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Development of Dynamic Well Plate System for Cell Culture with Mechanical Stimulus of Shear Stress and Magnetic Field

Songwan Jin[1], Jae-Hun Kim[2], and Won-Soo Yun[1,#]

1 Department of Mechanical Engineering, Korea Polytechnic University, 237, Sangidaehak-ro, Siheung-si, Gyeonggi-do, 429-793, South Korea
2 Department of Mechanical System Engineering, Graduate School of Knowledge-Based Technology and Energy, Korea Polytechnic University, 237, Sangidaehak-ro, Siheung-si, Gyeonggi-do, 429-793, South Korea
Corresponding Author / E-mail: wsyun@kpu.ac.kr, TEL: +82-31-8041-0412, FAX: +82-31-8041-0419

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Investigating the effects of various physical stimuli on cells is important for improving the efficiency of tissue repair and regeneration. In this research, we developed a dynamic well plate system by integrating the advantages of conventional well plates and a bioreactor to provide simultaneous physical stimuli of shear stress and a static magnetic field. The dynamic well plate involving perfusion of culture medium can control hydrodynamic shear while retaining the inherent simplicity of conventional well plates. The specific well plate cover was designed to load shear stress on cells during cultivation and was built to fit over a standard six-well plate. Additionally, to investigate the effects of a magnetic field on cell proliferation, a static neodymium magnet was placed beneath each well. To assess the system developed, calf pulmonary artery endothelial (CPAE) cells were cultured using the developed system. CPAE cells under hydrodynamic shear stress conditions were elongated and aligned in the direction of the flow and the magnetic field enhanced CPAE cell proliferation. Simultaneous application of a magnetic field and shear flow in CPAE cell cultivation allowed the development of optimized culture conditions, initially for cell proliferation and then for functional expression, such as cell shape changes.

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NOMENCLATURE

EI = elongation index value
A = area of cell
P = perimeter of cell
τ = wall shear stress
Q_{out} = volume flux of media
μ = viscosity of media
r = radial position from center of a well of a well plate
h = distance between bottom surface of a plunger and top surface of a well

1. Introduction

Physical stimuli that mimic those to which a tissue is exposed in vivo play important roles in repair and regeneration of the tissue. Various responses to physical stimuli can be expected and the effects of the mechanical properties of developing tissue and cell division or differentiation rate have been addressed to date 1. The mechanical properties of developing tissues can be altered with or without proper physical stimuli, such as shear stress,[2-5] stretch force,[6] and compression.[7,8] Among these, shear stress resulting from blood flow on vascular endothelial cells has been shown to be an important regulator of vascular structure and function, such as elongation, proliferation, migration, and permeability. The effect of shear stress has been investigated using both macroscale[2,4] and microscale[3,5] systems. A macroscale system usually uses two plates (one moving and one fixed plate) to generate a constant shear stress. However, the set-up is complex and large and requires large amounts of culture medium and cells. Recently, microfluidic devices have been used. Due to laminar flow, characterized by low Reynolds numbers, constant and controlled shear flow can easily be generated in a microfluidic system. However, cell seeding and culturing inside a microchannel is delicate process that requires practice.

A static magnetic field is another important physical stimulus for tissue regeneration. A magnetic field can be classified by its intensity of magnetic flux density as weak (< 1 mT), moderate (1 mT to 1 T), strong (1~5 T), and ultrastrong (> 5 T). Moderate-intensity magnetic fields can result in increased proliferation and they have been studied as an important physical stimulus.[9,10] It is known that the static magnetic field can modulate signaling network[13] and biophysical properties of membranes[14] of the cell, and interact with moving charges[15] inside and outside the cell. Although there are several speculations on the mechanism and the effect of static magnetic field, it has remained unclear.

In this paper, a dynamic well plate system that can provide simultaneous stimuli of shear stress and a static magnetic field was developed to investigate the effects of those stimuli. One important characteristic and advantage of the developed system is the use of a conventional six-well plate. Conventional well plate culture systems are in widespread use because of their simplicity and well-established experimental protocols. However, the well plate is not ideal for controlling physical stimuli, such as shear stress, during cell culture. The use of bioreactors involving convective mixing and perfusion of culture medium enables precise control of the cellular microenvironment, which can enhance the effectiveness of cell and tissue cultures and mass transfer. However, such bioreactors are, in most cases, of much larger volume than well plates and their use requires extra technical skills, reagents, and equipment. Thus, in this study, we developed a dynamic well plate system, combining the advantages of the two culture systems, the well plate and the bioreactor. The cover of the dynamic well plate system was specifically designed to apply medium flow to cells and fit over a standard six-well culture plate. The cover contains plungers that extend into each well and are machined precisely to provide a defined shear stress to the cells. By simply replacing the well plate cover with the cover of the dynamic well plate, medium flow can be applied to the cells and hydrodynamic shear can be controlled.

