Clinical Application of a Microwell System to In Vitro Culture of Human Preimplantation Embryos

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Abstract: *During in vitro culture, embryo density in culture medium affects the developmental competence of the embryo. However, in many cases, it is necessary to culture human embryos individually or in small groups. A microwell provides a single embryo with a microenvironment suitable for its development (diameter, 500 μm; depth, 300 μm; and volume, 0.04 μl). In the present study, the clinical results of a microwell system were compared with those of a microdroplet system, and the clinical validity of the microwell system was considered for routine clinical use. In the microwell system, the percentage of embryos that reached the blastocyst stage and the subsequent clinical pregnancy rate (61.7% and 41.4%, respectively) were slightly higher than those in the microdroplet system (51.8% and 34.5%, respectively). Moreover, the microwell system was suitable for routine clinical use with respect to safety, convenience and commercial availability. In conclusion, the microwell system not only slightly enhanced the developmental competence of the individual embryos and the subsequent clinical pregnancy rate, but also made it possible to follow the course of development of an individual embryo. It may therefore be beneficial for conducting elective single embryo transfer to avoid the risks of multiple pregnancy.*

Key words: *Microwell, Microenvironment, Individualization, Human embryo, In vitro culture*

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Introduction

In vitro culture (IVC) of human embryos is affected by various factors such as co-culture with somatic cells [1], growth factors/cytokines [2, 3], and oxidative stress [4]. With the introduction of sequential media and improvements based on the above factors, it is possible to culture human embryos up to the blastocyst stage. However, the percentage of human embryos developing to the blastocyst stage *in vitro* is still low.

Embryo density during IVC is another important factor [5, 6]. In a group culture, interaction among embryos through paracrine action of embryonic factors may promote embryo development. However, in many cases, it is necessary to culture human embryos individually and in small groups, therefore the beneficial effects of group culture may be absent.

The well of the well (WOW) system, a type of microwell system, was developed by Vajta *et al.* for culturing embryos individually or in small groups, and the culturing of biopsied, nuclear-transferred or/and zona-free embryos [7]. In human [8], bovine [7] and porcine [9] embryo cultures, a suitable microenvironment created in microwells increased the autocrine/paracrine action of the embryos and enhanced their development to the blastocyst stage. Moreover, microwell systems such as WOW allow identification of oocyte/embryo origin [10] and also allow sequential assessment of individual oocytes/embryos. These features of the microwell system have potential in the selection of one embryo in elective single embryo transfer for avoiding risks of multiple pregnancy.

The purpose of the present study was to investigate

Table 1. Patient and retrieval cycle characteristics

	Microwell	Microdroplet
No. of patients	29	29
No. of treatment cycles	29	29
Mean (± SD) age (year)	35.9 ± 4.3	36.1 ± 3.7
Mean (± SD) previous ART* cycles (n.)	2.0 ± 2.1	2.4 ± 3.2
Insemination technique IVF/ICSI cases (cycle)	15/14	14/15
Ovarian stimulation antagonist/agonist cases (cycle)	25/4	24/5

No significant differences in any variable were noted between the two groups. *advanced reproductive technology.

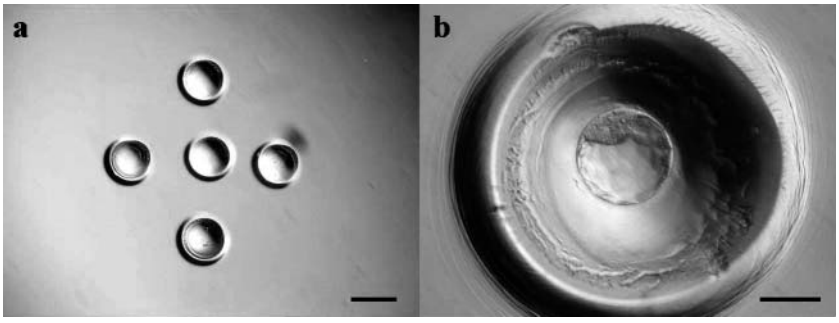

Fig. 1. (a) Microwells were prepared in the bottom of the culture dish. Scale bar = 500 μm. (b) An expanded blastocyst in a microwell. Scale bar = 100 μm.

the effect of a microwell system on the developmental competence of human embryos and the subsequent clinical pregnancy rate. We also evaluated its validity (safety, convenience, commercial availability and features) with regard to routine use in human infertility clinics.

