Application of a Microfluidic Chip with Mechanical Stimuli for Culturing Mouse Embryos *in vitro*

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Abstract: *To address the problems of a low blastocyst formation rate and poor embryo quality in vitro, we developed a microfluidic chip with mechanical stimuli (MS) to imitate the microenvironment of the oviduct and investigate its effect on early mouse embryo development. We constructed a novel system to apply MS similar to those generated inside the oviduct using poly (dimethylsiloxiane). We used this microfluidic chip to culture 2-cell stage mouse embryos produced by in vitro fertilization. There were large differences between control and microfluidic chip groups in the respective rates of development to morulae (63.1 ± 12.5% vs. 80.6 ± 17.9%) and blastocysts (58.6 ± 2.3% vs. 73.2 ± 2.8%) (all $p < 0.05$) and there were large differences in inner cell mass cell numbers in blastocysts between groups ($p < 0.05$). This culture device significantly improved the blastocyst development rate and quality of mouse embryos cultured in vitro. It is feasible for generating and applying MS similar to those generated inside the oviduct to cultured mammalian embryos.*

Keywords: *Microfluidic chip, Mechanical stimuli, Embryo culture, Mouse.*

Introduction

Developments in embryonic bioengineering technology, such as *in vitro* fertilization (IVF), cloning, and transgenic applications face problems of low blastocyst formation rates and poor embryo quality [14]. These problems restrict the development of embryo engineering. Studies have focused on the embryo culture media used for early embryo culture *in vitro*. However, they have ignored physical factors of embryo development in the oviduct. Based on the movement of cilia and the similar size between the lumen of the ampulla and isthmus and the diameter of the embryo (0.1 mm), fertilized oocytes might be subjected to mechanical stimuli (MS) from the tubal environment that are important for embryo development. The traditional method of early embryo culture can only provide a static, macroscopic two-dimensional growth microenvironment, so the growth environment *in vitro* is very different from that *in vivo*.

The mammalian embryo *in vivo* is exposed to biochemically and biophysically dynamic conditions when it traverses the oviduct [4]. In conjunction with fluid flow generated by the ciliated epithelium, the tube acts as a peristaltic pump with phasic contractions of the smooth muscle in the wall to transport the embryo towards the uterus [9]. Diffusion of growth factors and/or waste products is essential for the survival of embryos in culture [15]. Possible MS responsible for the observed movement of the embryos are shear stress (SS) caused by fluid dynamics, and compression of the embryos caused by interactions with the wall of the oviduct. We have developed a novel and simple system to generate and apply MS similar to those generated inside the oviduct to cultured mammalian embryos.

Matsuura's group showed that blastocyst development was significantly improved using a tilting embryo culture system (TECS) [3, 10]. However, there were some technical difficulties, namely microdrop collapse and spillage of mineral oil covering the microdrop when the angle of the TECS tilting plate was greater than 30°. Therefore, we aimed to construct a culture system to increase SS and apply other forms of MS during *in vitro* embryo culture. Our aim was to apply MS similar to those generated inside the oviduct to cultured mammalian embryos, using a device formed from poly (dimethylsiloxiane) (PDMS). We used this microfluidic chip embryo culture device to culture 2-cell stage mouse embryos *in vitro*, and to explore the effect of this microfluidic chip with MS on embryo development.

Materials and methods

Experimental animals

KunMing mice (male aged 12-16 weeks, female aged 6-8 weeks) were from the Department of Animal Reproduction, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, P. R. China (all mice were clean grade).

Chemicals and culture media

Sylgard-184 Silocone Elastomer (Lot No. 0005323071) was from Dow Corning (Midland, MI, USA). Other reagents including KCl, NaCl, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$ and polyvinyl alcohol were purchased from Sigma Aldrich China (Shanghai, P.R. China). Modified CZB medium [11] was used for culturing mouse embryos.

Experimental methods

Design of the air actuator

The system comprised a PDMS microfluidic device with channels for medium and air and a commercial mechanical actuator to mimic the structure of the oviduct (Fig. 1A). The device was placed in a humidified incubator at 37 °C, and the mechanical actuator was placed outside the incubator (Fig. 1B). A syringe for impelling air was connected to the mechanical actuator to control air pressure inside the PDMS channel and to deform the membrane.

