PULSATILE SHEAR STRESS AND HIGH GLUCOSE CONCENTRATIONS INDUCED REACTIVE OXYGEN SPECIES PRODUCTION IN ENDOTHELIAL CELLS

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ABSTRACT
A hemodynamic Lab-on-a-chip system was developed in this study. This system has two unique features: (1) it consists of a microfluidic network with an array of endothelial cell seeding sites for testing them under multiple conditions, and (2) the flow rate and the frequency of the culture medium in the microchannel are controlled by a pulsation free pump to mimic the flow profile of the blood in the blood vessel under different physiological conditions. The results demonstrate the advantage of utilizing this system over the conventional non-pulsatile system in the future shear stress related studies.

KEYWORDS
hemodynamic Lab-on-a-chip system, endothelial cell, reactive oxygen species

INTRODUCTION
Reactive oxygen species (ROS) are natural by-products of the normal aerobic metabolism in cells. The main source of ROS production is the mitochondrion during oxidative phosphorylation, which involves the electron transport chain. In the chain, electrons are passed through several proteins by different oxidation–reduction reactions. However, a small percentage of the electrons are leaked out from the mitochondrion electron transport chain, and subsequently superoxide anions are generated [1-2].

Hydrogen peroxide is a poorly reactive oxidizing agent but it is cell membrane-permeable [3]. It is generated by several enzymatic systems inside the cells. Under normal metabolic condition, hydrogen peroxide is involved in the regulation of the signal transduction events. However, excess hydrogen peroxide will cause cellular toxicity, causes the loss of homeostasis. The level of oxidative stress of the cell can be determined by monitoring the morphological change of the mitochondria. Normally, mitochondria in endothelial cells form a filamentous reticular network and constantly undergo morphological changes through fusion and fission. Fig. 1 shows the cellular responses of endothelial cells interact with physical and biochemical microenvironments. The shear stress is imposed directly on the apical surfaces of the ECs and the blood soluble molecules send chemical cues to the cells.

There are several reasons for the excess levels of ROS production in cells [4]. First, short term elevation of ROS level occurs after exhaustive exercise. Second, the presence of exogenous ROS sources can also elevate the ROS level, such as ultraviolet light, ionizing radiation, inflammatory cytokines, environmental toxins, etc. Third, diseases can also increase the ROS level, such as hypertension, renal failure, diabetes, cardiac disease, etc [5]. Therefore, researchers have put in efforts to investigate the relationship between the elevated ROS level and the ROS-related diseases, and further search for potential drugs with antioxidant ability [6]. For example, high glucose level in diabetes patients and high sodium level in renal failure patients have been proven to increase the ROS production level in cells. In addition, different antioxidants or ROS scavengers were studied and shown to reduce ROS production level. However, these studies were conducted under either a static condition in culture dishes or under a constant shear stress, which were different from the physiological condition with pulsatile shear stress [7].

In this paper, the designed Lab-on-a-chip system mimics the flow rate of blood in the artery at the resting condition or after an exhaustive exercise. Different concentrations of glucose were added into the flow to mimic hyperglycemic conditions in diabetes patients. The shear induced cellular responses under different extracellular condition were studied by investigating the ROS production level in the endothelial cells and by
monitoring the morphological changes of mitochondrion using fluorescence imaging technique.

MATERIALS AND METHODS

The design of the hemodynamic Lab-on-a-chip system is shown in Fig. 2. The chip consists of three-layer structures, namely bottom layer with a microfluidic network for cell loading and liquid injection, middle layer with pneumatic valves and top layer with pneumatic connecting channels to control a series of pneumatic valves simultaneously. Each branch of the microchannel has 4 cell culture sites to perform a series of time course experiments. Each cell culture site has a width of 600 μm and a height of 150 μm. The downstream of each cell culture site is connected to a common outlet and has a pneumatic valve for flow switching. Three conditions with different glucose concentration can be achieved with the concentration gradient network. A pulsation free precision pump (Nemesys, Cetoni) was used for cell loading and media injection. The flow profile of the pump was configured to mimic the blood flow in a blood vessel.

Initially, endothelial cells were loaded, cultured and stained prior to the application of shear stress and chemical treatments as shown in Figure 3a. Harvested cells were concentrated by centrifugation and then resuspended in the growth medium at the concentration of about 1×10^6 cells ml^-1. The chips were then incubated in the cell culture incubator with medium replenishment every 24 hours until the cells in the microchannel reached almost full confluence for the experiments as shown in Figure 3b.

The intracellular ROS level was measured using a cell-permeable fluorescent dye of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). Prior to the chemical and shear stress experiments, the cultured endothelial cells in the microfluidic chips were incubated in culture medium with 10 mM H₂DCFDA for 45 min to establish a stable intracellular level. The cells were then being exposed under several chemical and shear conditions with the same concentration level of H₂DCFDA dissolved in culture medium. With the presence of ROS in the cells, the H₂DCFDA retained in the cytoplasm was oxidized and became fluorescent as illustrated in Figure 3(c-d). For each condition, 300 cells were measured to obtain the average fluorescence intensity which is related to the ROS concentration.