2. Dynamic Well Plate System

2.1 System set-up

The dynamic well plate system consists of a multichannel peristaltic pump (520U, Watson Marlow Pump Ltd.), six-well plate, plunger, and neodymium magnet and magnet jig (Figs. 1(a), (b)). We replaced the existing six-well plate cover with the designed device, which consists of a plunger and micrometer head, as shown in Fig. 1(b). A narrow gap was created between the bottom surface of the plunger and the well plate. The medium, circulated by a peristaltic pump, flowed radially, from the edge to the center of plunger through the gap and the shear stress was controlled by changing the height of the gap or the flow rate of the medium. The gap was controlled by the micrometer head and the cover, which is assembled with the six-well plate. Using a multichannel peristaltic pump and six-well plate, a maximum of six different flow rates can be tested in one experiment.

The wall shear stress generated under current experimental conditions is calculated by[12]

$$\tau = \frac{3}{2}\mu\frac{Q_{out}}{\pi r h^2} \quad (1)$$

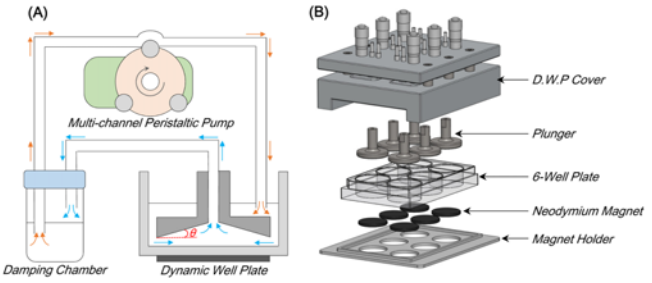

Fig. 1 Schematic illustration of the system (a) and dynamic well plate (b)

where Q_{out} is the volume flux of the medium, μ is the viscosity of the medium, r is the radial position, and h is the gap distance between the two plates. As indicated in Eq. (1), the shear stress is a function of r and h, and if the bottom surface of the plunger is parallel to the well plate, the shear stress decreases with an increase in r. To prevent this problem and generate a constant shear stress over a wide range of different radial positions, we designed the plunger to have a slope of θ, as indicated in Fig. 1(a). To determine the angle of plunger, we performed theoretical calculation of shear stress using Eq. (1) and 0.35~0.45 was the best angle to minimize shear stress variation over the wide range of r. We manufactured the angle of plunger to have that range and the slope angle of the manufactured plungers, as measured by a coordinated measuring instrument (Victor 101208, Duckin Co., Ltd., Korea), was 0.39±0.05°. The plunger and cover are made of stainless steel (SUS-316) and Teflon, respectively. Also, we added a damping chamber between a well and the channel of the peristaltic pump to reduce or remove pulsations generated by the peristaltic pump in the medium flow.

In addition, to stimulate the cultured cell with a magnetic field, a disk-shaped neodymium magnet fixed with a magnet jig was placed under each well of the plate. Test cells were cultured on the surface of the well plate. The distance between the upper surface of the neodymium magnet and the cell culture well plate surface was 1 mm.

2.2 Evaluation of the physical stimuli

Fig. 2 shows the physical stimuli generated by the dynamic well plate system. The theoretically calculated shear stress (using Eq. (1)) is shown as a solid line in Fig. 2(a) as a function of r when the flow rate was 64 mL/min and the gap between the plunger and the well plate at the edge of the plunger (h) was 180 μm. A numerical simulation was also performed using Comsol Multiphysics 4.2 (Comsol, Burlington, MA) to confirm the result of theoretical calculation. As shown in Fig. 2(a), there was no significant different between the theoretically and numerically calculated shear stress, and the shear stress was 1.0±0.2 Pa between r=8~14. In the human vascular system, typical shear stress on endothelial cells ranges widely, from 0.1 to 7 Pa.[1] The shear stress generated by the dynamic well plate system can cover that wide range by adjusting the gap distance and flow rate.

The magnetic flux density was measured on the bottom surface of the six-well plate with a gauss meter (TM-701PRB, Kanetec), as shown in Fig. 2(b). The measured magnetic flux density was 38~125 mT over the each well; thus, the magnetic flux density generated by system was

