Biomedical research application of a novel double-layer parallel-plate flow chamber

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Abstract

Since integrity and functions of vascular endothelial cells are greatly affected by shear stress, a variety of *in vitro* systems to subject endothelial cells under precisely controlled fluid conditions has been developed. Complicated designs of the conventional flow devices, however, have impeded such implementation. In the present study, we designed and developed a novel parallel-plate flow chamber (PPFC). It consists of multiple layers of different materials to adjust the required geometries of the chamber and provide a wide span of biomedical research applications. Because the chamber stacks separate layers to constitute the flow channel, different pieces can be easily removed or replaced. Moreover, the multilayer design only requires 2D cutting, which is easier and faster to manufacture. It is also capable of accepting up to four glass slides facing each other so that the flow within the channel is exclusively formed by endothelial cells. Furthermore, it minimizes the pressure loss across the chamber while maximizing the effective area of endothelial cells up to 96 cm^2. Results from mathematical analysis and dye injection experiments showed that a uniform magnitude of shear stress is applied throughout the entire surface of endothelial cells. In addition, the morphological changes and attenuated gene expression of pro-inflammatory mediators were observed in endothelial cells exposed to the physiologically

relevant shear stress. These findings indicate that our newly designed PPFC can provide a better *in vitro* system for versatile applications of biomedical research.

The reperfusion of blood flow occurred in a number of conditions such as stroke and organ transplantation immensely augments tissue injury and can cause more severe damage than prolonged ischemia. The injuries caused by cessation and reperfusion of blood flow are closely related to the inflammatory reactions involving in endothelium-leukocyte cascade responding to a shear stress exerted by the flow. Shear stress is also known to play an important role in human chronic diseases including atherosclerosis, neurological disorders, and cancer metastasis. Therefore, it is important to investigate the transmission of mechanical stimuli such as shear stress to various complex endothelial cell signaling pathways which process as a whole is often referred as mechanotransduction. Shear stress-mediated signaling pathways have been known to trigger endothelial cell responses and contribute to the pathophysiology of human vascular diseases. The present study was designed to apply the novel PPFC to biomedical research, especially ischemia/reperfusion injury. The changes in mRNA and protein expression of inflammatory mediators in endothelial cells were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. RBE4 and HMEC-1 cells were either maintained in continuous laminar flow condition (*Normal Flow*) or subjected to 1 h of flow cessation followed by reperfusion of flow (*Ischemia/Reperfusion*) for 24 h. *Ischemia/Reperfusion* significantly up-regulated expression of inflammatory mediators, such as IL-6, MCP-1, ICAM-1, VCAM-1, and E-selectin, in microvascular endothelial cells. Furthermore, antioxidant pyrrolidine dithiocarbamate (PDTC) significantly attenuated ischemia/reperfusion-induced overexpression of pro-inflammatory mediators. These data indicates that our newly designed PPFC provide a better *in vitro* system for versatile applications of biomedical research.

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Introduction

1.1. Role of endothelium in the development of cardiovascular disease

The endothelium consists of a monolayer of vascular endothelial cells and lines the internal surface of the blood vessels. In normal physiological conditions, the vascular endothelium has been known to exert crucial roles in functionality and maintenance of the vessel walls by modulating vascular tone, providing a selective barrier for macromolecular permeability, and regulating blood flow (Ross, 1993, Sumpio et al., 2002, Fujiwara et al., 1998). In addition, the presence of membrane-bound receptors such as proteins, metabolites, hormones and specific junction proteins allows the endothelium to serve a role in gate-keeping (Cines et al., 1998). It has been also reported that physiological blood flow regulated by endothelial cells (ECs) can contribute to the inhibition of platelet adhesion and clotting (Choi et al., 2003). Furthermore, one of the most important functions of the vascular endothelium is to regulate inflammatory reactions. The inflammatory responses are generally considered a self-defense mechanism in response to the extracellular stimuli including infectious agents. However, in some case, inflammatory response can lead to severe tissue damages if the immune system is improperly motivated. Indeed, it is widely believed that excessive inflammatory reactions are closely associated with vascular pathophysiology including the progression of atherosclerotic plaque formation (Lee and Hirani, 2006).

