Research Article

A microfluidic in vitro cultivation system for mechanical stimulation of bovine embryos

This work demonstrates a novel microfluidic in vitro cultivation system for embryos that improves their development using a partially constricted channel that mimics peristaltic muscle contraction. Conventional photolithography and a PDMS replica molding process were used to make straight or constricted microchannels. To investigate the effects of constriction geometry on embryonic development, different constriction widths of the channel were designed. Bovine embryos were loaded and incubated by simply placing them on a tilting machine to provide embryo movement via gravity. The fertilized embryos were cultivated on the microfluidic in vitro cultivation system until the blastocyst, hatching, or hatched blastocyst stages. To confirm the quality of blastocysts in the microfluidic channel, double staining was performed and compared with bovine embryos cultivated by the conventional droplet method. The proportion of eight-cell development among total embryos in the constricted channel (56.7 ± 13.7%; mean ± SD) was superior to that in the straight channel (23.9 ± 11.0%). This suggests that the effect of constriction is vital for the early development of bovine embryos in assisted-reproduction research.

Keywords:
Bovine embryos / In vitro cultivation / Mechanical stimulation / Microfluidics / Peristaltic constriction

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1 Introduction

Emerging reproductive biotechnologies have been developed in most in vitro production (IVP) fields for decades and applied to the treatment of human infertility [1], embryo manipulation [2], and nuclear transfer [3]. IVP techniques are particularly important in livestock breeding because embryo manipulation is directly related to economic aspects of the agricultural industry [4]. To improve the developmental efficiency and quality of embryos, many studies have addressed the chemical modification of medium [5] and its components, as well as environmental changes in physiological conditions, such as temperature [6], pH, shear stress [7], and osmolality [8]. Although these efforts have revealed many factors that are critical to developing embryos, the development rate of embryos, especially of large animals such as porcine and bovine species, still remains low [9]. This low efficiency is believed to be due to the different environments of in vitro and in vivo conditions.

Therefore, trials to identify key factors that positively affect embryo development in an in vivo environment are extremely important, and several efforts to mimic in vivo conditions have been proposed [10, 11].

Microfluidic technology is largely considered as an optimal strategy for creating a biological microenvironment and investigating microscale physical behavior [12, 13]. Microfluidics-based IVP has been applied in studies on spermatozoa sorting [14, 15], the removal of cumulus cells [16, 17], in vitro fertilization (IVF) [18], and in vitro cultivation (IVC) [19, 20]. For IVC in particular, microfluidics-based embryo culture systems represent a more physiological microenvironment than the volumetric conventional culture method; thus, novel culture systems using Braille-display [21], static microchannels [19], and dynamic flow channels [22] have been introduced. However, although these studies were innovative trials over the concept of chemical modification, few reports have considered dynamic physical stimulation in an oviduct, such as peristaltic constriction, which moves embryos from the fallopian tube to the uterus.

The present study demonstrates a novel microfluidic IVC system based on mechanical stimulation derived from peristalsis. To investigate the effects of mechanical stimulation on the development of bovine embryos in a microfluidic channel, the development rates of embryos cultivated...
in a straight channel were compared with those cultivated in channels with constricted areas of various widths. In addition, because IVC is a long-term culture process, a micro-modulated tilting machine was adopted to realize a simple dynamic flow culture system using gravity. This work represents the first trial that mimics the peristaltic constriction that occurs in vivo in the fallopian tube.

2 Materials and methods

2.1 Design of a mechanically stimulated microfluidic IVC channel

During transport, embryos are under the influence of two different mechanics, ciliary motility, and muscle contractility [23]. Muscular contraction is related to the transport, stimulation, and denudation of embryos due to deformation and squeezing of the tubal wall [24]. There are three different muscle layers in the isthmus, which is the narrowest part of the oviduct: the outer longitudinal or spiral-shaped uterine layer, the intermediate circular layer, and the innermost longitudinal layer. Segmental contractions in the isthmus are generated by intermediate circular muscle (Fig. 1A). The embryo is pressed against mucosal folds through the contracted oviductal segment [25]. In this study, the effect of the oviductal intermediate circular muscle was mimicked by designing microchannels with constricted areas (Fig. 1B).