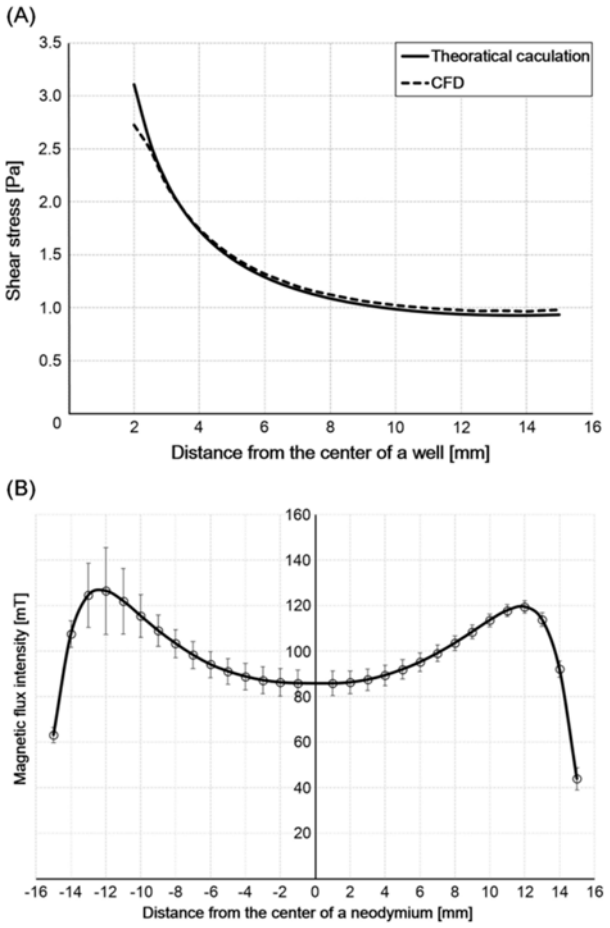

Fig. 2 Shear stress (a) and magnetic flux density (b) profile in the dynamic well plate with Q_{out} = 64 mL/min and h_e = 180 μm

classified as 'moderate' density. Also, we saw no sign of significant interference among the magnets.

3. Cell Culture using the Dynamic Well Plate System

3.1 Experimental procedure

Calf pulmonary artery endothelial (CPAE) cells were purchased from the Korea Cell Line Bank. Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin was used as the culture medium. All equipment was sterilized using an autoclave or cleaning with 70% ethyl alcohol followed by 12 h of UV exposure. In total, 120,000 CPAE cells were seeded in each well of a six-well plate and stabilized by incubating in the humidified incubator at 37°C with 5% CO_2 without physical stimuli for 6 h after seeding. After stabilization, the cells were exposed to shear stress and static magnetic fields by flowing culture medium at a constant flow rate and placing the neodymium magnets under the well plate, respectively. The whole dynamic well plate system, including the peristaltic pump, was placed inside the incubator to maintain culture conditions.

After 12 and 24 h of culture, CPAE cells were fixed using 3.7% formaldehyde and stained with Alexa Fluor 488 phalloidin to observe F-actin filaments and capture the morphology of the cells. In addition, at 0 h (after stabilization) cells were also fixed and stained using the same method.

3.2 Image processing

Morphological and numerical changes in the cells under dynamic culture conditions were evaluated by image processing. The images of cells at nine different positions between r = 8 and 14 with varied r were taken. An inverted microscope (Olympus, IX71) with a ×10 objective lens and SCMOS CCD camera (2400×1600, Andor) were used to capture images. The field of view of the image was approximately 1600×1300 mm^2.

For quantitative evaluation of degree of elongation of CPAE cells, elongation index (EI), a dimensionless parameter of cell roundness with value '0' for a perfect circle and '1' for a straight line, is defined as[11]

$$EI = 1 - \frac{4\pi A}{P^2} \tag{2}$$

where A is the area and P is the perimeter of the cell. The cell area, A, and perimeter, P, were measured with the ImageJ software using the images taken. The number of cells was also counted from each image.

3.3 Effects of physical stimuli of shear stress and a static magnetic field

To investigate the effects of shear stress and a magnetic field on cell culture, we divided the experimental group into single stimulus and multiple stimuli groups. Fig. 3 shows images of CPAE cells cultured under different conditions, including: (a) static culture (control group without physical stimulus), (b) single stimulus of shear stress (1.5 Pa), (c) single stimulus of moderate static magnetic field (38~125 mT), and (d) simultaneous stimuli of shear stress and moderate static magnetic field (1.5 Pa, 38~125 mT), for 0 (left panels), 12 (middle panels) and 24 (right panels) h. For quantitative evaluations of cell elongation and proliferation, the numbers of cell and elongation indexes, measured using images taken, are plotted in Figs. 4(a) and (b). As shown in Figs. 3(b) and 4(b), cell alignment and elongation along the flow direction increased with shear stress and these results are in accordance with many previous studies.[5] However, the number of cells decreased significantly. The number of cells decreased further after 24 h under shear stress (Fig. 3(b) right panel) whereas that in the static culture conditions increased (Fig. 3(a) right panel, Fig. 4(a)). This result seems reasonable because cells become detached when the adhesion force of the cells is not strong enough to withstand the forces generated by the shear stress.

