Improved Development of Mouse and Human Embryos by Tilting Embryo Culture System

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Abstract

Mammalian embryos experience not only hormonal but also mechanical stimuli, such as shear stress, compression, and friction force, in the fallopian tube before nidation. In order to apply mechanical stimuli to embryos in a conventional IVF culture system, we developed the Tilting Embryo Culture System (TECS). The observed embryo images from the TECS suggest that the velocities and shear stresses of TECS embryos are similar to those experienced in the oviduct. Use of TECS enhanced the development rate to the blastocyst stage and significantly increased the cell number of mouse blastocysts ($P<0.05$). Although not significant, human thawed embryos showed slight improvement in development to the blastocyst stage following culture in TECS compared to static controls. Rates of blastocyst formation following culture in TECS were significantly improved in low quality embryos and those embryos cultured under suboptimal conditions ($P<0.05$). Here, we propose that the TECS could be a promising approach to improve embryo development and blastocyst formation by exposing embryos to mechanical stimuli similar to in the fallopian tube.

Key Words: mechanical stimuli, shear stress, tilting embryo culture system, embryo development, blastocyst

Introduction

Mammalian embryos are transported to the uterine cavity through the fallopian tube during cell cleavage, blastomere, and blastocyst development (Halbert et al. 1976, Eddy et al. 1980). In conjunction with ciliated epithelium, the fallopian tube acts as a peristaltic pump due to phasic contraction of the smooth muscle in the wall to transport the embryo (Zervomanolakis et al. 2007, Lyons et al. 2006). Based on the movement of cilia and the similar size between the tubal lumen of ampulla and isthmus (Table 1) and the diameter of the embryo (0.1 mm), fertilized oocytes may be subject to a mechanical influence from the fallopian tube lumen, such as compression and shear stress (SS) from the tubal fluid. It is hypothesized that these mechanical actions in the fallopian tube might play an important role in embryo development. However, conventional *in vitro* static culture conditions do not mimic mechanical stimuli to embryos. Providing mechanical stimuli to developing embryos in vitro, similar to that experienced in the fallopian tube, may improve development.

From the 1980s, some groups reported *in vitro* non-static culture results (Staigmiller et al. 1984, Nagai et al. 1993). However, these reports did not discuss the precise effect on embryo development from cleaved embryo to blastocyst. Recent papers have reported that a microfluidic dynamic embryo culture system with media flow improved mouse embryo development (Cabrera et al. 2006). The report proposed the importance of the physical/mechanical environment on embryo development. Because the systems are complex to handle, clinical application of the culture system is quite difficult. In addition to ease-of-use, another consideration regarding mechanical stimulation during embryo development is avoiding excess stress. Excess mechanical stimuli would damage embryos. It was found that SS over 1.2 dynes/cm^2 caused lethality within 12 hours for blastocysts

(Xie et al. 2006). Over-handling of embryos, such as excess pipetting, caused elevation of phosphorylated stress-activated protein kinase, and may cause rapid transient changes in hundreds of proteins and mRNA. (Xie et al. 2007).

In order to construct a dynamic culture system that can apply a mechanical stimulus which, can be easily adapted to conventional static culture platforms, we developed the Tilting Embryo Culture System (TECS). Placing a conventional culture dish or plate on a tilted plate makes embryos in culture move along the bottom of the dish by gravity. We first conducted animal model experiments using TECS for the i*n vitro* culture of mouse 2-cell stage embryos. We then conducted a human study using thawed embryos cultured on TECS.