Materials and Methods

Patients

In 58 patients, 58 cycles were examined retrospectively from December 2006 to April 2007. A microwell system and a microdroplet system were each used in 29 cycles respectively. There were no significant differences between the two systems with respect to mean age and previous advanced reproductive technology cycles of the patients (Table 1). IVF cycle and ICSI cycle were treated as a single group, because there was no significant difference between the two cycles in embryo development and clinical pregnancy rate in our clinical results (data not shown). All patients gave their informed consent to participate in the study.

Preparation of zygotes

The patients were treated with gonadotrophin-releasing hormone (GnRH) antagonist or agonist protocol. A dose of 5,000 IU human chorionic gonadotrophin (hCG) was administered when two or more follicles reached 18 mm in diameter. Oocytes were collected transvaginally after approximately 36 h after hCG injection, and then inseminated by conventional-IVF or piezo-ICSI [11]. After 16–18 h of insemination, oocytes extruding the second polar body and forming two pronuclei were considered to be normally fertilized. The zygotes (hereafter referred to as embryos) thus formed were used in the present study.

Preparation of microwells and in vitro culture

Microwells were prepared using an aggregation needle (DN-10; BLS®, Budapest, Hungary) [12]. The sterilized needle was pressed into the bottom of the culture dish (1007, Falcon®), while making a circular movement with the free end of the needle we were holding. This movement created a tiny scoop of about 500 μm in diameter and 300 μm in depth with a clear, smooth wall. A 50-μl droplet of the culture medium was coated on the microwells, and then overlaid with mineral oil (Fig. 1a, b).

For IVC, two different culture systems were designed: one was 1–5 microwells in a 50-μl droplet (microwell

Table 2. Effect of the in vitro culture systems on embryo development and clinical pregnancy rate

	Microwell	Microdroplet
No. of fertilized oocytes	138	144
No. (%) of cleaved oocytes	135 (97.8)	143 (99.3)
No. (%) of good quality embryos	66 (48.9)	79 (55.2)
No. of sequential cultured oocytes until Day 5	81	85
No. (%) of embryos that reached morula + blastocyst stage	56 (69.1)	49 (57.6)
No. (%) of embryos that reached blastocyst stage	50 (61.7)	44 (51.8)
No. of embryo transferred cycles	29	29
Mean (± SD) embryos per transfer (n.)	1.9 ± 0.5	2.0 ± 0.6
No. (%) of implanted embryos	15 (27.8)	15 (26.3)
No. (%) of clinical pregnancies per embryo transfer	12 (41.4)	10 (34.5)

No significant differences in any variable were noted between the two groups.

Table 3. Favorable characteristics of the microwell system for routine clinical use

Formation	Diameter = ~ 500 μm
	Depth = ~ 300 μm
	Volume = ~ 0.04 μl
Safety	Equal to the conventional microdroplet system
Convenience	Does not need any specialized technique
Commercial availability	Possible to use current culture dish
Features	Individualization of oocytes/embryos and sequential evaluation for elective single embryo transfer

system) and the other was a conventional 50-μl droplet with flat-bottom (microdroplet system). In the microwell system, a single embryo was placed in each microwell. The same numbers of embryos (1–5 per drop) were placed in the microdroplet system. The culture medium used was potassium simplex optimized medium (KSOM) with amino acids (Global®; LifeGlobal) [13] containing 5 mg/ml human serum albumin (Irvine Scientific). Embryos were incubated for a maximum of 5 days (Day 0 was the day of insemination) at 37°C in 5% O_2, 5% CO_2, 90% N_2, and maximum humidity. Cleavage rate and morphological evaluation were observed on Day 3. A good quality embryo was defined as an embryo with more than seven even-sized blastomeres and less than 15% fragmentation. Development to the morula and blastocyst stages was recorded on Day 5.

