



# A modified hydrostatic microfluidic pumpless device for *in vitro* murine ovarian tissue culture as research model for fertility preservation

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This study aimed to compare the efficacies of conventional and non-conventional (modified hydrostatic microfluidic pumpless device, MHPD) systems on ovarian tissue culture and *in vitro* follicle growth using a mouse model. A total of 56 ovarian cortical tissues retrieved from seven wild-type mice were divided into three groups: 1) fresh control, 2) conventional culture system (control), and 3) non-conventional system with MHPD. Ovarian tissues were cultured for 96 hours and evaluated for follicle morphology, developmental stage, intact follicle density, and relative gene expression levels (proliferating cell nuclear antigen, insulin like growth factor 1, BAX, and Bcl-2). Our major data demonstrated that the mean percentage of primary follicle development was increased by the MHPD ( $P < 0.05$ ). In addition, this device could maintain and support follicle development better than the conventional culture systems. However, the overall outcomes were not significantly improved by our first-design prototype. Consequently, next-generation platforms should be developed as alternative medical tools for fertility preservation research.

**Keywords:** Ovarian follicle; *In vitro* technique; Crofluidics; Fertility preservation

## Introduction

Fertility preservation in cancer patients of childbearing age has grown expeditiously over the last decade [1,2]. Among the established fertility preservation techniques in women [1-3], compared to ovarian tissue transplantation, *in vitro* follicle growth (IVG) by ovarian tissue culture was recently introduced as an alternative approach to restore fertility without malignancy reintroduction [4]. In this method, ovarian cortical strips are cultured as flattened sheets under supporting matrix conditions [4-6]. The sequential change in culture media daily or on consecutive days is generally performed (static culture system) [7]. Although this technique allows tissues to maintain their microenvironmental properties, the static culture system lacks medium circulation that mimics natural vascularization [8]. To improve the culture system, non-static culture systems such as microfluidics have recently been introduced [8,9]. Conceptually, microfluidics is an engineered design of microscale devices that precisely

manipulates fluid-flow dynamics using an external pumping machine [10]. A promising approach using microfluidics in reproductive medicine research was revealed in mice when the designed platform adequately supported isolated follicle growth and completed the first meiotic division [8]. In humans, the pre-antral follicle diameter was maintained after 8