Cardiovascular disease caused by atherosclerosis is the leading cause of illness and death in the United States. A number of *in vitro* and *in vivo* studies have demonstrated that atherosclerosis, a hardness of an arterial wall due to the atherosclerotic plaques such as accumulation of fatty substances, cholesterol, and calcium, is a slow and progressive inflammatory disease of the vessel wall. It has been also proposed that activation or dysfunction of the vascular endothelium plays a critical role in the early events in the development of atherosclerosis. For example, formation of atherosclerotic lesions can be occurred by injury to the endothelium in response to a number of pathopysiological stimuli. Previous studies have also indicated that the close interactions between endothelial cells and blood leukocytes can initiate thrombotic occlusion *via* rupturing atherosclerotic plaques (Figure 1) (Ross, 1993).

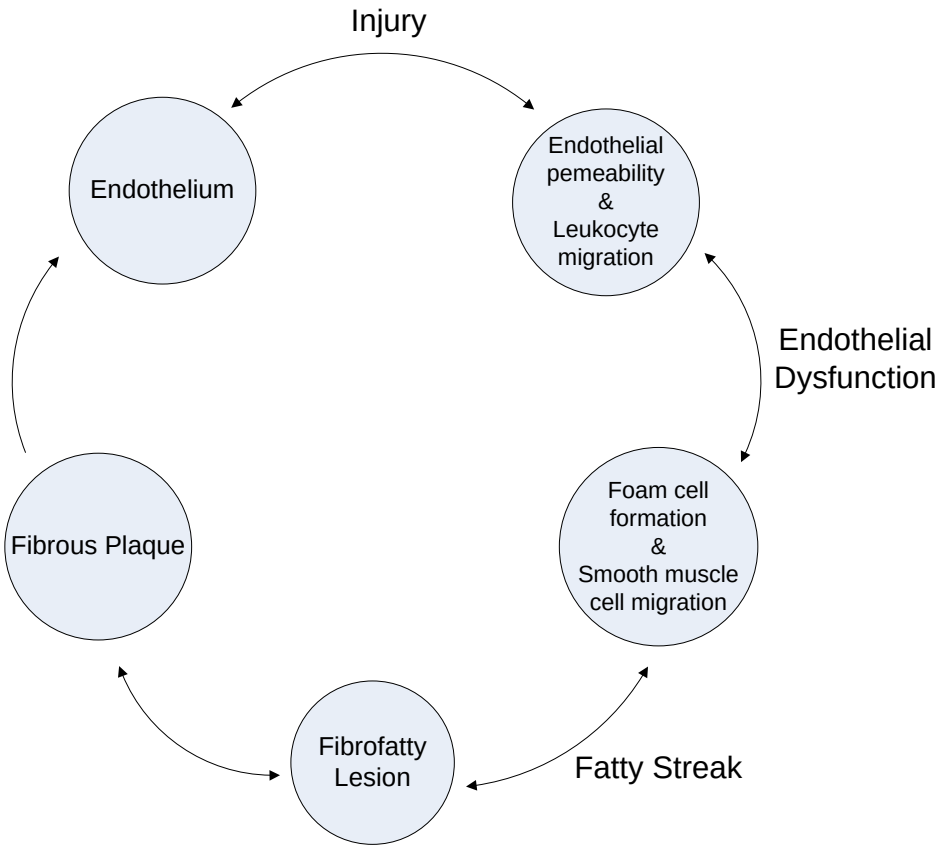


Figure 1. Causal relationship of endothelial dysfunction and development of atherosclerosis (data obtained from Ross, 1993).

