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A monolayer microfluidic device supporting mouse spermatogenesis with improved visibility

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ABSTRACT

In our previous study, we produced a microfluidic device (MFD) which successfully maintained spermatogenesis for over 6 months in mouse testis tissues loaded in the device. In the present study, we developed a new MFD, a monolayer device (ML-D) with a barrier structure consisting of pillars and slits, which is simpler in design and easier to make. This ML-D was also effective for inducing mouse spermatogenesis and maintained it for a longer period than the conventional culture method. In addition, we devised a way of introducing sample tissue into the device during its production, just before bonding the upper layer of polydimethylsiloxane (PDMS) and bottom glass slide. The tissue can obtain nutrients horizontally from the medium running beside it and oxygen vertically from above through PDMS. In addition, the glass slide set at the bottom improved the visibility of the sample tissue with an inverted microscope. When we took photos of cultured tissue of the *Acr-Gfp* transgenic mouse testis in ML-D sequentially every day, morphological changes of the acrosome during spermiogenesis were successfully recorded. The ML-D is simple in design and useful for culturing testis tissue for inducing and maintaining spermatogenesis with clearer visibility. Due to the new method of sample loading, tissues other than testis should also be applicable.

1. Introduction

In 2011, we cultured mouse testis tissues with the classical organ culture method and succeeded in the production of fertile sperm from spermatogonial stem cells [1,2]. In addition, testis tissue could be cryopreserved, and offspring were produced with sperm grown *in vitro* from the cryopreserved samples [3]. However, the efficiency and duration of this *in vitro* spermatogenesis were low and limited, respectively, being totally incomparable with those *in vivo*. In order to improve the culture conditions, we adopted microfluidic technology in our testis organ culture system. The material of the MFD has been polydimethylsiloxane (PDMS) since the 1990s, which has the advantages of marked gas-permeability and plasticity for fabricating minute and delicate designs [4]. Thus, MFDs have been applied for handling and culturing cells from a variety of sources [5–7]. Recently, tissue specimens, such as brain or liver slices, have also been cultured in MFDs to maintain their function more favorably than conventional culture methods [8,9]. In our trial to introduce the microfluidic system, we initially placed testis tissue in the microchannel where medium was flowing. In that experiment, however, spermatogenic induction was poor. On the contrary, we found that testis tissues separated from the flowing medium showed the better progression of spermatogenesis. Then, we manufactured a MFD which incorporated a porous membrane (PM) that separates the cultured tissue and a flowing medium [10]. This initial device, here named membrane-based multilayer device

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(MM-D), produced culture conditions suitable for testis tissue, which led to the more efficient spermatogenesis and its maintenance for periods as long as over 6 months. There are three possible advantages that the MM-D has conferred on the culturing environment: efficient molecular exchange through PM between the flowing medium and tissue owing to the wide-flat design of the tissue space, enhanced para- and auto-cline effects through the isolation of tissue by PDMS and PM, and lowered oxygen toxicity allowing adequate supply of oxygen owing to the sealing in PDMS. We regarded these advantages as essential features of our MFD. In the present study with the aims of producing another type of MFD that is also suitable for testis organ culture, we markedly changed the device design from MM-D. In the MM-D, nutrients come to the tissue from flowing medium running above, while oxygen comes mainly from below through PDMS. In the new device presented below as ML-D, we changed the flow direction of these: Namely, nutrients come horizontally and oxygen vertically. This basic design of ML-D might be reminiscent of some devices previously reported [11–14]. All of them, however, were used for cell cultures. It was not sure if ML-D works for culture of the tissues, particularly for the testis tissue. Naturally, change of design leads to a trade-off, bringing advantages and disadvantages. Through the present study, we found that the ML-D offered the same efficiency regarding spermatogenesis induction and maintenance. In addition, it has become possible to set a glass slide at the bottom of the tissue space, which would make visualization of the cultured tissue finer and clearer with an inverted microscope. These results indicated that ML-D is another useful MFD for testis organ culture.