To determine appropriate constriction widths (CWs), a range of applied forces that positively affected embryos was calculated relative to embryo deformation (Fig. 2). Based on a previous study on human embryos [26], the maximal force to maintain elastic deformation was set as a critical threshold value for mechanical stimulation where the nature of bovine embryos was assumed to be similar to that of human embryos. In general, the diameter of bovine embryos in the morula stage ranged from 140 to 170 μm. Based on the results shown in Fig. 2, the CWs used for mechanical stimulation were 150 and 160 μm (Figs. 3A and B).

Each microchannel was 200 μm × 50 mm × 200 μm (w × l × h) and contained (except for the control) ten constriction areas. The channels also included two side reservoirs, two channel necks, and an in/outlet port (Fig. 3A). Channel necks were 50 μm in width to prevent embryos from leaking. The in/outlet port was positioned at the middle of each channel for loading embryos.

2.2 Fabrication of the straight microfluidic IVC channel

The device was prepared by conventional photolithography, PDMS (Sylgard 184; Dow Corning, Midland, MI) replica molding, and aligning processes. The channel features were fabricated using an SU-8 2100 (MicroChem, Newton, MA) photosist. Briefly, the spin speeds were set at 500 rpm for 10 s and 1450 rpm for 30 s to make a fluidic channel 200 μm in height. Then samples were soft baked at 65 °C for 25 min and 95 °C for 90 min, exposed to ultraviolet light for 36.1 s at 650 mJ/cm², and then hard baked at 65 °C for 1 min and 95 °C for 30 min. Finally, the resultant wafer was immersed into SU-8 developer for 30 min and rinsed with isopropanol for 15 s. The patterned silicon wafer was sufficiently silanized and then submersed in PDMS and cured. After

Figure 1. Schematic view and design of a channel mimicking segmental contractions in an oviduct. (A) Segmental contractions with cross-section view and longitudinal sectional view (not to scale): Segmental contractions are generated by the intermediate circular muscle layer. A fertilized egg is pressed against mucosal folds in the segmental contraction. (B) Peristalsis-mimicked schematic with constricted area and CW: the oviductal intermediate circular muscle layer can be mimicked by designing a microchannel with a constricted area.

Figure 2. Relationship between the applied force and embryo deformation determined by embryo diameter and CW. The critical force applied to embryos (0.2 μN) and stiffness of embryos was determined according to [28].
punching side reservoirs and the in/outlet port, the device was bonded with a glass slide by the O₂ plasma asher (270 W, 30 s). As shown in Fig. 3C, a mineral oil reservoir made of PDMS was attached to both side reservoirs using the O₂ plasma asher (270 W, 30 s). Then PDMS was pasted outside of the reservoirs and allowed to cure. The final device is shown in Fig. 3D.

2.3 In vitro maturation (IVM), IVF, and the preparation of bovine embryos

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Bovine oocyte maturation medium (IVM medium) [27], Percoll [28], SP-TALP [28], Fert-TALP [27], CR1aa medium supplemented with 0.3% BSA (IVC–BSA medium) [29], and CR1aa medium supplemented with 10% fetal bovine serum (FBS) (IVC–FBS) [29] were used as reported previously. Collected bovine ovaries were transported to the laboratory in 0.9% normal saline in a thermo flask containing 150 μg/mL penicillin and 100 μg/mL streptomycin. The selected oocytes were incubated in IVM medium at 38.5°C for 22 h. After incubation, the oocytes were washed to remove cumulus cells that were growing around the oocytes. To prepare IVF, frozen bovine semen was included in a two-phase Percoll tube to separate motile sperm cells from others and centrifuged at 2000 rpm for 20 min. Motile sperm cells from the bottom of the tube were sprinkled on SP-TALP and centrifuged at 1000 rpm for 10 min. Prepared oocytes were loaded on Fert-TALP with 2 μL heparin and 2 μL sperm cells. The IVF process was continued with incubation at 38.5°C for 22 h. Fertilized oocytes were prepared for both conventional microdrop [29] and the microfluidic IVC device procedures. All procedures were performed according to the ethical guidelines of the Institutional Review Board at KAIST.