Performance of the air actuator

The components of the mechanical drive system included a controller, a motor (made by our laboratory), and a plastic syringe (Yuandong Co., Ltd., Changzhou, P. R. China). The motor was driven by the controller. The mechanical system is illustrated in Fig. 2A. A representative motion cycle of the actuator is shown in Fig. 2B. The motion of the syringe is reciprocal to that of the actuator, with the syringe stroke considered as a linear uniform motion (Fig. 2B). The reciprocal motion of the syringe continued until the power was turned off. We confirmed continuous motion of the device for 72 h of culture.

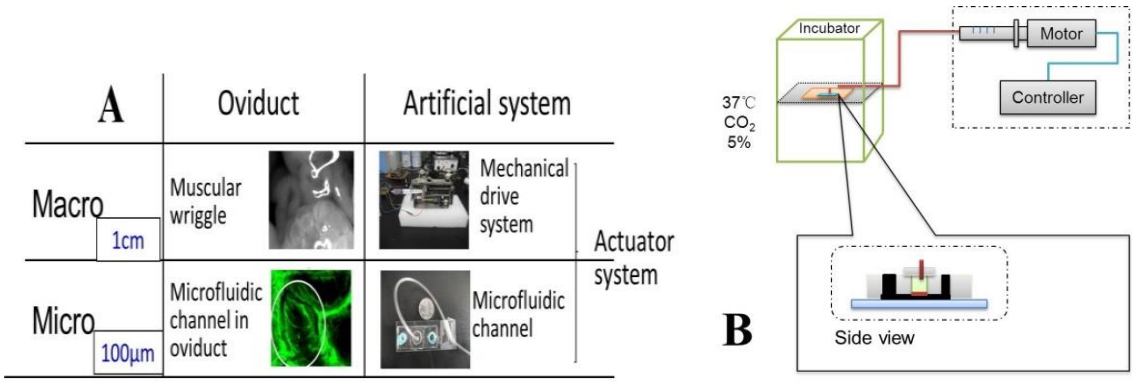

Fig. 1 (A) Summary of the oviduct and artificial system; (B) Schematic view of a system combining the mechanical drive system and PDMS microfluidic channel [8].

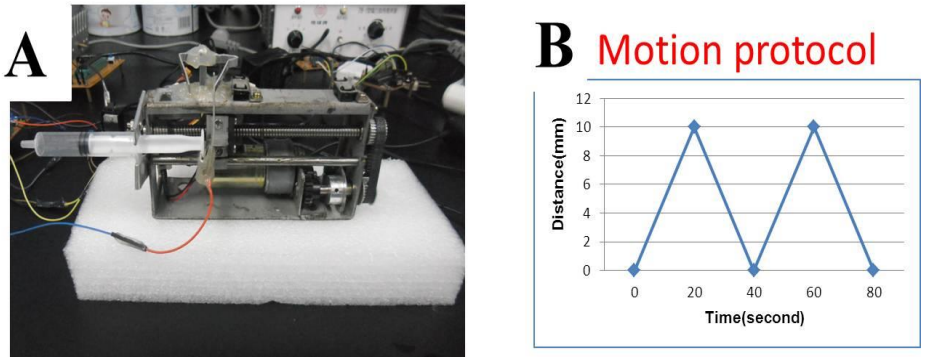

Fig. 2 (A) Picture of mechanical drive system (Picture of 5 ml plastic syringe with air combined with mechanical system); (B) Representative motion protocol.

Preparation of the PDMS microfluidic device

The microfluidic device consisted of a reservoir unit with a 0.1-mm-thick membrane and a microfluidic channel unit manufactured by glass corrosion carving. The reservoir units were fabricated by soft lithography of PDMS (Fig. 3). The mold of the reservoir unit was made of poly(methylmethacrylate) (PMMA) and prepared using a microdrilling device. Liquid PDMS, (Sylgard-184 Silocone Elastomer) free of air bubbles was injected into the PMMA mold using a vacuum pump for 10 min. A glass slide was used to cover the liquid PDMS in the mold under normal air pressure to prepare the membrane. The liquid PDMS was cured in an incubator at 70 °C for at least 1 h [2]. To cover the medium with sterile mineral oil, PMMA pipes were connected to the cured PDMS mold (Fig. 3A, medium reservoir).

Preparation of mouse embryos and protocol for culturing in the microfluidic channel

Two-cell-stage embryos recovered from Kunming mice following IVF were used in the culture experiments. The culture medium was covered with sterile mineral oil and incubated in a humidified environment of 5% CO_2 in air at 37 °C for 72 h to grow blastocysts [15]. The syringe velocity was set at 0.05-1.0 mm/s and SS was set at 0.03-0.9 dyn/mm^2 [8].