1.2. Pro-oxidative and pro-inflammatory mechanisms of vascular endothelial dysfunction

Inflammatory reactions in the vascular endothelium are primarily regulated through the production of pro-inflammatory mediators (Lee et al., 2004b, Ross, 1999). In fact, enhanced expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), chemokines such as monocyte chemoattractant protein-1 (MCP-1), and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin in vascular endothelial cells, and their close interactions facilitate recruiting and adhering inflammatory cells into the endothelium, and thus stimulate transendothelial migration, which can be considered an early atherogenic process (Davies et al., 1993, Reape and Groot, 1999). For example, ICAM-1 and VCAM-1 stimulate adhesion and transmigration of blood leukocytes onto and across the vascular endothelium. Both MCP-1 and, to a lesser extent, TNF-α are potent chemoattractive factors, which play a significant role in recruiting blood-born inflammatory cells into the vessel wall. In addition, TNF-α and IL-6 are strong inducers of expression of a spectrum of pro-inflammatory mediators as well as of inflammatory reactions in vascular endothelium (Lee and Hirani, 2006, Lukacs et al., 1995, Pober, 1998, Strieter et al., 1989, Toborek et al., 2002a).

Oxidative stress has been implicated in the initiation and progression of atherosclerosis (DiCorleto and Chisolm, 1986, Gimbrone et al., 1990, Hennig et al., 1996) and vascular endothelial cells are particularly sensitive to disturbances in the redox steady state (Hennig and Chow, 1988). It is also well known that oxidative stress may induce changes in cellular membrane structure, fluidity, transport, and antigenic characteristic as well as in disturbances in fibrinolytic pathway and prostacyclin synthesis (Hempel et al., 1990, Holvoet and Collen, 1994). These abnormalities may ultimately contribute to endothelial cell injury, which is one of the crucial steps in the development of atherosclerotic lesion formation (Ross, 1993). In addition, it has been suggested that increase in intracellular oxidative stress may regulate the expression of redox-sensitive genes, including those encoding for pro-inflammatory mediators (Yla-Herttuala, 1992).

Indeed, recent evidences from our group and others have demonstrated that pro-inflammatory cytokine interleukin-4 (IL-4) can increase the oxidation potential of various cell types. For example, IL-4 treatment of human umbilical vein endothelial cells (HUVEC) markedly enhances the intracellular oxidizing potential, as indicated by an increase in 2',7'-dichlorofluorescein (DCF) fluorescence (Lee et al., 2001c, Lee and Hirani, 2006).

Previous studies have demonstrated that the expression of pro-inflammatory mediators is regulated at the transcriptional level through activation of specific transcription factors (Lee et al., 2001a, Lee et al., 2001b, Lee et al., 2003a, Stanimirovic et al., 2001, Toborek et al., 2002b). As illustrated in Figure 2, the promoter regions of pro-inflammatory genes contain potential binding sites for a variety of transcription factors, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), cAMP responsive element-binding protein (CREB), sequence specific protein-1 (SP-1), and signal transducers and activators of transcription (STAT-1 and STAT-6), etc. Compeling body of evidence has also indicated the critical role of oxidative stress in molecular regulatory pathways leading to activation of these transcription factors and subsequent gene expressions. It is now generally accepted that oxidative stress up-regulates the expression of pro-inflammatory mediator genes and produces pro-inflammatory vascular environments *via* activation of redox-responsive transcription factors. Indeed, activation of NF-κB and AP-1 are considered to be a part of a general regulation of a number of inflammatory gene expressions by cellular oxidative stress and/or intracellular glutathione levels (Arrigo, 1999, Bouloumie et al., 1999, Lakshminarayanan et al., 1998, Schreck et al., 1992, Wung et al., 1997). For example, expression of the inflammatory cytokine TNF-α gene is induced by increased oxidative stress through activation of NF-κB and AP-1 (Guha et al., 2000, Lee et al., 2001a, Lee et al., 2001b, Rahman and MacNee, 2000, Verhasselt et al., 1998). It was also found that oxidative stress-mediated activation of NF-κB resulted in overexpression of IL-6 gene (Uzzo et al., 2006). These findings provide compelling evidence that oxidative stress-mediated pro-inflammatory pathways in the vascular endothelium can be critically involved in endothelial dysfunction and may play a significant role in the pathogenesis of cardiovascular disease.

