SD)

implantation rate did not differ significantly between the groups (30.6% vs. 27.2%; $\chi^2(1) = 1.54$, $P = 0.214$; Table 4).

Abortion rate was similar in the two groups (18.4% vs. 18.8%; $\chi^2(1) = 0.01$, $P = 0.937$). Live birth rate was 41.5% in the group culture and 32.9% in the individual culture groups. The difference was slightly significant ($\chi^2(1) = 3.86$, $P = 0.0496$). Length of pregnancy, birth weight of newborns and the male/female ratio did not differ significantly between the groups (Table 4).

Embryos suitable for cryopreservation were available in 44.3% of cycles in the microwell group culture and in 32.9% of cycles in the individual culture group ($\chi^2(1) = 6.76$, $P = 0.009$). Thus, embryo cryopreservation rate and embryo utilization rate were significantly higher in the group culture than in the individual culture (Table 5).

Embryo morphological evaluation needed less time when using the microwell group culture dish compared to a conventional Petri dish (19.2 ± 8.2 s/embryo vs. 23.8 ± 8.2 s/embryo; $t(141) = 8.60$, $P < 0.0001$). The average difference between the two groups was 31 s per IVF cycle.

Discussion

Culturing embryos individually in microdrop in a conventional, widely used Petri dish was compared to the group culture of embryos in a microwell group culture dish. The main outcome measure was clinical pregnancy, and further clinically important endpoints were included. Overall, we showed that microwell group culture dish resulted

Table 4 Pregnancy, implantation and delivery rates in the group culture and individual culture groups

	Microwell group culture		Individual culture		P value
No. of cycles with ET	246		249		
No. of ET on day 2	68/246	27.6	70/249	28.1	$\chi^2(1) = 0.01$, $P = 0.907$
No. of ET on day 3	178/246	72.4	179/249	71,9	$\chi^2(1) = 0.01$, $P = 0.907$
Number of embryos transferred [a]	2.24	± 0.77	2.21	$\pm 0,76$	$t(493) = 0.45$, $P = 0.651$
Positive β-hCG test	137/246	55.7%	116/249	46.6%	$\chi^2(1) = 4.11$, $P = 0.043$
Clinical pregnancy rate	125/246	50.8%	101/249	40.6%	$\chi^2(1) = 0.02$, $P = 0.022$
Multiple pregnancy rate	41/125	32.8%	48/101	47.5%	$\chi^2(1) = 5.07$, $P = 0.024$
Abortion rate	23/125	18.4%	19/101	18.8%	$\chi^2(1) = 0.01$, $P = 0.937$
Live birth rate	102/246	41.5%	82/249	32.9%	$\chi^2(1) = 3.86$, $P = 0.0496$
Length of pregnancy (weeks)[a]	37.6	± 2.6	37.2	± 3.0	$t(182) = 0.87$, $P = 0.387$
Number of newborns	137		114		
Male/female rate	74/63	54.0%	54/60	47.4%	$\chi^2(1) = 1.10$, $P = 0.294$
Birth weight (g)[a]	2815.4	± 720.3	2737.4	± 728.6	$t(249) = 0.85$, $P = 0.396$
Implantation rate	169/552	30.6%	150/551	27.2%	$\chi^2(1) = 1.54$, $P = 0.214$

Clinical pregnancy rate = No. of cycles with fetal heart activity at 6 weeks of gestation/No. of ETs

Multiple pregnancy rate = No. of multiple pregnancies/No. of clinical pregnancies

Abortion rate = No. of pregnancy losses (after the 6th week of gestation)/No. of clinical pregnancies

Live birth rate = No. of live births/No. of ETs

Implantation rate = No. of embryos transferred/No. of fetal sacs with heart activity

[a]Numbers represent averages ($\pm$SD)

Table 5 Cryopreservation and embryo utilization in the group culture and individual culture groups

	Microwell group culture		Individual culture		P value
No. of embryos cryopreserved[a,b]	4.9	± 3.0	5.2	± 3.2	$t(203) = 0.56$, $P = 0.573$
No. of cycles with cryopreservation	109/246	44.3%	82/249	32.9%	$\chi^2(1) = 6.76$, $P = 0.009$
Cryopreservation rate	584/1409	41.4%	452/1337	33.8%	$\chi^2(1) = 17.05$, $P \leq 0.001$
Embryo utilization rate	1136/1409	80.6%	1003/1337	75.0%	$\chi^2(1) = 12.52$, $P \leq 0.001$

Cryopreservation rate = No. of embryos cryopreserved/No. of normally fertilized oocytes

Embryo utilization rate = No. of embryos transferred or cryopreserved/No. of normally fertilized oocytes

[a]Numbers represent averages ($\pm$SD)

[b]Cycles with cryopreservation only

in multiple benefits that were resulted in increased embryo utilization rates as well as increased clinical pregnancy and live birth rates.

The first report on the beneficial effects of group culture in human IVF treatment was published by Moessner and Dodson (Moessner and Dodson 1995). Embryos in group culture may facilitate their own development by producing embryotropins that act as communication messengers. Secretion can be an active procedure, a passive outflow or act via messengers. Inter-embryo communication is regulated by a wide range of biochemical messengers, including growth factors, proteins, lipids, neurotransmitters, saccharides, and microRNAs, all of which can be exchanged among embryos when cultured together in group culture. Different types of messenger molecules influence embryo development by triggering different pathways. Embryotropins, such as insulin-like growth factor-I (IGF-I) or platelet activating factor (PAF), or other molecules, including lipophilic autocrine factors such as prostaglandins or fatty acids, activate different pathways that are important for embryo survival or growth or have anti-apoptotic functions (reviewed by O'Neill 2008; Wydooghe et al. 2015).