2. Materials & methods

2.1. Device fabrication

The MFD was produced by conventional photolithography and soft lithography techniques [15–17]. A mold master made of a negative–type photoresist material (Micro Chem Co., SU-8 2100) was prepared, serving as a mold for the production of the upper PDMS layer (Fig. 1A). The SU-8 was first poured on a 4-inch wafer and spin-coated over it to achieve the target thickness over the wafer. They were then pre-baked, and patterned by UV light exposure through a photomask for several seconds to carve a pattern of channels, followed by post-baking. The photomask was designed with CAD software (AutoCAD: Autodesk, Inc.) and fabricated with a laser lithography system. The baked mold master was developed by incubation in (1-methoxy-2-propyl) acetate (Godo/Tokyo/Japan) for 10 min, followed by rinsing in isopropanol (Kanto Chemical/code 32435-70/Kanto/Japan) for 3 min. For the bottom layer, a plate of glass was used (Fig. 1A). For the production of each device, PDMS prepolymer was mixed with a curing reagent (Silpot 184, DowCorning) at a 10:1 wt ratio and poured over the mold master. After curing, solidified PDMS was peeled off the master. Holes were drilled into the upper layer of PDMS for the medium inlet and outlet. Silicone tubes were attached for the medium outlet and fixed by PDMS. A medium reservoir tank, which was the cut half of a polypropylene tube (TPP, TubeSpin® Bioreactor 15), was also fixed by PDMS at the medium inlet. The other end of the silicone outlet tube was connected to a fluorinated ethylene propylene tube (Dupont FEP tubing 1527, IDEX, inner diameter: 0.25 mm), which was connected to a disposable syringe set in a syringe pump (MFS-SP10X, Microfluidic System Works Inc.). White micro slide glass (Matsunami, S9111) was used as the glass bottom layer. It was cleansed by ultrasonic cleaning using Cicaclean LX-IV (Kanto Chemical Co., INC, 07593-82). Both upper and bottom layers were flushed with DDW to remove dusts and dried. After being sterilized by ethylene oxide gas, they were stored in a sterilization bag (AS ONE, PMB-6).

2.2. Animals

Acr-Gfp transgenic mice provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan were used as the testis tissue source [18]. The spermatogenic germ cells of this mouse express GFP at the middle-pachytene stage onward. The GFP in the cytoplasm gradually aggregates into the Golgi complex, which develops into an acrosome, observed as a GFP-dot adjacent to the nucleus. As acrosome formation proceeds during spermiogenesis, the GFP-dot shape changes into a cap-like and then skewed crescent-like form [19]. For mating, males homozygous for *Acr-Gfp* were used as sires, while females were either homozygous, heterozygous, or the wild-type. Mice were housed in a specific pathogen-free, air-conditioned room, maintained at $24 \pm 1\,^{\circ}C$ and $55 \pm 5\%$ humidity, with a 14-h-light and 10-h-dark lighting cycle. Commercially made hard pellets (MF, Oriental Yeast, Japan) were fed ad libitum. Drinking water was acidified to pH 2.8–3.0 using HCl. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committees of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

2.3. Culture of testis tissues

Testes of neonatal mice at 0.5–5.5 days postpartum (dpp) were used, except when otherwise stated. They were decapsulated and used whole or cut into several pieces for culturing (Fig. 1D–F). Before loading the sample tissues into the MFD, both upper and lower layers were placed in a plasma cleaner (Harrick Scientific, PDC-32G) initially to decompress for more than 30 min to remove gases solving in PDMS. This procedure helps to absorb and remove air bubbles trapped in the circuit, which could hinder the flow of medium [20]. After this degassing, the bonding surfaces of both upper and lower layers were treated with plasma for cleaning. Immediately, the sample tissue was set in the tissue chamber space of the upper PDMS sheet (Fig. 1G–I). Then, the bottom layer, glass slide, was bonded to the upper layer (Fig. 1J). After filling the tank with culture medium, it was drawn through the outlet by the syringe pump at 0.05 μL/min (Fig. 1K and L). The MFD was placed in an incubator, which was supplied with 5% CO_2 in air and maintained at 34 °C, while the syringe pump was set outside next to the incubator. Tissues for the control experiment using an agarose gel method (AG) were cultured on agarose stands (1.5% w/v) placed in wells of a 12-well culture plate (CELLSTAR® Tissue Culture Plates, Greiner Bio-One). Each gel was loaded with 1–3 testis tissue fragments. The amount of medium was adjusted so that it would come up to about half of the height of the agarose gel (approximately 0.5 mL/well). Medium change was performed once a week. The culture medium was α-minimum essential medium (α-MEM) (Invitrogen: 12000-022) supplemented with AlbuMAX (ThermoFisher Scientific: 11020021) at a 40 mg/mL final concentration.

2.4. Evaluation by live imaging

Tissues in culture were observed every 7 days under a stereomicroscope (Leica, Leica M 205 FA) to evaluate the GFP-expression area occupancy. Areas showing GFP expression per total area of each sample when observed from above, 0–100%, were scored visually. Pictures were also taken. An inverted microscope (Olympus IX 73; Olympus, Tokyo, Japan) was also used to observe

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