2.4 Operation of the microfluidic IVC system

Before the bovine embryos were loaded on the device, each channel was filled with 50 μL IVC–BSA media via the in/outlet port and 200 μL mineral oil was poured into the oil reservoirs to prevent media evaporation. The fertilized oocytes were collected and loaded through the in/outlet port, which was blocked by the inlet cap during incubation (Fig. 3C). The microfluidic IVC device with the IVF oocytes was incubated at 38.5°C for 44 h on the tilting machine, which made a cyclic 7° 10° 1° tilt over 1 min (Figs. 3E and F).

After oocytes had completed early development, a new device filled with 50 μL IVC–FBS medium was prepared for later-stage development. At this point, cell number was recorded for each stage and both four-cells and eight-cells were loaded rapidly. The device was also incubated at 38.5°C for 1 wk to get bovine blastocysts on the tilting machine.

2.5 Blastocyst double staining

Blastocyst quality was examined by double staining. The collected blastocysts were treated by pronase to remove zona pellucida, and then incubated in rabbit anti-pig whole serum for 1 h. Both propidium iodide (Invitrogen, Carlsbad, CA) and Hoechst (Invitrogen) were applied for 1 h and
then stained blastocysts were fixed on glass slides. A fluorescent microscope (Olympus 1 × 51, Hachioji, Japan) was used to detect the number of cells and the color of stained cells.

2.6 Data analysis

Twenty fertilized embryos were inserted into each microfluidic channel and incubated. The embryos were cultivated for 3 days and counted at every two-, four- and eight-cell stage via microscopic inspection. Cell numbers at each stage were summarized, and independent tests were performed six times. The cell number at each stage was divided by 20 (total embryos) and multiplied by 100, representing the percent for the stage among total embryos.

3 Results and discussion

3.1 Microfluidic embryo manipulation for IVC

Fertilized embryos are sensitive to temperature [30], and thus the cell manipulation time for IVC outside of CO₂ incubators should be minimized. Hence, a simple cell-loading technique using micropipettes was adopted. Connecting external equipment to a microfluidic device may change the temperature of the CO₂ incubator, even if the embryo injection time outside the CO₂ incubator is minimized. Therefore, a micro-modulated tilting machine as shown in Fig. 3E was used to create a driving force to move embryos, rather than using a conventional syringe pump, which requires tubing connections. By simply inserting the embryos through the in/outlet port of the microfluidic device and using a tilting machine, preparation for IVC culturing was completed simply and rapidly within 1 min.

The plane angle of the tilting machine affected the velocity of embryo movement. To apply a peristalsis effect to the embryos, they must penetrate the constricted areas. The penetration of embryos occurs when the moving force of embryos ($F_1$) is larger than the frictional force ($F_2$):

$$F_1 = (\rho_{\text{embryo}} - \rho_{\text{medium}}) V_{\text{embryo}} g \sin \theta - 6 \pi r v_{\text{embryo}}$$

