Effect of micro-vibration culture system on embryo development

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Received: 10 March 2013 / Accepted: 26 April 2013 / Published online: 9 May 2013
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Abstract

Purpose Micro-vibration culture system was examined to determine the effects on mouse and human embryo development and possible improvement of clinical outcomes in poor responders.

Materials and methods The embryonic development rates and cell numbers of blastocysts were compared between a static culture group ($n=178$) and a micro-vibration culture group ($n=181$) in mice. The embryonic development rates and clinical results were compared between a static culture group ($n=159$ cycles) and a micro-vibration culture group ($n=166$ cycles) in poor responders. A micro-vibrator was set at a frequency of 42 Hz, 5 s/60 min duration for mouse and human embryo development.

Results The embryonic development rate was significantly improved in the micro-vibration culture group in mice ($p<0.05$). The cell numbers of mouse blastocysts were significantly higher in the micro-vibration group than in the static culture group ($p<0.05$). In the poor responders, the rate of high grade embryos was not significantly improved in the micro-vibration culture group on day 3. However, the optimal embryonic development rate on day 5 was improved in the micro-vibration group, and the total pregnancy rate and implantation rate were significantly higher in the micro-vibration group than in the static culture group ($p<0.05$).

Conclusions Micro-vibration culture methods have a beneficial effect on embryonic development in mouse embryos.

Capsule Micro-vibration culture system can improve mouse embryo development rate and clinical outcomes in poor responders.

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In poor responders, the embryo development rate was improved to a limited extent under the micro-vibration culture conditions, but the clinical results were significantly improved.

Keywords Embryo culture · Micro-vibration · Static culture · Dynamic culture

Introduction

A major consideration in assisted reproductive technology (ART) is providing an environment that is similar to the internal body conditions during germ cell development *in vitro*. Technologies have been improved for maintaining the temperature and the appropriate *in vivo*-like pH range [1]. Moreover, for several decades, researchers have been developing technologies to make culture media and additives that can facilitate germ cell development [2–6].

Nevertheless, despite efforts to improve the quality of ART, one of the unresolved issues regarding current embryonic culture conditions is the static culture system. Fertility clinics worldwide culture embryos in the static state. Before implantation, the oocytes and zygotes within the oviduct and uterus are constantly moving and being stimulated by the surrounding environment during natural pregnancy [7–9]. Moving and stimulating sources that influence embryonic development include shear stress by tubal fluid flow, compression by peristaltic tubal wall movement, buoyancy and kinetic friction forces between the embryo and cilia [10, 11]. The combination of these *in vivo* mechanical forces leads to an average ovum velocity of 0.1 $\mu\text{m/s}$ and a maximum velocity of up to 8.6 $\mu\text{m/s}$ [12–15]. These *in vivo* mechanical forces are believed to induce cell-to-cell communication, exert beneficial effects by refreshing the fluid surrounding the embryo and eliminate metabolites emitted from embryos [16–19]. Studies to achieve higher quality embryos by external stimuli using machines (i.e., tilting machines and micro-vibrators) to replicate *in vivo* mechanical forces have been recently reported [20, 21]. Embryonic culture using a micro-vibrator as an external stimulus has been actively investigated [22]. The present study investigated the effects of a micro-vibrator on embryo development.

Poor responders who require large doses of stimulant medications and who produce less than an optimal number of oocytes remain a significant challenge in ART [23]. Poor responders exhibit advanced age, previous ovarian surgery or pelvic adhesions [24, 25], all of which can contribute to a reduced oocyte and embryo quality in comparison with normal responders. To improve the pregnancy rate in poor responders, various approaches have been attempted, but a satisfactory solution has not been reached [26, 27]. However,

most of the approaches have focused on specific aspects by refining induction protocols in poor responders to increase the number of collected oocytes and the quality of the embryos [28, 29]. The purpose of the present study was to improve embryo quality in poor responders using a unique culture method.

The aim of this investigation was to evaluate the effect of micro-vibration on mouse and human embryonic development and the effect of micro-vibration on the clinical results in poor responders.