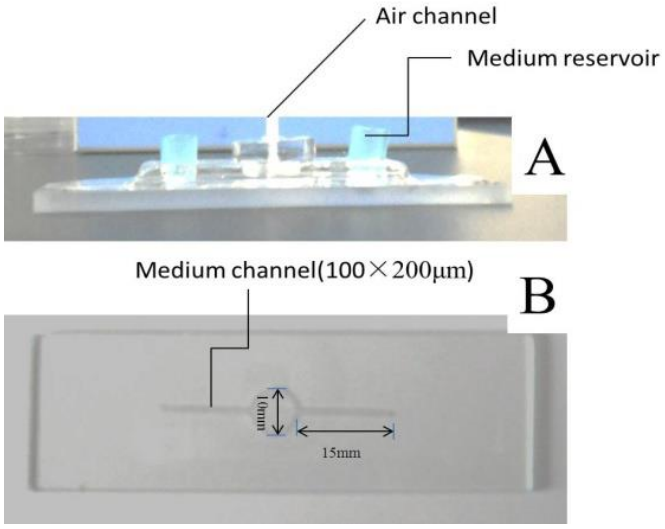

Fig. 3 (A) Side views of the PDMS microfluidic channel, respectively;
(B) Presentation of the top view of the channel.

Experimental designs

Effects on the rate of early embryo development in vitro

Embryos were divided randomly into two groups: the control group was subjected to conventional microdroplet culture in mCZB medium with 5 mM glutathione; the experimental group was placed in the microfluidic chip for embryo culture with the same medium. The results of the two groups were compared statistically.

Effects on inner cell mass (ICM) cell numbers in mouse blastocyst

Blastocysts from the control and microfluidic chip groups were stained with Hoechst 33342 for DNA, and the cell numbers of the ICM were observed and recorded using fluorescence microscopy (Fig. 4).

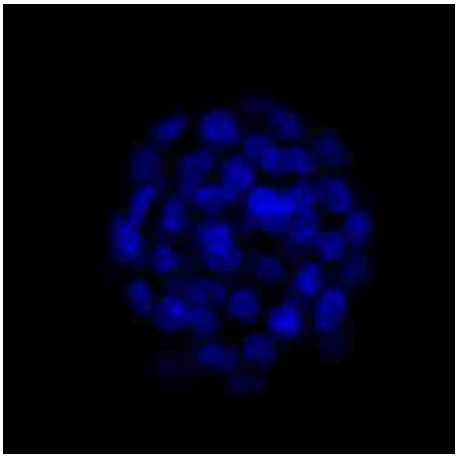

Fig. 4 Blastocyst from microfluidic chip group
was stained with Hoechst 33342

Statistical analysis

Data from four replicated trials were analyzed by one-way analysis of variance (ANOVA) using STATVIEW software (Abacus Concepts, Inc., Berkeley, CA, USA). If the *p* value was less than 0.05 in ANOVA, Tukey-Kramer Honest Significant Difference (HSD) tests were

carried out using the same program. All data are expressed as the mean ± the standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Effects on the rate of early embryo development in vitro

Table 1 shows that there were large difference in development rates of > 4-cell stage embryos between the control and microfluidic chip groups ($p < 0.05$). There were also significant increases in the rates of development to the morula (80.6 ± 17.9% vs 63.1 ± 12.5%) and blastocyst stages (73.3 ± 2.8% vs 58.6 ± 2.3%) in the microfluidic chip group compared with controls ($p < 0.05$). Clearly, using the chip system improved late mouse embryo development *in vitro*. One reason was that toxic products of embryo metabolism were probably eliminated under MS conditions. Another factor was that growth factors from embryos were probably distributed more efficiently [1, 12]. Microfluidic devices offer the ability to replenish culture media and remove harmful byproducts, which is not possible with simple agitation/tilting devices. Furthermore, the major advantages of these fluid-flow systems lie in their ability to automate the flow of medium and allow its replenishment in a seamless fashion, without manipulations outside the incubator and associated stresses, which can be detrimental during manual exchange [6].

Table 1. Effect of a microfluidic chip with mechanical stimuli for embryo culture on the rate of embryo development *in vitro*

Items / Group[2]	Number of embryos examined	Percent (%)[1] of 2-cell stage embryos developed to		
		> 4-cell stage	morula stage	blastocyst stage
Control	115	78.5 ± 2.8^{b}	63.1 ± 12.5^{b}	58.6 ± 2.3^{b}
Microfluidic chip	128	95.2 ± 7.5^{a}	80.6 ± 17.9^{a}	73.3 ± 2.8^{a}

[1]Percentage based on the total number of embryos examined.
[2]Control was static droplet culture group, which was 20-30 embryos in 50 µl medium. A microfluidic chip with mechanical stimuli was used for embryo culture in microfluidic chip group. Data are given as mean ± SEM from four replicated experiments. Values with different superscripts within column are significantly different ($p < 0.05$).