(1)

$$F_2 = \mu f_n = \mu K \delta$$

(2)

where $\rho_{\text{embryo}}$ and $\rho_{\text{medium}}$ are the densities of the embryo and medium, respectively, $V_{\text{embryo}}$ is the volume of an embryo, $g$ is the acceleration of gravity, $\theta$ is the plane angle of the tilting machine, $\eta$ is the fluid viscosity, $r$ is the Stokes radius of the embryo, $v_{\text{embryo}}$ is the terminal velocity of the embryo, $\mu$ is the coefficient of friction, $f_n$ is the normal force exerted on embryo deformation, $K$ is the stiffness of an embryo, and $\delta$ is embryo deformation. Thus, the angle of the tilting machine and the difference between the CW and diameter of an embryo are the principal parameters for mechanical stimulation. As shown in Figs. 4A and B, a tilt of $10^\circ$ achieved over 1 min was sufficient to penetrate the constriction area of embryos even though they were of various sizes. Therefore, this angle was applied and maintained for 5 min, and then reversed $10^\circ$ over 1 min, to resemble a seesaw.

Because the fertilized embryos were sticky due to the presence of a zona pellucida [31], surface modification was considered to maintain the movement of embryos in the microfluidic channels. However, BSA was presented in the IVC medium so that the bovine embryos moved well along the straight channel without interacting with the PDMS walls, even though they were attached and stuck to each other. Evaporation may change the osmolality of a medium, and prevent embryonic development. Thus to minimize evaporation, water was filled in the petridish containing a device and mineral oil was

Figure 4. Bovine embryo development in the microfluidic IVC system. (A) Picture of an embryo before penetrating a constriction area. (B) Picture of an embryo after penetrating the constriction area. (C) Picture of embryos developed for 1 wk. Black solid arrow indicates an embryo in the blastocyst stage. (D) Picture of embryos developed for 10 days. Dotted arrow and solid arrow indicate hatched blastocyst and inner cell mass of the blastocyst, respectively. All scale bars are 100 μm. (E) Double-stained blastocyst cultivated in the microfluidic IVC system. (F) Double-stained blastocyst cultivated using the conventional microdrop method. Blue and red colors represent trophoblasts and the inner cell mass, respectively.
poured into the side media reservoirs. In addition, the hole of the in/outlet port was capped with a PDMS fragment.

3.2 Microfluidic IVC with a peristalsis effect for bovine embryo development

Microfluidic channels were filled with IVC media, and fertilized bovine embryos were inserted. The medium volume was 50 μL, which was the same as the amount used in the conventional microdrop method.

The embryos loaded into the IVC device were cultivated in IVC–BSA media for 44 h. Cleaved embryos at the four- and eight-cell stages were collected from the in/outlet port using a micropipette and transferred to another device filled with IVC–FBS media. The transferred embryos were cultivated for 5–7 days. Figure 4C shows bovine embryos cultivated in a microfluidic channel for 7 days. The black arrow indicates an embryo in the blastocyst stage of up to 180 μm in size. Because this embryo was too big to penetrate the constriction areas, it moved partially between adjacent constriction areas. When cultivated for a further 1–2 days, such embryos continued development until hatching, or the hatched blastocyst stage, as indicated by the dotted arrow of Fig. 4D. Although a hatched zona pellucida >200 μm in size sometimes blocked the movement of embryos (as shown in Fig. 4D), this did not cause any major problems because this only happened at the end of the IVC.

On the contrary, because the size of fertilized embryos does not differ significantly until the early morula stage, we simply observed the penetration of embryos in the microfluidic channels by comparing their initial and final positions.

To verify the quality of embryos cultivated in the microfluidic channel with mechanical stimulation, they were stained with Hoechst (blue, trophoblast) and propidium iodide (red, inner cell mass) (Fig. 4E). Figure 4F shows an embryo in the blastocyst stage cultivated using the conventional microdrop method. The high proportion of inner cell mass over trophoblast and more staining reflects the higher quality of the blastocysts [32]. Actually, it was doubted whether a blastocyst cultivated in the microfluidic channel was normally developed and comparable with that from the conventional culture method in quality. However, the double-staining method revealed that embryos cultivated in the microfluidic IVC device were comparable with those cultivated using the conventional method.