Methods

Mouse embryonic culture and Hoechst staining

All animal care and use procedures were approved by the Institutional Animal Care Committee of Kangwon National University.

The protocol for handling the mouse embryos followed previous laboratory methods [30]. Briefly, 6-week-old B6D2 F1 mice were superovulated with pregnant mare

Fig. 1 a Appearance of the micro-vibrator installed in the incubator. b 3-dimensional movement of the micro-vibrator (according to the product specifications provided by Nepa Gene)

Fig. 2 Comparison of mouse embryo quality between the static culture group and the micro-vibration culture group on day 3 (**a**, **b**) and day 5 (**c**, **d**) and a comparison of the cell numbers between the static culture group and the micro-vibration culture group (**e**, **f**) **a** Static cultured embryos on day 3, magnification $\times 200$ **b** Micro-vibration cultured embryos on day 3, magnification $\times 200$ **c** Static cultured embryos on day 5, magnification $\times 200$ **d** Micro-vibration cultured embryos on day 5, magnification $\times 200$ **e** Stained blastocyst from the static culture, magnification $\times 400$ **f** Stained blastocyst from the micro-vibration culture, magnification $\times 400$

serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG). Twenty hours after hCG injection, the female mice were sacrificed, and single-cell zygotes were collected and cultured into mouse tubal fluid (MTF) +4 mg/mL human serum albumin medium at 37 °C in an atmosphere of 5 % CO₂. Each 50 μ L MTF droplet contained 5 mouse embryos and was cultured as a static culture group ($n=178$) and a micro-vibration culture group ($n=181$).

Observation of mouse embryonic development was performed on days 3 (at 70 h post hCG) and 5 (at 120 h post

hCG). On day 3, the embryos were evaluated for their quality and cell stages. On day 5, the blastocyst was graded as a hatching blastocyst, expanded blastocyst, intermediate expanded blastocyst, early blastocyst or pre-morula stage.

After observation on day 5, the blastocysts were stained with Hoechst 33342 (Sigma-Aldrich, B2261) to determine the effect of micro-vibration stimuli on mouse embryonic development. Mouse blastocysts were washed in phosphate buffered saline (PBS), stained for 15 min with 0.5 μ g/mL Hoechst 33342 and subsequently washed 3 times in PBS (0.1 % polyvinyl alcohol). The blastocysts were fixed by Permunt solution (Vector Laboratories, Burlingame, USA) and examined under a fluorescence microscope (Olympus BX51-FL, Japan) [31, 32].

Ovulation induction and embryonic culture in poor responders

This study was approved by the Maria Fertility Hospital Institutional Review Board. Certain protocols for various poor responders are routinely utilized to maximize the numbers of eggs in this hospital. Some patients were administered a GnRH agonist on the second or third day of the menstrual cycle, but its administration lasted for 3 days, and a GnRH antagonist was then administered to prevent premature ovulation. Some patients were administered only a low dose of the GnRH agonist. Some patients were administered the GnRH agonist during the luteal phase, but the agonist administration was discontinued when the menstrual cycle started. The other patients followed a modified natural cycle. The poor responders were randomly distributed into a static culture group or a micro-vibration culture group during the same period (July to December 2012).

Fertilization was assessed 15–18 h after insemination based on the presence of 2 pronuclei. The zygotes were washed and cultured in groups of less than 5 in a 50 μ L micro-droplet (Sydney IVF Cleavage Medium-CM; COOK, Brisbane, Australia) for 48 h, and the embryos were subsequently selected for transfer. Some surplus embryos were culture in a 50 μ L micro-droplet (Sydney IVF Blastocyst

Table 1 Comparison of the optimal development rate on day 3 and day 5 between the static culture group and the micro-vibration culture group in the mouse embryonic culture