Dynamic fluid movements might benefit embryo development by the removal of harmful metabolic byproducts, replenishment of substrates, disruption of environmental gradients, and physical stimulation or activation of signaling pathways [13]. We demonstrated here that medium flowing with a velocity of 0.05-1.0 mm/s during culture had a favorable effect on mouse embryo development. However, it is important to note that the pattern and intensity of such movements are critical factors that can influence fluid dynamics and thus embryo development. Embryos can sense SS, and excessive MS can damage cells [18]. The flowing environment applied in this study might have been too intense, causing SS or excessive displacement of beneficial embryo-secreted factors. The pattern of fluid movement should also be considered in achieving an optimal dynamic culture environment. Matsuura [10] utilized a rocking embryo culture system with various angles at various speeds to try to mimic forces experienced by embryos *in vivo*.

As promising as dynamic culture appears, one of the limitations of these systems is their relative complexity in comparison with static culture devices. Several criteria and

considerations must be met before any dynamic culture system receives widespread implementation, and these include ease-of-use and lab compatibility. Biocompatibility is also paramount. When dealing with dynamic systems, there is a specific concern with SS [5]. SS over 1.2 dyn/cm^2 resulted embryo degeneration within 12 h [18]. In this study, SS was set at 0.03-0.9 dyn/mm^2 and the mouse embryos did not degenerate. Of course, movement or flow rates as well as the pattern of movement (e.g., motion paths, continuous or periodic) are obvious factors influencing SS. Another approach to culturing embryos within microfluidic devices employed not only dynamic media flow rates, but also co-culture. Wang et al. [16] adopted a "womb-on-a-chip" design, in which endometrial cells are grown in a lower chamber, while embryos are cultured in an upper chamber, separated from the lower by a thin membrane, thus allowing embryos to interact with secreted factors from the endometrial cells. In that preliminary study, the authors demonstrated that mouse oocytes fertilized in and cultured in these devices showed similar cleavage to the 2-cell stage and similar blastocyst formation rates compared with control microdrop cultures and the results were similar to Hickman´s results (2002) [7].

Effects on ICM cell numbers in blastocysts

In this experiment, there were significant increases in blastocyst ICM cell numbers in the experimental group compared with controls ($p < 0.05$) (Table 2). Thus, mechanical forces affected the ICM cell numbers in this microfluidic chip system [6].

Table 2. Effect of a microfluidic chip with mechanical stimuli for embryo culture on the cell number of ICM in mouse blastocyst

Group[1]	Number of embryos examined	Number of ICM
Control	46	38.3 ± 6.5^a
Microfluidic chip	58	48.4 ± 6.3^b

[1]Control was static droplet culture group. A microfluidic chip with mechanical stimuli was used for embryo culture in microfluidic chip group.
Data are given as mean ± SEM from four replicated experiments.
Values with different superscripts within column are significantly different ($p < 0.05$).

The microfluidic embryo culture system provides a unique physical environment for embryo culture, in which the constricted culture space more closely mimics the *in vivo* situation compared with a standard microdrop embryo culture. Using this microfluidic system, we could culture mouse embryos with success rates and blastocyst development rates and quality exceeding that of our standard microdrop system. This microfluidic chip with MS resulted in a significantly higher development rates of 2-cell mouse embryos to the morula and blastocyst stages, while producing higher numbers of normal embryos compared with controls; this result was similar to a previous report [17].

Microfluidic embryo culture systems have been used successfully for the culture of pig embryos produced by parthenogenetic activation and resulted in significant improvement in blastocyst formation [18, 19]. They also resulted in significant improvements in the blastocyst formation for *in vivo*-derived mouse zygotes and human embryos [11]. Our study also demonstrated that there were improvements in the rates of blastocyst formation and ICM blastomere numbers produced from a microfluidic chip embryo culture system compared with that of a conventional microdrop culture system.

Conclusions

We used a microfluidic chip with MS to culture mouse embryos *in vitro*. This mimics the peristaltic movement of the oviduct. The culture device could significantly improve the quality and blastocyst development rate of Kunming mouse embryos *in vitro*. This novel and simple system is feasible for generating and applying MS similar to those generated inside the oviduct for cultured mammalian embryos.

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