Embryo transfer, luteal support, and assessment of pregnancy

One to three embryos were transferred into the patient's uterus on Day 3 or 5. After embryo transfer, a progesterone vaginal suppository was administered to provide luteal support. Clinical pregnancy and implantation were observed by ultrasonic detection of the gestational sac in the uterine cavity.

Statistical analysis

Data from each culture system were analyzed by the χ^2 test and Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results

In order to develop a better culture system for human embryos, we compared the efficiencies of microwell and microdroplet systems. Data on embryo development and clinical pregnancy rate for the two systems are shown in Table 2. There were no significant differences between the microwell and microdroplet systems for the rate of cleaved oocytes, morphologically good quality embryos and implanted embryos. In the microwell system, however, the percentage of embryos that reached the blastocyst stage and the subsequent clinical pregnancy rate (61.7% and 41.4%, respectively) were slightly higher than those for the microdroplet system (51.8% and 34.5%, respectively). The results of the present study do not provide firm evidence regarding the enhancement of the developmental competence of human preimplantation embryos in the microwell system, because the sample size was small, only 58 cycles. However, for routine clinical use, the microwell system is convenient in practice (Table 3), its safety is equal to that of the conventional microdroplet

system, it does not need any specialized techniques, and the current type of culture dish can be used.

Discussion

In humans, individual embryos or small groups have been used in IVC. Reduction of embryo density in culture medium is avoided in order to prevent a decline in the developmental competence, because the paracrine action of various growth factors/cytokines is believed to promote embryo development. Such embryonic factors have been detected in human preimplantation embryos [14]. In the present study, we used microwells having the following approximate dimensions: diameter, 500 μm; depth, 300 μm; and volume, 0.04 μl. The microenvironment created by the microwell should allow a suitable concentration of growth factors/cytokines, allowing increase in the autocrine action of embryos and the paracrine action of blastomeres. The accumulation of toxic substances such as ammonia [15] and oxygen-derived free radicals [16] in the microenvironment lead to late developmental anomalies. However, the open condition of the microwell system may dilute these metabolized toxic substances. In this clinical study, we found that the rates of development to morula + blastocyst and blastocyst (69.1% and 61.7%, respectively) in the microwell system were slightly higher than those in the microdroplet system (57.6% and 51.8%, respectively). However, in the microwell system, the percentage of morphologically good embryos (48.9%) on Day 3 was slightly lower than that in the microdroplet system (55.2%) (Table 2). Taka *et al.* [9] considered that some growth factors/cytokines secreted at the morula and blastocyst stages might accelerate blastocyst formation in porcine embryo cultures. Similarly, in the present clinical study, stage-specific growth factors/cytokines might have enhanced the development to the morula and blastocyst stages.

Morphological evaluation at different developmental stages is a powerful tool for predicting the developmental and implantation competence of the embryos, e.g., morphology of the first polar body at metaphase II [17], the Z-score at the pronuclear stage [18], and the scoring systems of Veeck *et al.* [19] and Gardner *et al.* [20]. By collating such morphological information about the oocyte/embryo, we might be able to predict its developmental fate more accurately. This would require sequential morphological observations of individual oocyte/embryos at different stages, and the microwell system may be helpful for this purpose.

In conclusion, the microwell system slightly improved the rate of development of embryos to the blastocyst stage and the subsequent clinical pregnancy rate, and it is convenient for routine clinical use. The microwell system might be valuable in elective single embryo transfer for reducing the risk of multiple pregnancy.

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