Cell stages	Day 3				Day 5				
	8-7 cell	6-5 cell	4 cell	< 3 cell	> HB	EdB	MeB	ErB	< Mor.
Static culture Group (%) ($n=178$)	21 (11.8)	27 (15.2)	114 (64.0)	16 (9.0)	32 (18.0)	70 (39.3)	32 (18.0)	10 (5.6)	34 (19.1)
Micro-vibration culture group (%) ($n=181$)	48* (26.5)	43 (23.8)	73 (40.3)	17 (9.4)	67* (37.0)	65 (35.9)	17 (9.4)	13 (7.2)	19 (10.5)

HB hatching blastocysts (including hatched blastocysts); EdB expanded blastocysts; MeB intermediate expanded blastocysts; ErB early blastocysts; * $p<0.05$

Fig. 3 Comparison of the average cell numbers of mouse blastocysts between the static culture group and the micro-vibration culture group. Error bars represent the standard error of the mean

Medium-BM; COOK, Brisbane, Australia) for blastocyst development, and the others were cryopreserved or discarded. All culturing of the embryos was performed in a $\text{CO}_2\text{:O}_2\text{:N}_2$ (6 %:5 %:89 %) environment.

Micro-vibration

In the present study, a micro-vibrator (NSSB-300, Nepa Gene, Ichikawa, Chiba, Japan) was set at a frequency of 42 Hz, 5 s/60 min duration for mouse and human embryo development based on the previous reports [21, 22, 33]. Micro-vibration was applied to the single-cell zygote stage in the mouse. Moreover, in the poor responders, micro-vibration was applied to the pronuclear zygote stage. The micro-vibrator installed in an incubator and its unique 3-dimensional movements are illustrated Fig. 1.

Embryo transfer and clinical results in the poor responders

The static culture group underwent 159 cycles, and the micro-vibration group underwent 166 cycles. Embryo selection for transfer was performed on day 3. The number of embryos for transfer was limited to 2. Some poor responders

received 3 embryos, depending on their clinical history (i.e., repeat failures or old age). Generally, the cell division rate in poor responders is slower than in good responders because of the poor-quality oocytes and sub-optimal number of collected oocytes in the former [24]. Therefore, based on the report by Ziebe et al. [34], the embryos were divided into 3 grades. Grade A was the 6–8 cell stage (10 % > fragmentation), grade B was the 4–5 cell stage (10 % ≤ fragmentation < 30 %), and grade C was the less than 4 cell stage (30 % ≤ fragmentation). Some of the surplus embryos after transfer were cultured for blastocyst development under the same conditions. Others were cryopreserved at the cleavage stage, and the remaining embryos were discarded. The blastocysts were evaluated on day 5 and 6 according to Gardner's grading methods and were cryopreserved or discarded based on their quality [35]. Pregnancy was assessed by serum hCG 14 days after the administration of progesterone, and implantation was confirmed by the presence of a gestational sac.

The clinical data and results were summarized into 2 types to compare the effect of micro-vibration stimulus on human embryonic culture. The first type included whole sets of clinical data and the results from poor responders for entire study periods. The second type included partial data and the results from the more severe poor responders, from whom fewer than 6 oocytes were collected. It was difficult to compare the effect of micro-vibration on human blastocyst development in the more severe poor responders because there were an insufficient number of surplus embryos for blastocyst development after transfer. The data included mildly poor responders, from whom more than 6 oocytes were collected; these results were useful to compare the effect of micro-vibration on blastocyst development but did not exhibit a marked influence on the clinical results.

Statistical analysis

The mouse and human embryonic quality and development rates were analyzed with the χ^2 test and *t* test. Clinical data in the poor responders were compared using the χ^2 test.

Table 2 Patient characteristics of the poor responders

	Static culture (<i>n</i> =159)	Micro-vibration culture (<i>n</i> =166)	<i>p</i>
Age (year)	38.5±4.1	37.9±4.4	n.s.
Mean number of oocytes	5.2±3.9	5.4±3.5	n.s.
Fertilization rate	76.1 % (579/761)	80.0 % (670/837)	n.s.
Mean number of zygote	3.6±2.7	4.0±2.6	n.s.
The rate of high grade embryos on day 3 (grade A)	60.4 % (317/525)	66.4 % (397/598)	n.s.
Mean number of transferred embryos	2.2±0.9	2.3±0.7	n.s.