Several previous studies have reported microfluidic IVC of mouse embryos, the process of which is well developed compared with the IVC of large animals [19], while only a few studies have attempted IVC of bovine embryos in a microfluidic environment [33]. Here, we have demonstrated the successful and efficient bovine embryo cultivation in a mechanically stimulated microfluidic channel. This IVC platform is expected to be a fundamental process included in future microfluidic platforms for assisted-reproduction research of large animals.

3.3 Effect of mechanical stimulation on the early development of bovine embryos

Conventional embryology has focused on the chemical composition of the culture environment, and modifications thereof, to enhance embryo development [6, 34]. Recent findings of the key factors that positively affect embryo development in the in vivo environment have been critical and efforts to mimic in vivo conditions have been progressed. However, few studies have considered dynamic physical stimulation, such as peristaltic constriction, in an oviduct.

By comparing the development ratio of embryos cultivated in a straight channel to those cultivated in channels with constricted areas, the effects of mechanical stimulation on the early development of bovine embryos were examined. Figure 5 shows the percent of embryos at each early developmental stage relative to total embryo numbers, such that the summed percent of each stage (the two-, four- and eight-cell stages) should be 100% (n = 6). Therefore, a larger percent in the eight-cell stage represents a better environment for embryo development. The percent of embryos in the straight channel and constricted channels with 150 and 160 μm CWs in the eight-cell stage were 23.9 ± 11.0 (mean ± SD), 37.8 ± 11.0, and 56.7 ± 13.7%, respectively. As seen in Fig. 5, the microchannels with constricted areas were superior to the microchannel without mechanical stimulation. We also examined embryonic development in a microchannel with a 140 μm CW; however, a preliminary experiment indicated that only about 10% of the embryos developed until the eight-cell stage and many of them did...
not exhibit healthy morphology. In addition, when more than 0.2 μN force was applied to human embryos, they but did not return to their original state after deformation, i.e. they were severely damaged [26]. As shown in Fig. 2, embryos greater than 150 μm were exposed to harsh external forces in the microchannel with a 140 μm CW. Assuming that bovine embryos are similar to human embryos, it can be inferred that this stress negatively affected embryonic development. This also corresponds to the results shown in Fig. 5. Although the microchannel with a 150 μm CW improved embryo development more than did the control channel with no mechanical stimulation, the condition was not an optimal range of stimulation, representing that the force caused by the dimension was relatively big condition to embryos when compared with the result of 160 μm width.

Many factors affect embryo cultivation, such as the angle of the tilting machine, number of constricted areas, CW, time required to achieve the angle, and time held at the angle. Because IVC requires long culture times, we could not examine embryonic development to optimize each of these factors. Therefore, more detailed studies are needed to realize more effective conditions for embryonic development. In addition, because the system was designed for transferring cleaved embryos into a new device filled with IVC–FBS medium, unexpected contamination or disappearance of embryos sometimes occurred. Because contamination was also possible during the media-transfer process, the samples were handled very carefully. No contamination or disappearance of embryos was observed in the main experiments used for data analysis. However, an advanced concept to eliminate the transfer process of embryos and the need to remove the device from the CO₂ incubator would be desirable to develop an improved IVC system.

4 Concluding remarks

Microfluidics is very useful for studying sophisticated biological environments and for customizing experiments. We demonstrated that mechanical stimulation could affect and encourage the early development of bovine embryos. Conventionally, a syringe pump is used to generate fluid movement [22, 35]. In contrast, we used a micro-modulated tilting machine to create dynamic IVC conditions and realize simple and rapid cell manipulation. To the best of our knowledge, this is the first trial to examine the effects of mechanical stimulation on embryonic growth using constricted channel design to mimic peristaltic constriction in vivo. Although much supplementary work should follow this systemic concept, this work is expected to provide new insight into the development of microfluidic platforms for assisted-reproduction research.

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5 References