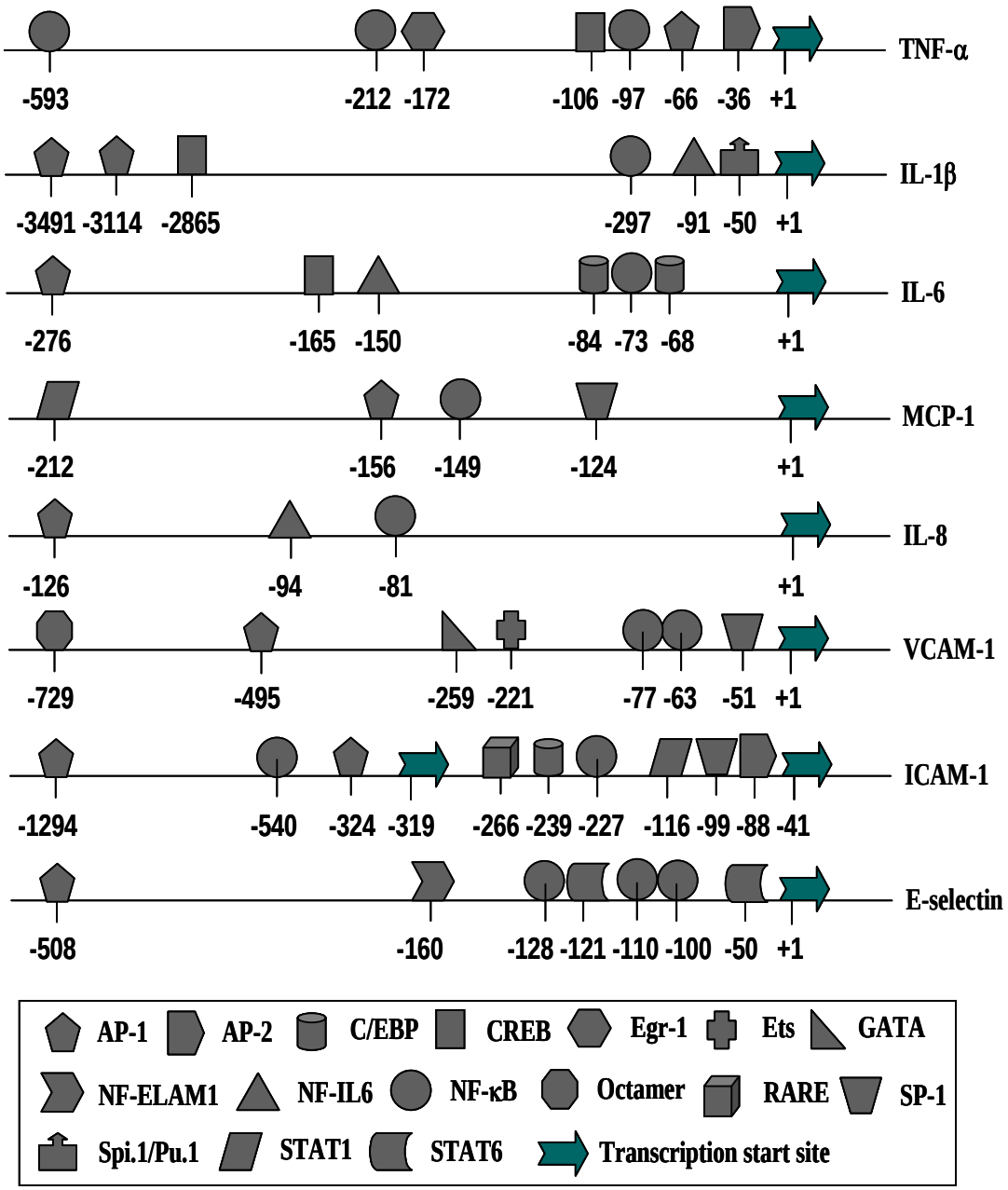


Figure 2. Schematic representation of promoter regions of the human inflammatory gene showing the location of the transcription factor binding sites. Abbreviation: TNF-α, tumor necrosis factor-α; IL-1β, inerleukin-1β; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin-8; VCAM-1,vascular cell adhesion molecule-1; ICAM-1,intercellular adhesion molecule-1 (data obtained from Lee and Hirani, 2006).

1.3. Shear stress-mediated mechanotransduction in vascular endothelial cells

Since the vascular endothelium forms an interface between the blood and the underlying layers of the vessel walls, its unique location allows endothelial cells to be constantly exposed to the shear stress generated by blood flow. Growing body of evidence has indicated that vascular endothelial cells act as mechanoreceptors by sensing and responding to shear stress. Indeed, endothelial cells are able to detect changes in hemodynamic shear stress using multiple sensing mechanisms and convert mechanical stimuli into intracellular signals. Then, shear stress-mediated activation of signaling pathways subsequently triggers a variety of responses of endothelial cells that affect their structure and functions, such as endothelial cell proliferation, apoptosis, migration, permeability, and remodeling of cytoskeleton, as well as modulation of gene expression (Davies, 1995, Isenberg et al., 2006, Ando and Kamiya, 1996, Chien et al., 1998, Davies et al., 1984, Li et al., 2005).

Even though the effects of shear stress on vascular endothelial cells have extensively been investigated, many difficulties arise in studying the *in vivo* responses of endothelium to shear stress due to the complex structures of the blood vessels and lack of resolution of the measurements with current technologies (Chung et al., 2003, Helmlinger et al., 1991). To overcome such limitations of *in vivo* approaches as well as to study the dynamic response of vascular endothelial cells to controlled levels of fluid shear stress, various types of *in vitro* systems that allow cultured endothelial cells to be exposed to well-defined flow conditions have been developed. These systems include a cone-plate apparatus (Dewey et al., 1981), orbital shakers (Dardik et al., 2005), capillary flow