Materials and Methods

OBSERVATION OF EMBRYO MOTION AND SHEAR STRESS CALCULATION

To estimate the shear stress (SS) applied to embryos, mouse embryo motion in the microdrop on the tilting plate of a prototype TECS was observed by objective lens (20x) attached to the bottom of the tilting TECS plate. To estimate the flow velocity of media in the microdrop, we also observed the motion of microspheres (diameter: 5-0.5 μm) in the microdrop covered with mineral oil by tilting inverted microscope (Eclipse, Nicon, Japan). We focused on the motion of microspheres at the centre of the bottom where embryos in the microdrop were moving. When we tilted the microscope, the particles moved in the same direction as the leaning microscope. We calculated the average particle velocities that were considered as the velocity of the medium in the microdrop. On holding the tilt and static condition, Brownian motion of the particles was dominant.

Images of moving embryos and microspheres were recorded by a charge-coupled device camera connected to a personal computer. The frame rate of the recording was 30 frames/sec.

The observed maximum velocity of the embryos and the velocity of the medium were V(E) and V(M), respectively. The velocity to estimate maximum shear-stress (V(SS)) was calculated from the difference between V(E) and V(M) (1).

$$V(SS) = |V(E) - V(M)| \quad (1)$$

The maximum SS applied to embryos during the tilting was calculated from the following equation (2);

$$SS = 6\pi\mu r V(SS)/4\pi\mu r^2 = 6V(SS) \text{ dynes/cm}^2 \quad (2)$$

where μ: viscosity of the medium, r: radius of the embryo.

In an attempt to provide as accurate an estimate as possible regarding sheer stress experienced by embryos, we attempted to determine the flow velocity of fluids using microspheres. The formula (1) and (2) are applicable in the flow condition. Generally, we can neglect the effect of Brownian motion in ’flow’ for the shear-stress calculation. However, it would be difficult to determine accurate fluid velocity by particle image velocimetry (PIV). Although forces such as friction come into play with this approach, it provides us with a rough estimation for our final calculations.

The experimental methods to observe the embryo motion in extirpated mouse oviducts was summarized. Female ICR mice (Charles River Japan, Yokohama, Japan and Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan; 8-12 weeks old) were injected with 5 IU PMSG (Aska Pharmaceutical Co. Ltd. Tokyo, Japan), followed by 5 IU hCG (Aska Pharmaceutical Co., Ltd.) 48 hour later and mated with males. Successful mating was determined the following morning by the detection of a vaginal plug. After 2 days from the mating, we extirpated the oviduct with embryos

and the oviduct was sandwiched with a cover slip and a slide glass to observe the embryo in the oviduct. The velocity of the embryo in extirpated mouse oviducts in the table was calculated from the movie of the embryo motion. Animal use protocols were approved by Okayama University Animal Investigation Committee.

SPECIFICATION AND MOTION PROGRAMS OF TECS

TECS (STREX Inc., Osaka, Japan) is an electrical device with a power cord that is designed to be used in a humidified incubator. This device consists of a control unit (Fig. 1A, upper) and a motor unit with a tilting plate (Fig. 1A, lower). 4-well chambers and/or dishes are set on the plate. The TECS motor unit is controlled and DC-powered by a cord connected to the control unit through an access hole in the incubator. If the incubator does not have an access hole, a flat-type cable extends tightly through the door of the incubator in order not to alter temperature, gas concentration, and humidity inside the incubator. The motor unit is water-proof. We input the motion parameters by the controller outside the incubator.

A representative motion program of TECS is shown in Figure 1B and 1C. We can control the parameters of the uniform radial velocity (V_r), the maximum tilt angle (θ_r), and the holding time at the maximum tilt angle (T_h). The tilting time (T_r) is calculated from $2\theta_r/V_r$ (sec). First, the plate is tilted to the plus maximum tilt angle ($+\theta_r$) (M1). Second, the tilting plate is held for T_h with no motion (M2). Third, the plate is tilted to the minus maximum tilt angle ($-\theta_r$) (M3). Last, the tilting plate is held for T_h with no motion (M4). This is a cycle of the TECS motion summarized in Figure 1C, and the cycle continues until the power is turned off.