Mitochondrial morphology can be visualized by staining the mitochondria inside the cells using a green fluorescent dye (MitoTracker Green FM, Invitrogen) as shown in Figure 3(e-f). Prior to the chemical and shear stress experiments, the endothelial cells grown in the microfluidic chips were incubated in culture medium with a working concentration of 200 nM of MitoTracker dye for 45 min. For each chemical and shear treatment condition, 300 cells were examined to determine the morphological changes of the mitochondria. Each data point was expressed as a mitochondrial fission ratio, which is the ratio of the number of cells with diffused mitochondria to the total number of cells evaluated.

RESULTS AND DISCUSSIONS

Three different shear treatment conditions were selected to investigate the shear-induced responses on
cultured endothelial cells in the microchannel: (1) a constant shear stress of 30 dyne cm\(^{-2}\) with a steady flow rate of 8.46 mL s\(^{-1}\), (2) a normal physiological pulsatile shear stress of 15 dyne cm\(^{-2}\) (mean) with a frequency of 70 bpm and (3) a fast pulsatile shear stress of 30 dyne cm\(^{-2}\) (mean) with a frequency of 140 bpm to mimic the condition under exhaustive exercise.

The temporal responses of the endothelial cells in ROS production level under different shear treatment conditions are shown in Fig. 4. The static condition without a fluidic flow serves as a negative control of the experiment and it can be seen that the fluorescence intensity increased when the dye molecules stained the cells for longer than 15 min. To exclude this intensity increment, the normalized fluorescence intensities for the other three shear treatment conditions were subtracted with that of the negative control. The intracellular ROS level when the endothelial cells were being exposed to pulsatile shear stress of 30 dyne cm\(^{-2}\) for different time periods. The ROS level was increased at the first 60 min by nearly 4-fold. Prolonged exposure of the endothelial cells to the shear flow up to 2 hours resulted in a sustained elevation in the ROS levels, which is similar to the one reported previously. The intracellular ROS level when the endothelial cells were being exposed to pulsatile shear stress of 30 dyne cm\(^{-2}\) for different time periods. The ROS level was increased at the first 60 min by nearly 4-fold.

For normal people, the concentration of glucose in the blood is ranging from 4 to 6 mM. For diabetes patients, however, the concentration of glucose in the blood is above 6 mM and may be up to 20 mM. In these experiments, 10 mM of glucose was added to mimic the plasma condition of a diabetes patient and the ROS level of the endothelial cells was monitored under normal physiological pulsatile shear stress condition or under exhaustive exercise. The experimental results for the normal physiological pulsatile shear stress condition are shown in Fig. 5. When the endothelial cells were being exposed to 10 mM glucose under the normal pulsatile shear stress of 15 dyne cm\(^{-2}\), the ROS level was quickly increased for over 4-fold in the first 60 min and stabilized afterward. By comparing the ROS level at the 60 min time point between the cells treated with 10 mM glucose in a static state and that under a normal shear stress of 15 dyne cm\(^{-2}\), the ROS level was increased 1.7 fold (0.81/0.49). It can be concluded that although high concentration of glucose can elevate the level of intracellular ROS, a much higher level of ROS can be detected if the glucose solution is delivered under a normal pulsatile shear stress condition.
To investigate the mitochondrial morphological changes of endothelial cells, 20 mM of glucose was added and the endothelial cells were being exposed under different shear treatment conditions for 4 hours. The fluorescent images of the endothelial cells after being exposed for 4 hours and the mitochondrial fission ratios monitored for the first 4 hours are shown in Fig. 6. For endothelial cells treated with 20 mM glucose for 4 hours, mitochondrial fragmentation was prevalent with a mitochondrial fission ratio of 20%. When the endothelial cells were being exposed with 20 mM glucose plus the pulsatile shear stress of 15 or 30 dyne cm\(^{-2}\), the mitochondrial fission ratio was further elevated from 20% to 25% and 26%, respectively. The results show that the chemical treatment (i.e. high concentration of glucose) produced a more dominant effect in promoting mitochondrial fission than the physical treatment (i.e. pulsatile shear stress), especially in such an extreme case of 20 mM of glucose.

CONCLUSIONS
In this paper, the intracellular ROS level and the mitochondrial morphology of endothelial cells under physiological pulsatile shear stresses and different glucose concentrations were investigated using a hemodynamic Lab-on-a-chip system. The intracellular ROS level was studied using real-time fluorescence microscopy with the measurement of the oxidation of H\(_2\)DCFDA by hydroxyl radicals or hydrogen peroxide molecules. The elevated cellular ROS level led to morphological changes of mitochondria from filamentous reticular networks to diffused and short fragments. The mitochondrial morphology was studied via fluorescent MitoTracker staining. The shearinduced cellular responses of endothelial cells under glucose concentration of 10 mM or 20 mM were realized and studied by mimicking the physiological pulsatile flow profiles in the blood vessel, i.e. during resting and exhaustive exercising. The results show that ROS level was elevated during exhaustive exercise (shear stress of 30 dyne cm\(^{-2}\)) and with high glucose concentration (diabetes patient). The results also show that pulsatile shear stress is an essential element to mimic the physiological conditions in the blood vessel, which also highlights the potential of using the developed Lab-on-a-chip system in future hemodynamic studies as compared to the conventional experimental setup.

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REFERENCES


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