tubes (Olesen et al., 1988, Jacobs et al., 1995), and parallel-plate flow chambers (PPFC) (Usami et al., 1993, Ruel et al., 1995, Chiu et al., 1998, Lawrence et al., 1987). Among *in vitro* systems employed to study the effects of flow conditions on endothelial cells, PPFC have been the most commonly used to achieve a fully developed laminar flow within the channel of the chamber. A PPFC sets two plates in parallel to constitute a thin rectangular flow channel in between where a monolayer of cells is seeded on an either side. The simplicity of the concept and usage had evidently attributed to its wide spread.

Consequently, a number of variant designs of PPFC have been developed for different applications of biomedical research such as platelet adhesion (Lawrence et al., 1987, Cao et al., 1997), cell morphology (Levesque and Nerem, 1985, Albuquerque et al., 2000, Gray et al., 2004), gene and protein expression (Miyagi et al., 2005, Peters et al., 2002, McCormick et al., 2000, Ando and Kamiya, 1996, Ando et al., 1995, Chiu et al., 1997, Morigi et al., 1995) and cell responses under special conditions (Ruel et al., 1995, Yee et al., 2006, Ivarsson et al., 1989, Chiu et al., 1998, Chen et al., 2006). These conventional PPFC, however, have shown weaknesses and problems in several aspects of its design. For example, the chambers are usually too difficult to handle and perform experiments which during the process erroneous outcomes may result. Indeed, the difficulty as well as the complexity in connecting tubes between the components of the system impede the progression of the experiment and evoke problems by either letting the cells stay outside the incubator for an extended period of time or causing considerable variations between each chamber. Setting up the chamber system bubble-free is also a difficult task with conventional PPFC that it often requires multiple trials to remove these bubbles. In addition, the success rate of the experiments is significantly decreased by unexpected crack on the glass slide which later can cause the media to leak. Furthermore, some designs are simply too difficult to manufacture which requires it to be fabricated by highly trained technicians and makes it inconvenient to implement further adjustments and improvements to the design.