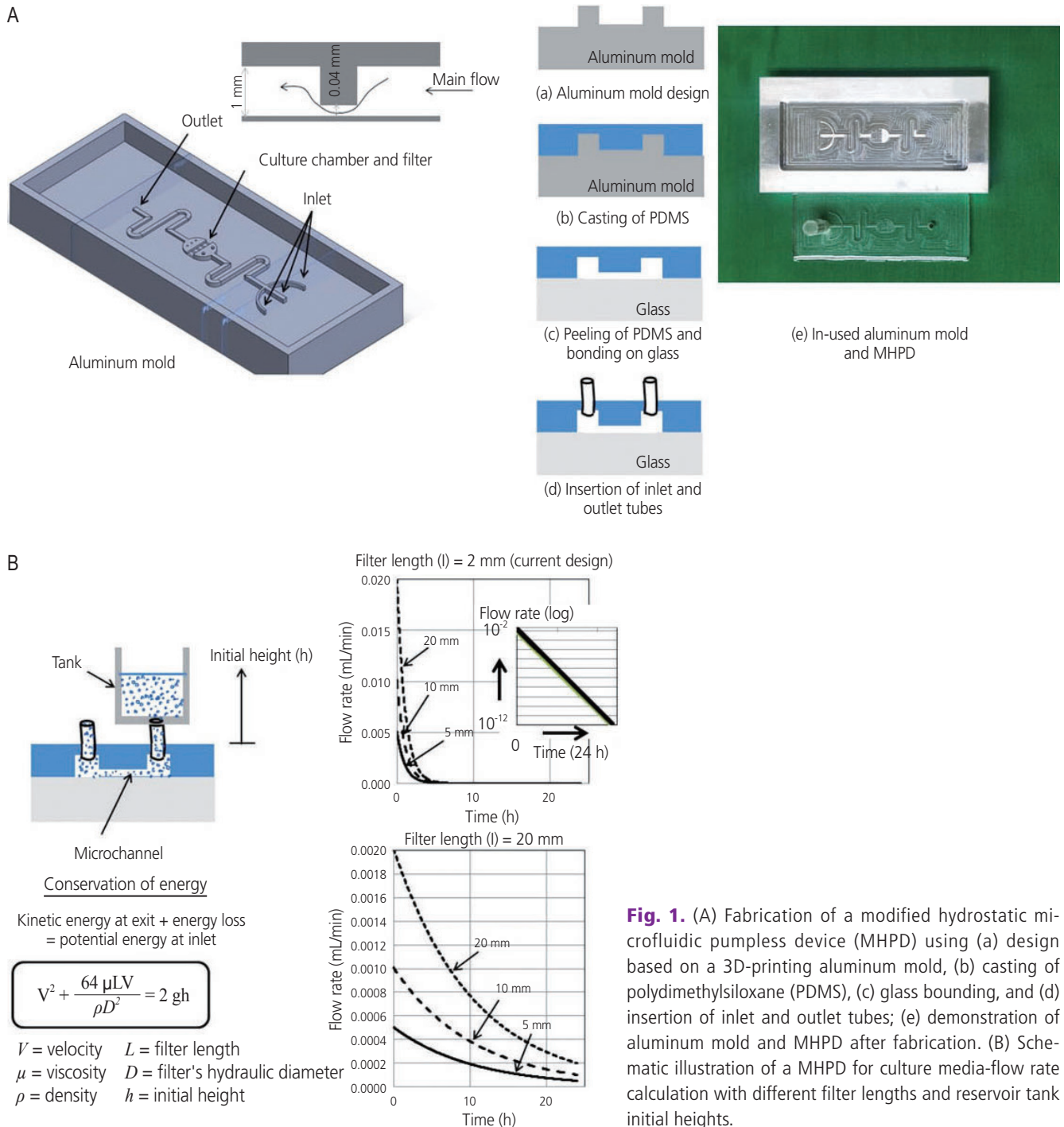
Received: 2022.01.03. Revised: 2022.03.07. Accepted: 2022.03.22.  
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days of *in vitro* culture using microfluidics compared to that with the static culture system [11]. However, *in vitro* ovarian tissue culture using microfluidics in human research models has not been reported. Additionally, the requirement for an external syringe pump in the currently designed microfluidic platforms presents disadvantages, that is, vast amounts of

culture media is filled in the syringe or culture media temperature is uncontrollable [8,11]. Hence, our study aimed to preliminarily investigate the efficacy of microfluidics driven by a hydrostatic force (syringe pump-free device or modified hydrostatic microfluidic pumpless device [MHPD]) on *in vitro* ovarian tissue culture and IVG using mice as the research model.



**Fig. 1.** (A) Fabrication of a modified hydrostatic microfluidic pumpless device (MHPD) using (a) design based on a 3D-printing aluminum mold, (b) casting of polydimethylsiloxane (PDMS), (c) glass bounding, and (d) insertion of inlet and outlet tubes; (e) demonstration of aluminum mold and MHPD after fabrication. (B) Schematic illustration of a MHPD for culture media-flow rate calculation with different filter lengths and reservoir tank initial heights.