Table 3 Clinical outcomes and blastocyst development rates in the surplus embryos

	Static culture	Micro-vibration culture
No. of embryo transfer cycles	159	166
Total pregnancy rate	13.2 % (21)	22.9 % (38)*
Implantation rate	6.7 % (23)	11.6 % (44)*
No. of surplus embryos	195	261
Blastocysts development rate on day 5	7.4 % (14)	16.9 % (44)*
Total blastocysts development rate on day 5 and 6	25.6 % (50)	32.2 % (84)

* $p < 0.05$

Results

Illustrations of the mouse embryonic development between the static culture group and the micro-vibration culture group on days 3 (A, B) and 5 (C, D) are shown in Fig. 2. A comparison of the cell numbers of mouse blastocysts (E, F) between the static culture group and the micro-vibration group is also shown in Fig. 2.

In both culture groups, differences in the optimal development rate on days 3 and 5 were demonstrated. On day 3, 48 (26.5 %) embryos in the 7–8 cell stage were present in the micro-vibration culture group, whereas only 21 (11.8 %) were present in the static culture group. On day 5, 67 (37.0 %) embryos had reached or passed the hatching blastocyst stage in the micro-vibration culture group, whereas only 32 (18.0 %) had reached or passed this stage in the static culture group (Table 1). The number of embryos that developed at the optimal rate in the micro-vibration group was significantly higher than in the static culture group.

A comparison of the cell numbers of stained blastocysts between the static culture group and the micro-vibration culture group is shown in Fig. 3. The cell numbers of mouse blastocysts in the micro-vibration culture group were significantly higher than in static culture group.

In the poor responders, there were no significant differences in the age, number of oocytes, fertilization rate, zygote number and embryo transfer number between the 2 groups. The rate of high grade embryos was not improved significantly in the micro-vibration culture group on day 3. However, the total pregnancy and implantation rates were significantly higher in the micro-vibration culture group. Moreover, the blastocyst development rate in the micro-

vibration culture group on day 5 was higher than in the static culture group. The total blastocyst development rate on day 5 and 6 was not significantly different between the 2 groups (Tables 2 and 3).

In the more severe poor responders, in whom fewer than 6 oocytes were collected, the total pregnancy rate was significantly higher in the micro-vibration culture group than in the static culture group (Table 4). The implantation rate was increased in the micro-vibration culture group but not significantly.

Discussion

In this study, micro-vibration stimulus during mouse embryonic culture had a positive effect on the development rates. During mouse embryonic development, the number of mouse embryos in a 50 μ L MTF droplet was limited to 5 to minimize the influence of group culture [36, 37] and to maximize the effect of the experimental conditions between the static culture and the micro-vibration culture [20]. The mouse embryonic development rate when the O_2 pressure is low (5 %) is commonly faster than when the O_2 pressure is high (20 %) [38, 39]. These conditions, including limiting the number of mouse embryos in a droplet and high O_2 pressure, did not have a positive effect on the mouse embryonic development. Even under these adverse conditions, the mouse embryonic development rate was increased significantly in response to the micro-vibration stimulus.

Although the beneficial effects of micro-vibration have been reported already in human in vitro fertilization, basic information of micro-vibration culture condition on various

Table 4 Clinical results of a limited population (no. of oocytes ≤ 5) in poor responders

	Static culture	Micro-vibration culture
No. of embryo transfer cycles	96	95
Age (year)	39.3 $\pm$ 4.1	38.7 $\pm$ 4.2
Mean number of oocytes	2.7 $\pm$ 1.5	2.9 $\pm$ 1.3
Fertilization rate	84.8 % (207/244)	84.9 % (219/258)
Mean number of transferred embryos	1.9 $\pm$ 0.9	2.0 $\pm$ 0.8
Total pregnancy rate	10.4 % (10)	21.1 % (20)*
Implantation rate	6.2 % (11/178)	11.3 % (22/194)

* $p < 0.05$

mammalian embryo cultures is not enough. First study of micro-vibration on porcine immature oocytes encouraged researchers to apply to mammalian embryos. In this study, it was approved that the mouse embryo development was significantly improved under the micro-vibration conditions. Studies of various mammalian embryos culture include this study on micro-vibration culture condition would be useful information for dynamic culture or embryo activation.