According to earlier studies, embryo-to-embryo distance is an important factor affecting the alleged beneficial effects of embryo cross-talk in group culture. It has been shown that distance between embryos cultured together should be < 250 μm (Stokes et al. 2005; Gopichandran and Leese 2006), and embryos outside this distance may not experience any potential effects of group culture. This finding suggests an effective zone of embryotrophic ligands that accumulate around the embryos and may act in autocrine and paracrine manners in group culture (Reed et al. 2011).

In an artificial environment, inter- and intra- embryo communication can be facilitated by modifying culture conditions, eg. introducing microwells with a morphology purposely designed to facilitate the autocrine effect. The volume of the 9-well petridish is 0.11 μl which may result the accumulation of autocrine factors around the embryo (Kelley and Gardner 2017). A study specifically targeting microwell morphology concluded that the concentration of secreted small molecules and macromolecules was two- to threefold higher in a narrow microwell than on a flat surface. The author concluded that the microwell culture system is better than conventional group culture because of the increased final concentration of autocrine factors (Matsuura 2013).

The successful application of narrow and deep microwells that are located in a matrix close to each other has been previously published (Vajta et al. 2000, 2010; Pribenszky 2010a, b) The microwell group culture dish (Fig. 1) allows the individual follow up of embryo development, while narrow and deep (310 μm) wells holding the embryos within 80 μm from each other. This enabled the collection of morphological data from each embryo

cultured together in the same dish while obtaining the alleged benefits of group culture due to paracrine and autocrine effects.

An important factor to consider during group culture is embryo density, which is defined as the embryo-to-volume ratio. Generally, 3–15 μl of medium is used per embryo in a group culture of human embryos (Ebner et al. 2010; Rebollar-Lazaro and Matson 2010; Spyropoulou et al. 1999; Moessner and Dodson 1995; Lehner et al. 2017); however, beneficial effects have been reported using either extremely low (< 0.5 μl/embryo) or high (40 μl/embryo) embryo densities in animal studies (Reed et al. 2011). The embryos in our study were cultured in 25 μl of culture medium both in individual and in group culture, results in densities of between 2.8 μl and 25 μl per embryo. This density can supply the nutritional and buffer needs during early embryo development (Gardner et al. 2002).

The fertilization method used may affect the outcome of IVF treatment, however, the fertilization rates following IVF and ICSI treatment were similar in the present study. Overall fertilization rate during the study period achieved the competence values (IVF: 60%, ICSI: 65%) (Alpha and ESHRE SIG Embryology 2011).

We observed a significantly higher fertilization rate in ICSI cycles, while fertilization rate in conventional IVF treatments were the same. The difference in the two fertilization method regarding culture system is that all biological processes following sperm penetration occurred in the respective culture dishes in case of ICSI treatment. We hypothesize that the important physiological procedures after fertilization is enhanced by the fact that zygotes are in a microwell compared to keeping them in a regular Petri dish.

The higher cell number of embryos in group culture refers to a faster embryo development, which can indicate better embryo viability and higher implantation potential. This finding is consistent with the earlier observation of Moessner and Dodson (1995); however, other publications did not confirm these results (Ebner et al. 2010; Spyropoulou et al. 1999). The positive effect of group culture is not evident in terms of embryo morphology during the first 2–3 days of culture (Ebner et al. 2010; Spyropoulou et al. 1999; Vajta et al. 2008). This was confirmed by our study because embryo morphology was similar between the groups. The presence of multinucleation is associated with a lower implantation potential. In our study the number of multinucleate embryos was also lower in microwell culture group.

Previous reports comparing pregnancy and implantation rates in individual and group culture reported different findings. (Rebollar-Lazaro and Matson 2010; Spyropoulou et al. 1999; Ebner et al. 2010; Vajta et al. 2008). The ten percent higher clinical pregnancy rate found in our study indicates that culture in microwell group culture dishes may result in more viable embryos.

The other major advantage of microwell group culture in our study was the higher number of embryos that were available for cryopreservation. Embryo morphology did not differ between the two groups, but the higher cell number and the lower rate of multinucleation might affect the suitability of the embryos for cryopreservation. The number of transferred embryos was similar between the two groups, but more cycles occurred with cryopreservation; thus, the embryo utilization rate was also significantly higher when a microwell group culture dish was used. This finding confirms previous reports, where authors found more blastocysts for cryopreservation and transfer in case of group culture (Rebollar-Lazaro and Matson 2010; Vajta et al. 2008).

The morphological evaluation of embryos in the conventional culture system is time consuming because the distance between microdrops is larger than the field of view of the microscope. In the microwell group culture dish, all of the embryos can be fitted into the same microscope field of view. This can reduce the time needed for embryo evaluation by 30 s on average per patient. Faster evaluation may result in lower fluctuations of temperature and pH during morphological assessment.

Conclusions for future biology

The present study has shown that the use of a microwell group culture dish has a beneficial effect on embryo development and viability in human IVF treatment compared to the conventional individual microdrop culture. The microwell group culture system resulted in higher clinical pregnancy rate and a tendency of higher live birth rate as well. Furthermore, group culture resulted in higher fertilization rate in ICSI cycles and faster embryo development, a higher embryo utilization rate and also reduced the time needed for embryo evaluation compared to individual embryo culture.

Author contributions PF: Study design, execution, data analysis, manuscript drafting, critical discussion. CP: Study design, manuscript drafting, critical discussion. AL: Study design, execution, data analysis. AM: Execution, manuscript drafting, critical discussion. ZK: Data analysis, manuscript drafting. AN: Manuscript drafting, critical discussion. JU: Study design, execution, critical discussion.

Declarations

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