In present study, there are two major objectives to examine the effects of dynamic shear stress on the structure and function of endothelial cells *in vitro*:

Objective I: **Design and construction of a novel double-layer parallel-plate flow chamber (PPFC).**

Objective II: **Biomedical research applications of PPFC: a novel *in vitro* ischemia/reperfusion injury model.**

Design and Construction of A Novel Double-Layer Parallel-Plate Flow Chamber (PPFC)

2.1. Abstract

Since integrity and functions of vascular endothelial cells are greatly affected by shear stress, a variety of *in vitro* systems to subject endothelial cells under precisely controlled fluid conditions has been developed. Complicated designs of the conventional flow devices, however, have impeded such implementation. In the present study, we designed and developed a novel parallel-plate flow chamber (PPFC). It consists of multiple layers of different materials to adjust the required geometries of the chamber and provide a wide span of biomedical research applications. Because the chamber stacks separate layers to constitute the flow channel, different pieces can be easily removed or replaced. Moreover, the multilayer design only requires 2D cutting, which is easier and faster to manufacture. It is also capable of accepting up to four glass slides facing each other so that the flow within the channel is exclusively formed by endothelial cells. Furthermore, it minimizes the pressure loss across the chamber while maximizing the effective area of endothelial cells up to 96 cm^2. Results from mathematical analysis and dye injection experiments showed that a uniform magnitude of shear stress is applied throughout the entire surface of endothelial cells. In addition, the morphological changes and attenuated gene expression of pro-inflammatory mediators were observed in endothelial cells exposed to the physiologically relevant shear stress. These findings indicate that our newly designed PPFC can provide a better *in vitro* system for versatile applications of biomedical research.

2.2. Introduction

Even though effects of shear stress on vascular endothelial cells have been investigated, the current technologies have shown many difficulties in studying the *in vivo* responses of endothelium to shear stress due to the complex structures of the blood vessels and lack of resolution of the measurements (Chung et al., 2003, Helmlinger et al., 1991). To overcome such limitations, various types of *in vitro* systems that allow cultured endothelial cells to be exposed to well-defined flow conditions have been developed. Among these systems, PPFC have been the most commonly used, and various

designs of PPFC have been developed for different applications of biomedical research. The conventional PPFC, however, have shown weaknesses and problems in several aspects of its design. In the present study, we designed and developed a new double-layer PPFC to eliminate these problems and thus provide a better system that is easy to manufacture, simple to use, and convenient to adjust and improve upon its geometries.

2.3. Materials and Methods

We designed and constructed a novel double-layer PPFC to study the dynamic response of vascular endothelial cells to controlled level of shear stress. To verify design of the novel double-layer PPFC, mathematical analysis and dye injection experiments were performed. In addition, Rat brain microvascular endothelial cells (RBE4) and human microvascular endothelial cells (HMEC-1) were used to examine the effects of fluid shear stress on the structure and function of endothelial cells.

2.3.1. Cell cultures

Rat brain microvascular endothelial cells (RBE4) were a generous gift from Dr. Michael Aschner (Vanderbilt University, Nashville, TN). RBE4 were cultured in 1:1 Ham's F10-MEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml of streptomysin, 1 ng/ml of basic fibroblast growth factor (bFGF), 2 mM L-glutamine, and 12.5 mM HEPES (Mediatech). The cells were maintained in a humid 5% CO_2 atmosphere at 37 °C (Figure 3).