To apply conventional culture dishes/approaches to the TECS, spill out of the mineral oil should be prevented. The angles at which mineral oil begins to spill out are different according to

the size of the dishes. In our mouse embryo experiments, we used dishes of 35 mm diameter, and the maximum tilt was 20 degree. However, in the human embryo study, the plate could not be tilted over 10 degree due to the use of dishes of 60 mm diameter.

We surveyed maximum tilt angle (θ_r) to spill out mineral oil, and the limit of the maximum tilt angle was approximately 20 degree in the microdrop setup. Furthermore, excess uniform radial velocity (V_r) also induces spill out of the mineral oil. We investigated the minimum V_r at which the mineral oil spilt out from the 35mm dish. The V_r was 240 deg/sec when θ_r = 20 deg. While the oil did not spill out when θ_r = 10 deg. It is necessary to increase θ_r and V_r to move embryos in the microdrop. However, in this study, we used θ_r and V_r that did not result in the spill out of the mineral oil and the observation of embryo motion in the video rate recording. Thus, we used the condition in which θ_r and V_r were approximately 10-20 deg and 1deg/sec, respectively.

TILTING MOUSE EMBRYO CULTURE

Frozen 2-cell stage embryos of ICR mouse (Arc Resources Inc. Japan) were thawed and cultured in 50 and 500 μl of potassium simplex optimized embryo culture medium (mw: Daiya Shiyaku Inc. Japan) covered with mineral-oil for 3 days in a humidified environment of 5% CO_2 in air at 37^{o}C. In the TECS group, we cultured the mouse embryos at a 20 degree tilt (θ_r) for 1 min (T_h) using the TECS. The plate was tilted at 1 degree/second (V_r) to reach a total tilt of 20 degrees. To count cells in the mouse blastocyst, the cells were stained with Hochest 33342, as previously reported (Hardy et al. 1989, Mottla et al. 1995). The stained blastocysts were observed by confocal microscopy (FV-1000 Olympus, Japan) and a 3D image was constructed.

HUMAN EMBRYO STUDY

We used 3-11-cell stage embryos frozen by the slow method 3 days after collection from May 2000 to December 2004 (Cohen J et al. 1985), and extended the prospective study of 220 fertilized human embryos that were to be discarded with consent after pregnancy of patients.

This study was approved by the Ethics Committee of the Okayama University Graduate School of Medicine. The frozen embryos were thawed with THAW-KIT1^{TM} (Vitrolife, Sweden), and the viability of the thawed embryos was approximately 80%.

One thawed human embryos were cultured in a 20μl microdrop of Global$^{®}$medium (LifeGlobal, Canada) covered with mineral-oil for 48 hours. The thawed embryos with morphologically regular cleavage were divided so that there would be the same percentage of the embryos with regular cleavage (33%) in both the TECS group and the Static control (CTRL) group. The viable cell number of the thawed human embryos was from 3 to 11. The dishes were placed on the tilting plate of TECS in a 50L multi-gas incubator (ASTEC, Japan). The thawed human embryos were cultured at a 10 degree tilt (θ_r) for 10 min (T_h). The velocity of the tilt was 1 degree/second (V_r).

The blastocysts were morphologically evaluated according to the classification of Gardner et al. (Gardner et al. 2000). The cells in the human blastocyst were stained with Hochest 33342, the blastocysts were observed by confocal microscopy (FV-1000 Olympus, Japan), and a 3D image was constructed.

STATISTICAL ANALYSIS

χ^2 squared test and Student's t-test were used to determine differences in the blastocyst

development rate and in the number of cells in the blastocysts between the groups, respectively. A P-value <0.05 was considered significant.