## Materials and methods

### 1. Animals, experimental design, and chemical substances

Wild-type institute of cancer research female mice at the an-oestrous stage (age, 4 weeks; n=7) were caged under standard conditions. Ovaries were immediately collected from euthanized animals that were donated by another research project (approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University, Thailand). The ovaries were transported to the laboratory within 30 minutes. Eight ovarian cortical tissues from each mouse (total of 56 tissue fragments with an average size of 1.0×1.0×1.0 mm) were randomly divided into three groups (fresh control, conventional culture system [experimental control], and non-conventional system [MHPD]) and cultured for 96 hours. For the control, the tissues were placed on top of 1.5% (w/v) agarose gel submerged with 500 µL of culture medium (α-MEM medium with 5% fetal bovine serum, 1 IU/mL penicillin-streptomycin, 0.05 mM ascorbic acid, 0.5 µg/mL insulin, 0.4 µg/mL transferrin, 0.5 ng/mL selenium, 100 mIU/mL follicle-stimulating hormone, and 0.1% polyvinyl alcohol) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The culture medium was replaced every alternate day. For MHPD, ovarian tissues were placed in the device culture chamber. A total of 1 mL of the culture medium was loaded into the tank reservoir and refilled every other day. The evaluation criteria were performed on days 1 and 5: histology (follicle morphology, developmental stage, and density) [12] and relative

gene expression levels (proliferating cell nuclear antigen [PCNA], insulin like growth factor 1 [IGF-1], BAX, Bcl-2, and glyceraldehyde-3-phosphate dehydrogenase) by quantitative real-time polymerase chain reaction (quantitative polymerase chain reaction).

All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2. Fabrication of the microfluidic device

The device was fabricated with polydimethylsiloxane using a soft lithography technique, and mainly comprised media flow channels (inlet and outlet) and a culture chamber (Fig. 1). The beginning of the inlet flow channel (1.0×1.0 mm; width×height) was connected to the culture media tank reservoir (cutting edge, 1 mL tuberculin syringe; 1 cm-height), whereas the terminal end of inlet flow channel was connected to the culture chamber. The semi-circular culture chamber was of 7.0 mm and 1.0 mm (width×height), connected with five filter tubes (0.5×0.04×2.0 mm; width×height×length) and a waste reservoir. When the tank reservoir was completely filled, the legitimated flow rates were as follows: 0-8 hours, 10<sup>-4</sup>-10<sup>-1</sup> mL/min; 8-16 hours, 10<sup>-8</sup>-10<sup>-4</sup> mL/min; and 16-24 hours, 10<sup>-12</sup>-10<sup>-8</sup> mL/min (Fig. 1). The flow rate changed significantly when the length of the filter tube was varied. For example, the flow rate varied between 0.0002-0.002 mL/min when the filter length was changed to 20 mm.

### 3. Statistical analysis

Data analyses were performed using the Statistical Analysis

**Table 1.** Follicle quality assessment and developmental stages in mouse ovarian tissues that were cultured *in vitro* using two different systems, static culture (control) and non-static culture (MHPD) systems (n=7 animals)

Parameter	Fresh control (day 1)	<i>In vitro</i> culture (day 5)	
		Control	MHPD
Follicle quality			
Morphologically normal follicles (%)	79.8±1.3 <sup>a)</sup>	73.9±2.1 <sup>b)</sup>	71.5±1.5 <sup>b)</sup>
Follicle density (number of intact follicles/mm <sup>2</sup> )	31.6±1.3 <sup>a)</sup>	27.9±0.6 <sup>b)</sup>	27.6±1.9 <sup>b)</sup>
Follicle developmental stage			
Primordial follicle (%)	59.7±1.5 <sup>a)</sup>	55.1±2.4 <sup>a)</sup>	54.7±3.2 <sup>a)</sup>
Primary follicle (%)	22.6±3.7 <sup>a),b)</sup>	26.8±3.6 <sup>a),b)</sup>	28.9±2.8 <sup>b)</sup>
Preantral follicle (%)	17.7±3.5 <sup>a)</sup>	18.1±3.7 <sup>a)</sup>	16.4±2.9 <sup>a)</sup>

Values are presented as mean±standard deviation.

MHPD, modified hydrostatic microfluidic pumpless device.

<sup>a),b)</sup>Different letters indicate statistical differences within the same row (*P*<0.05).

Systems software package (SAS 9.2, SAS Institute Inc, Cary, NC, USA). When data were normally distributed, differences in dependent variables between groups were compared using a *t*-test. Non-normally distributed data were analyzed using the Wilcoxon signed-rank test. All results are presented as the mean±standard deviation. The level of significance was set at  $P\leq 0.05$ .