Human microvascular endothelial cells (HMEC-1) were provided from Dr. Edwin Ades and Mr. Fransisco J. Candal of Center for Disease Control and Prevention (Atlanta, GA) and Dr. Thomas Lawley of Emory University (Atlanta, GA). HMEC-1 are an immortalized cell line obtained by transformation of human primary dermal microvascular endothelial cells with the SV40 large T antigen. These cells retain endothelial cell phenotype and functional characteristics (True et al., 2000). The cells were cultured in MCDB 131 medium (Mediatech) enriched with 10% fetal bovine serum

(FBS), 10 mM L-glutamine, 1% antibiotic-antimycotic, 1 μg/ml hydrocortisone (Sigma, St. Louis, MO), and 10 ng/ml epidermal growth factor (Roche, Indianapolis, IN) in 5% CO_2 atmosphere at 37 °C (Figure 3).

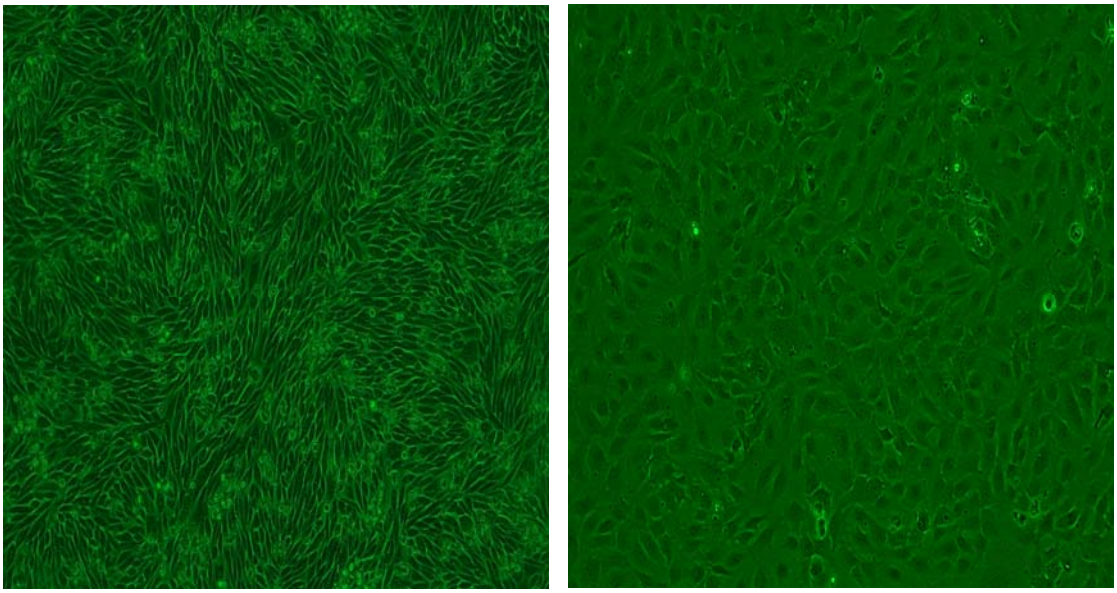


Figure 3. Micrographs of rat brain microvascular endothelial cells (RBE4; *Left panel*) and human microvascular endothelial cells (HMEC-1; *Right panel*) employed in the present study.

2.3.2. Parallel-plate flow chamber (PPFC) and flow apparatus

The chamber consists of clear cast acrylic sheets (ACRYLITE, CYRO Industries, Rockaway, NJ) with two different thicknesses of 0.5-inch and 0.08-inch. The silicone sheets (Specialty Manufacturing Inc., Saginaw, MI) of 0.01-inch and 0.03-inch in thicknesses were used for gaskets. Binding head machine screws made of 18-8 stainless steel with thread size of 8-32-inch and length of 1-1/2-inch were held tight with 18-8 stainless steel hex nuts of 3/32-inch height that correspond to the same thread size as the screws. The inlets and outlets were formed with male slip polypropylene fittings (Cole-Parmer Instrument Co., Vernon Hills, IL) with 1/16-inch hose barb. Each acrylic layer of the chamber was cut off by Laser Computer Aided Modeling and Manufacture