Fig. 3(c) shows the effects of the magnetic field on cell proliferation. When the CPAE cells were exposed to the static magnetic field, proliferation was enhanced, compared with the control group, but there was no change in cell shape. These results can be confirmed by the elongation index, as shown in Fig. 4(b). The different cells show different responses to the static magnetic field[13] and it has been reported that the moderate static magnetic field can promotes proliferation and osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs).[16]

There was no significant change between the elongation index of the control cells and those exposed to the single stimulus of a static

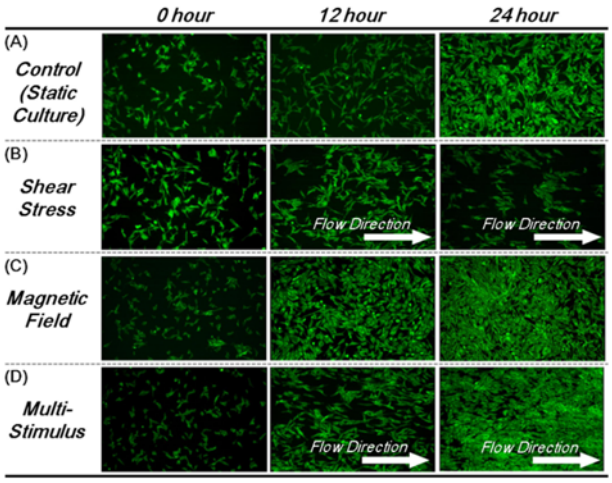

Fig. 3 Photographs of actin filaments of CPAE cells under static (a), single stimulus of shear stress (b), single stimulus of moderate magnetic flux (c) and simultaneous stimuli of shear stress and moderate magnetic flux (d)

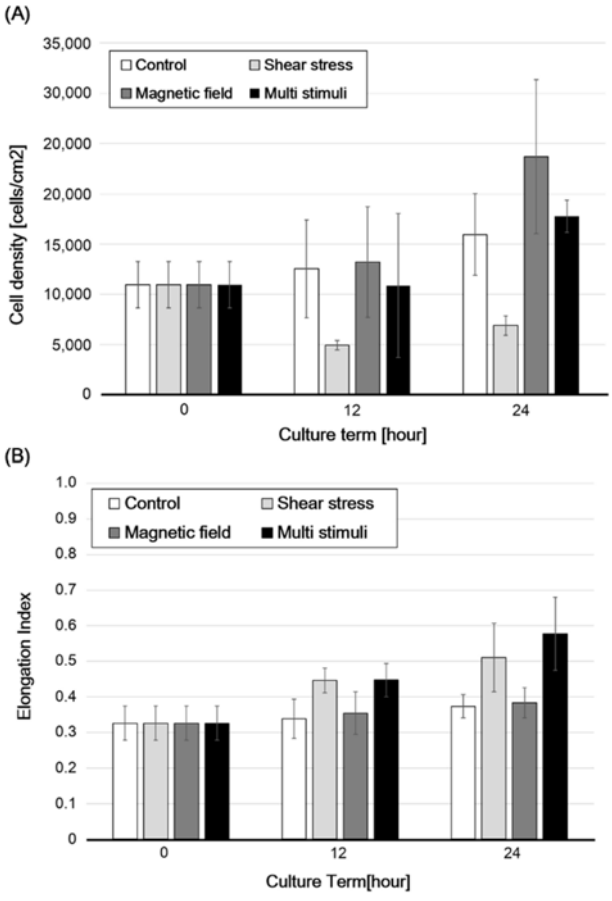

Fig. 4 Effects of physical stimuli on CPAE cell culture. (a) Number of cells. (b) Elongation index

magnetic field. Next, we examined the effects of both stimuli when applied simultaneously. After 24 h of culture, the number of cells in the multiple stimuli sample was less than that of the single stimulus of the static magnetic field. However, the number of cells was similar to that of the sample without physical stimulus and higher than with the single stimulus of shear stress. The elongation index of the multiple stimuli samples was the lowest of all samples. The elongation index of the multiple stimuli cells was smaller than that with the single stimulus of shear stress, indicating that CPAE cells in the multiple stimuli experiments were more elongated than with the single stimulus of shear stress.

4. Conclusion

We developed a dynamic well plate system that can be used for culturing cells with the stimuli of shear stress and a moderate magnetic field simultaneously in a standard six-well plate. We applied the individual and simultaneous stimuli of shear stress and a moderate magnetic field to CPAE cells using the dynamic well plate system developed. The simultaneous stimuli of shear stress and moderate magnetic field could change the cytoskeleton and elongate the CPAE cells without decreasing the cell number. The well plate system developed was easy to handle and user friendly. In addition, by investigating the effects of the simultaneous stimuli of shear stress and a magnetic field on CPAE cells, it was shown that the stimuli can affect growth of cells in the dynamic well plate system.

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