## Results

After ovarian tissue culture, the percentage of structurally normal follicles was significantly decreased during the culture period in both systems ( $P<0.05$ ) (Table 1). The normal follicle morphology in the MHPD system on day 5 was ~2% lower than the control that of the control (static culture system). However, markedly different data were not observed between the groups (Table 1). In accordance with the follicle morphology observations, our findings indicated that the average of four intact follicles per 1 mm<sup>2</sup>-ovarian tissue area declined after culture ( $P<0.05$ ). Regarding follicle developmental stage, the ratio between primordial follicles: primary stage follicles was slightly increased by the culture period (fresh control group [day 1], 1.0:0.37; control group [day 5], 1.0:0.48; MHPD group [day 5], 1.0:0.53). Although the percentage of primary follicles was increased by the MHPD culture system (day 1 vs. day 5) ( $P<0.05$ ), this device could only maintain follicle development comparable to that of the control (Table 1).

The relative expression levels of target genes related to cell proliferation (PCNA), granulosa cell differentiation (IGF-1), and cell apoptosis (BAX and Bcl-2) were significantly increased by culture time (days 1 to 5) ( $P<0.05$ ). An increase was observed in the expression levels of all target genes with average increases of 1.4 (PCNA), 1.6 (IGF-1), 1.4 (BAX), and 1.7 (Bcl-2) times in the control cohorts, and of 1.5 (PCNA, BAX, and Bcl-2) and 1.6 (IGF-1) times in the MHPD group. However, relative gene expression values did not differ between the two culture systems.

## Discussion

Our data from a mouse research model suggested that the current straightforward platform could maintain follicle vi-

ability, developmental competency, and gene expression levels related to cell proliferation and differentiation pathways, at levels comparable to those of the traditional static culture conditions. In the present study, microfluidics with hydrostatic pressure as the driving force to facilitate dynamic media flow was modified based on studies on human isolated follicles and mouse testicular tissue cultures [9,11]. Using this system, the culture media was stored in the reservoir tank and flowed through the microscale channels steadily (the average flow rate was 2  $\mu\text{L}/\text{min}$  with a filter size of 2 mm). In male mice, development of round spermatid was accomplished with testicular tissue culture [9]. The development competency of mouse oocytes was enhanced by the passive dynamic culture system compared to that with the active dynamic system with an external pump or static control [13]. The contradictory results between the previous and present studies might be attributed to the inconsistent flow rate because of hydrostatic pressure, which was occasionally 2 to 4 times higher than that reported previously (1  $\mu\text{L}/\text{min}$  [13] and average 0.5  $\mu\text{L}/\text{min}$  [9]). A study on human embryonic stem cell-derived mesenchymal progenitors (human mesenchymal stem cells, hMSCs) categorized media flow rate into three levels: slow, fast, and rapid (12, 120, and 600  $\mu\text{L}/\text{min}$ ) [14]. The results showed that optimal dynamic flow prominently influenced hMSCs function [14]. In addition, the thickness of each tissue culture was noted. A previous study indicated that an optimal thickness of mouse testicular tissue is a prerequisite for round spermatid development during long-term culture [15]. The major explanation is related to the physiology of the natural thickness of the seminiferous tubules in mice [15], whereas a thickness of 1 mm of the ovarian cortex is suitable for pre-antral follicle growth. Owing to the lack of data on the influence of dent depth that dictates ovarian tissue thickness during *in vitro* culture, this aspect should be thoroughly considered. Consequently, a good agreement should be maintained between the device design, dynamic flow rate, and cell or tissue types to mimic the optimal *in vitro* microenvironment and gas and nutrient exchange for cell growth and development.