Based on a study conducted on immature porcine oocytes and embryos, the blastocyst formation rate is significantly increased at a frequency of 20 Hz, 5 s/30 to 60 min duration [21]. Isachenko's group has reported several relevant results. In their 2010 study, the micro-vibration frequency and interval were 20 Hz and 5 s/60 min, respectively, and the clinical results were significantly improved [22]. Furthermore, in their 2011 study, the micro-vibration frequency and interval were 44 Hz and 5 s/60 min, respectively, and the clinical results were significantly improved [33]. In the present study, the mouse embryo development rate and clinical results of the poor responders were significantly improved at a frequency of 42 Hz, 60 min duration. These reports indicate that the frequency range that has a positive effect on mammalian embryo development is broad, namely, from 20 Hz to 44 Hz.

The parameters of movement were not directly transmitted to the embryos in the media droplet. Isachenko's group reported that the acceleration of cells is slower than these rates when the plate is vibrating [33]. There may be a frictional force between the dish and the plate, between the dish and the mineral oil, between the mineral oil and the media droplet and between the media and the embryos. The viscosity of the media should also be considered a frictional force. Reduced acceleration and frequency may cause frequencies of cilia beating that range from 5 to 20 Hz [40]. Cilia beating, pulsatile muscle contractions of the uterus and oviduct and sperm motility generate forces that move fluids [41]. The fluid motion increases the positive effect of the hormones and nutrients on the embryos [42, 43]. It is difficult to define the combined in vivo mechanical forces that produce various movement patterns in the oviduct fluid. Moreover, it is difficult to quantify each in vivo mechanical force. However, it is clear that in vivo mechanical forces exert a beneficial effect on natural pregnancy. Furthermore, a micro-vibration embryonic culture method that mimics in vivo mechanical forces would be a valuable approach to improving the quality of in vitro fertilization (IVF) treatment. Other dynamic culture systems have been recently reported, including a microfluidic dynamic embryonic culture system [44, 45] and a tilting embryonic culture system [20]. Each system has pros and cons and presents challenges for practical use [46]. With improvements in each unique culture system, a dynamic culture system will be ultimately established.

In normal responders, the human embryonic development and clinical results were significantly improved under the micro-vibration condition [22]. Because the better quality of oocytes and embryos in normal responders, the positive effect on embryonic development could be observed more easily. In poor responders, large doses of stimulant medication, sub-optimal numbers of oocytes, poor-quality embryos, old age and a defective endometrium are the various factors that influence a poor prognosis [23–25]. The human embryo development rate were improved to a limited extent, suggesting that it is difficult to improve the cell division rate in poor responders because of their poor-quality oocytes and sub-optimal numbers of collected oocytes. However, the total pregnancy and implantation rates were significantly increased when a micro-vibration stimulus was applied to the human embryonic culture. This finding indicates that a slight improvement of embryo quality in poor responders can significantly influence the clinical results. In conclusion, this study suggests that micro-vibration embryo culture condition would be another approach for poor responders that remain a significant challenge in IVF.

The micro-vibration embryonic culture method would be valuable for in vitro maturation (IVM) or repeated IVF failures. Micro-vibration did not influence the maturation of porcine immature oocytes. However, the blastocyst development rates were significantly improved by micro-vibration [21]. It is not known whether micro-vibration has a positive effect on the maturation of human immature oocytes or on the clinical results in IVM. Moreover, it is still unclear whether micro-vibration has a beneficial effect on embryo development and implantation after repeated IVF failures. Further investigations are necessary to determine the effects of micro-vibration on other poor prognoses in IVF.

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