Results

Observation of Embryos in Motion by TECS and Estimation of Shear-stress

To obtain detailed information on the embryo motion, we observed the blastomeres on the TECS during tilting and holding. Figure 2 shows cropped images of the mouse embryos in motion. When the TECS plate was held for 10 degree tilt (M2 and M4 in figure 1B), the embryo slid at a velocity of 0.03 mm/min (Figure 2C and D), which gave rise to a shear stress of 1.5 $x10^{-4}$ dynes/cm^2 in the medium, where the motion of medium at the center of the bottom was neglected in the motion phases. Between each 10 degree tilt (M1 and M3 in figure 1B), the TECS plate was tilted at 1 degree/second. The motion of the medium was observed in M1 and M3. The embryo moved at a velocity of 1.5 mm/min on average (Figure 2A and B), which gave rise to shear stress of $7.0x10^{-3}$ dynes/cm^2 at the bottom of the center in the microdrop at a velocity of 0.1 mm/min. These values are summarized in Table 2.

Mouse Embryo Development

Table 3 shows the blastocyst development rate from 2-cell stage mouse embryos cultured under several conditions. As shown in Figure 3, the blastocyst development rate was 79% in 10 embryos in a 50 μl microdrop using the TECS, which was not significantly different to the static culture controls (75%). Because mouse blastocyst development rate is reported to be influenced by the number of embryos in the culture medium (Melin et al. 2009) and in order to enhance the statistical difference between TECS and CTRL, we reduced the number of embryos in the

microdrop, and increased the medium volume to embryo which might simulate poor development condition such as low quality human embryos. The blastocyst development rate of 4-6 embryos in a 50 μl microdrop was less than that of 10 embryos, although in this poor condition, TECS significantly improved the blastocyst development rate (TECS 59% (n=145); Static Control (CTRL) 46% (n=151) $P<0.05$). When 10 embryos were cultured in 500 μl medium, the blastocyst development rate decreased to 27% (n=101) in the static control, whereas for those cultured in the TECS it was significantly higher (42% (n=99); $P<0.05$)). The average number of cells in the blastocysts cultured using the TECS was 77 ± 4 cells (n=34, ±SEM), while that of the CTRL was 66±4 cells (n=26, ±SEM), as shown in Figure 4. There was a significant difference in the average cell number between the two groups ($P<0.05$).

Human Embryo Development

As TECS enhanced blastocyst development rate in mouse embryos, we next conducted experiments on thawed human embryos. The development rates to the blastocyst stage of the TECS and CTRL groups were 53% and 45%, respectively (Table 4).

Due to the insufficient sample number of thawed human embryos, we investigated the cell number of the developed blastocysts instead of the blastocyst development rate. Figure 5 shows the comparison of average cell numbers in the blastocysts developed by the CTRL and the TECS. The mean cell number of developed blastocysts of Day 5 by the TECS was 43±3 cells (n=24, ±SEM), while that of the CTRL was 34±3 cells (n=18, ±SEM). There was a significant difference in the average cell number between the two groups ($P<0.05$).

Discussion

The fallopian tube is a multifunctional organ, involved in pick-up of ovulated oocytes, providing a suitable environment for fertilization and early development, and transporting embryos to the uterus. Here, we shed light on the mechanical properties of the fallopian tube that might influence early development of embryos during culture. Early studies showed that the fallopian tube is a mechanically-active organ, and can introduce as (1) SS by a tubal fluid flow, (2) compression by peristaltic tubal wall movement, (3) buoyancy, and (4) kinetic friction force between embryo and cilia. Punctuated velocities of maxima from 0.39 to 1.8 mm/min have been observed in rat oviducts for microspheres emulating the size of embryos (Xie et al. 2006). Inappropriate culture conditions could be detrimental. Indeed, embryos sense shear stress and development is compromised (Xie et al. 2006, 2007). Because SS is a function of the velocity of the embryo and flow and the contribution is important, to mimic the dynamic environment of the oviduct, we propose that the velocity of embryos should be made similar to those in the oviduct. Although the similarity in the velocities of the mouse embryos was found, we did not observe those in other species. The velocities *in* and/or *ex vivo* should be investigated for optimization.