In conclusion, the present study preliminarily addressed the application of a pump-free microfluidic device for ovarian tissue culture and IVG in a mouse model. Regarding the fabrication of biocompatible devices with the designed dynamic flow, the current MHPD platform can only maintain follicle development competency. However, this device has

some advantages, including simple architecture, ease of use, and medium-temperature control. However, a more accurate control of the flow rate using a well-designed resistance circuit (length, height, and width), tank reservoir, or insertion of an internal electromagnet valve should be further developed to create an adequate milieu for long-term culture. This promising technology presents a clear opportunity for cancer patients to preserve their fertility.

## Conflict of interest

The authors declare no conflicts of interest.

## Ethical approval

Institute of Animal Care and Use, Faculty of Medicine, Chulalongkorn University

## Patient consent

None.

## Funding information

This study was financially supported by the Research Unit of Reproductive Medicine and Fertility Preservation, Faculty of Medicine, Chulalongkorn University, Thailand.

## Author contributions

All authors designed the experiments while A.P. and P.T. designed microfluidic device. P.T. and F.T. performed the experiments. P.T., A.P. and P.S. prepared and revised the draft of manuscript.

## References

1. Ataman LM, Rodrigues JK, Marinho RM, Caetano JP, Chehin MB, Alves da Motta EL, et al. Creating a global

community of practice for oncofertility. *J Glob Oncol* 2016;2:83-96.

2. Anderson RA, Wallace WHB, Telfer EE. Ovarian tissue cryopreservation for fertility preservation: clinical and research perspectives. *Hum Reprod Open* 2017;2017:hox001.

3. Obermair A, Baxter E, Brennan DJ, McAlpine JN, Mueller JJ, Amant F, et al. Fertility-sparing treatment in early endometrial cancer: current state and future strategies. *Obstet Gynecol Sci* 2020;63:417-31.

4. Telfer EE. Future developments: in vitro growth (IVG) of human ovarian follicles. *Acta Obstet Gynecol Scand* 2019;98:653-8.

5. Smits J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update* 2010;16:395-414.

6. Filatov MA, Khramova YV, Kiseleva MV, Malinova IV, Komarova EV, Semenova ML. Female fertility preservation strategies: cryopreservation and ovarian tissue in vitro culture, current state of the art and future perspectives. *Zygote* 2016;24:635-53.

7. Salama M, Woodruff TK. From bench to bedside: current developments and future possibilities of artificial human ovary to restore fertility. *Acta Obstet Gynecol Scand* 2019;98:659-64.

8. Xiao S, Coppeta JR, Rogers HB, Isenberg BC, Zhu J, Olanakan SA, et al. A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle. *Nat Commun* 2017;8:14584.

9. Komeya M, Hayashi K, Nakamura H, Yamanaka H, Sanjo H, Kojima K, et al. Pumpless microfluidic system driven by hydrostatic pressure induces and maintains mouse spermatogenesis in vitro. *Sci Rep* 2017;7:15459.

10. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature* 2014;507:181-9.

11. Aziz AUR, Fu M, Deng J, Geng C, Luo Y, Lin B, et al. A microfluidic device for culturing an encapsulated ovarian follicle. *Micromachines (Basel)* 2017;8:335.

12. Lo BKM, Sheikh S, Williams SA. In vitro and in vivo mouse follicle development in ovaries and reaggregated ovaries. *Reproduction* 2019;157:135-48.

13. Sadeghzadeh Oskouei B, Pashaiasl M, Heidari MH, Salehi

- M, Veladi H, Ghaderi Pakdel F, et al. Evaluation of mouse oocyte in vitro maturation developmental competency in dynamic culture systems by design and construction of a lab on a chip device and its comparison with conventional culture system. *Cell J* 2016;18:205-13.
14. Campos Marín A, Brunelli M, Lacroix D. Flow perfusion rate modulates cell deposition onto scaffold substrate during cell seeding. *Biomech Model Mechanobiol* 2018;17:675-87.
15. Komeya M, Yamanaka H, Sanjo H, Yao M, Nakamura H, Kimura H, et al. In vitro spermatogenesis in two-dimensionally spread mouse testis tissues. *Reprod Med Biol* 2019;18:362-9.