The observed velocity of mouse embryos on a TECS culture plate was of a similar order to those proposed in the oviduct. The embryo motion in the microdrops on the TECS plate correlates to the plate motion. The observed embryo images on the TECS plate suggest that the velocities and shear stresses of embryos (Table 2) are similar to the TECS plate and in the oviduct. This comparative experiment suggests that the TECS can apply physiological mechanical stimuli to mouse embryos. In the case of human embryos, the embryo velocity in the microdrop on the TECS plate was the same order (approximately 1 mm/min) as those of mouse embryos and below 1.2 dynes/cm^2. Therefore, the TECS could apply physiological stimuli without an excess amount of SS

that might cause damage to the embryos. Furthermore, the detrimental or beneficial SS would be different at each developmental stage, and our future experiments will address this.

Embryo motion in fluid is affected not only by species differences but also the environment of the embryos. For example, when cumulus cells are coated with zona, embryos with the cells stick on the bottom of the dish. To optimize the parameters (θ_r and V_r) for each embryo condition, the relationship between the motions in fluid and physical characteristics of embryos (such as density, dimension, and zona surface structure) should be considered.

The TECS significantly improved development in low quality human embryos and suboptimal culture conditions in mouse embryos. The significance of the improvement by the TECS was dependent on the number of mouse embryos in the medium. As mentioned in the results, the blastocyst development rate significantly improved in the case of 4-6 mouse embryos in the microdrop. In the case of 10 embryos in the microdrop, blastocyst development rates of the CTRL and the TECS group were 75% and 79%, respectively, and the difference was not significant. A higher number of embryos in the medium can improve the blastocyst development rate. According to previous reports that the concentration and production of autocrine and/or paracrine factors enhance mouse embryo development (Kawamura et al. 2005, Contramaestre et al. 2008), a higher number of embryos in the medium can improve the blastocyst development rate. Our results in Figure 3 demonstrate that blastocyst development rate was significantly improved by the TECS, when using a lower mouse embryo to volume of media ratio. This may be due to diffusion of growth factors and/or waste products, which would be facilitated by the TECS motion in the mouse embryo culture. However, mechanical stimuli could also be beneficial. Indeed, in bone and endotherial cells, downstream transcription factors in the nucleus have been shown to be activated by mechanical stimuli such as shear stress and mechanotransduction, and gene transcription and DNA syntheses

would be also activated (Wang et al. 2006). Due to the enhancement of cell division induced by these activations, cell numbers would be increased without apoptosis.

Our results demonstrated that the cell numbers of the mouse and human blastocysts cultured in the TECS were greater than those cultured in the CTRL, and that TECS can improve the quality of those blastocysts. The increase in cell number of the mouse and human blastocysts suggests that TECS could enhance cell division of human embryos. According to published material (Xie et al. 2006, Cui et al. 2008), negative correlations between the percentage of TUNEL positive cells and cell numbers in mouse embryos have been suggested. Therefore, although not measured, reduction of necrosis and/or apoptosis may be one explanation for the increased cell number in blastocysts obtained from TECS culture. Future experiments will confirm or refute this theory.

Finally, we describe advantages of the TECS in clinical use. Although the culture conditions are different in each ART laboratory, a benefit of this system is its ability to be rapidly implemented because it can be adapted to multiple styles of culture dishes/approaches. These results of thawed mouse and human embryo development indicate that the clinical study of embryo culture using the TECS can be extended without problems. To demonstrate the clinical importance of the TECS in human embryo development, we are preparing a clinical multi-center study of human embryo development by the TECS, involving embryo-transfer. We would like to emphasize that improved quality of developed embryos by the TECS might contribute to enhanced pregnancy rates in clinical practice. As blastocyst cell numbers were increased by the TECS, pregnancy rates resulting from embryos cultured in this system might be improved.

In conclusion, the TECS enhanced blastocyst development rates of mouse embryos after the 2-cell stage and caused a significant increase of cell number in blastocysts. Thawed human embryos after the 3-cell stage tend to show an improved blastocyst development rate when cultured by the

TECS. In particular, the improvements made by the TECS were significant in low quality embryos and suboptimal culture conditions. We proposed that one possible reason for the improvements could be mechanical stimuli by embryo motion based on the comparison of both mouse and human embryo development results.

Table 1. Lengths and tubal lumen diameters (mm) of the ampulla and isthmus.

		Mouse	Human
Ampulla	Length	-[a]	50-80
	Diameter	~1	1-2
Isthmus	Length	-[a]	20-30
	Diameter	0.1-0.3	0.1-1
References		Suarez et al. 1987	Eddy et al. 1980

[a] The total length of the mouse oviduct is 18 mm (Fox et al. 2007).

Table 2. Observed velocity and calculated shear stress of mouse embryos

	Maximum velocity of embryo (mm / min)	Velocity of medium (mm / min)	Estimated maximum SS (dynes / mm^2)
TECS Tilting	1.5	0.1	0.7
TECS Holding the tilt	0.03	-	0.015
Static control	0.006	-	0.003
In extirpated mouse oviduct	0.6	0 ~ 6	0 ~ 3

TECS = Tilting Embryo Culture System.

Table 3. Mouse blastocyst development rates of the (Tilting Embryo Culture System) TECS and control groups.

Culture condition	TECS group n (%)	Control group n (%)	Significance
10 embryos/50 μl	96/121 (79)	96/128 (75)	None
5 embryos/50 μl	85/145 (59)	69/151 (46)	$P < 0.05$
10 embryos/500 μl	42/99 (42)	27/101 (27)	$P < 0.05$

Table 4. Human blastocyst development rates of the Tilting Embryo Culture System (TECS) and control groups.

	TECS group	Control group
Blastocyst (%)	52.9 (46/87)	43.5 (37/85)
High quality blastocyst (≥3BB) (%)	17.2 (15/87)	11.8 (10/85)

FIGURE CAPTIONS

Figure 1. A: Photograph and B: Motion programs of the Tilting Embryo Culture System. V_r and θ_r represent the radial velocity (deg/sec) and the maximum tilt angle (deg). T_h is the holding time at the maximum tilt angle (min). The range of 3 parameters are $0.1 \leq V_r \leq 1$ (deg/sec), $1 \leq \theta_r \leq 20$ (deg), and $1 \leq T_h \leq 60$ (min). C: Left: Graph of the time-dish angle. Right: Definition of the dish angle. M1~M4 correspond to those in Figure 1B, respectively.

Figure 2. Observed images and motions of mouse embryos by tilting microscope. When the plate of the Tilting Embryo Culture System was tilting, the mouse embryos in image A moved to the location in image B after 6 sec. On holding the tilt, the mouse embryos in image C moved to the location in image D after 40 sec. The arrows show the motion of the embryos.

Figure 3. Relationship of the ratio of the number of mouse embryos to the volume of media and mouse blastocyst development rates. Light and dark grey bars indicate the number of blastocysts of the control and Tilting Embryo Culture System groups, respectively. Error bars are SEM. Statistically significant differences are indicated: *P<0.05.

Figure 4. Comparison of average cell numbers of mouse blastocysts between the two groups. Light and dark grey bars are the percentages of the control (CTRL) and Tilting Embryo Culture System (TECS) groups, respectively. Error bars are SEM. Statistically significant difference is indicated: *P<0.05. The culture condition was 4-6 embryos in a 50 μl microdrop.

Figure 5. Comparison of average cell numbers of human blastocysts between the two groups. Light and dark grey bars are the number of blastocysts of the control (CTRL) and Tilting Embryo Culture System (TECS) groups, respectively. Error bars are SEM. Statistically significant difference is indicated: *P<0.05.

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