(LaserCAMM) machine. The system is a computerized, turn-key laser cutter that uses a laser beam to scribe or cut a wide variety of sheet materials into intricate patterns with a high degree of accuracy. In addition, since it integrates fully with Autodesk® AutoCAD®, any two-dimensional shape that is drawn on AutoCAD can be cut by LaserCAMM. More importantly, LaserCAMM rapidly creates accurate copies of a part which render numerous prototypes of different designs and can provide immediate feedback and help resolve manufacturing problems.

The flow loop apparatus system which consists of upper and lower reservoirs providing a constant hydrostatic pressure was established as described previously (Frangos, 1988)). The constant pressure head was maintained by continuously pumping the media from the lower reservoir to the upper reservoir by a peristaltic pump (Cole-Parmer) at rates faster than the flow through the chamber. The excess culture medium then overflowed down to the lower reservoir promoting gas exchange. Flow rate through the chamber was controlled with stopcocks installed on tubes leading into the inlets as the flow rates were monitored with flow meters connected from the outlets.

Endothelial cells were seeded on glass slides (38 mm × 75 mm, Fisher Scientific) coated with 0.025% Collagen Type I (BD Biosciences, Bedford, MA), and incubated for 5-7 days until they reach the confluent. The desired wall shear stress (τ_{wall}) was calculated by the Navier Stokes equation (Ulfman et al., 1999). The equation and real values measured in our experiment as following:

$$\tau_{wall} = \frac{6Q\mu}{wh^2}$$

where τ = shear stress (4 dyne/cm^2)

Q = volumetric flow rate of the medium (ml/s)

μ = viscosity coefficient of the medium (0.72cP at 37°C)

w = the flow channel with (3.2 cm)

h = channel height (0.0254 cm)

An inverted microscope equipped with 12-bit dynamics camera (The Axio Cam MR, Carl Zeiss Inc.) was used to observe morphological changes of endothelial cells in response to static and flow conditions.

2.3.3. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the protocol of the manufacturer. 1 μg of total RNA was reverse-transcribed at 25 ºC for 15 min, 42 ºC for 45 min, and 99 ºC for 5 min in 20 μL of 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/μL of recombinant RNasin ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase, and 0.5 μg of random hexamers. For quantitative PCR, amplifications of individual genes were performed on ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan® Universal PCR Master Mix, gene-specific TaqMan PCR probes and primers, and a standard thermal cycler protocol (50 ºC for 2 min before the 1st cycle, 95 ºC for 15 s, and 60 ºC for 1 min, repeated 45 times). For specific probes and primers of PCR amplifications, TaqMan® Gene Expression Assay Reagents for rat interleukin-6 (IL-6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene), as well as human intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and β-actin (housekeeping gene) were obtained from Applied Biosystems. The threshold cycle (C_T), which indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold, from each well was determined using by the Applied Biosystems Sequence Detection Software v1.2.3. Relative quantification, which represents the change in gene expression from real-time quantitative PCR experiments between static and flow-adapted groups as well as continuous and ischemia/reperfusion groups, was calculated by the comparative C_T method as described earlier (Livak and Schmittgen, 2001, Deng et al., 2003). The data were analyzed using equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ = [C_T of target gene - C_T of housekeeping gene] $_{\text{static or continuous flow group}}$ − [C_T of target gene - C_T of housekeeping gene] $_{\text{flow-adapted or ischemia/reperfusion group}}$. Evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in

gene expression, normalized to a housekeeping gene (GAPDH or β-actin) and relative to the control group. Both GAPDH and β-actin have been most commonly used housekeeping genes for gene expression analyses in a various type of cells.

2.3.4. Statistical analysis

Statistical analysis of data was completed using SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA). One-way ANOVA was used to compare mean responses among the treatments. For each endpoint, the treatment means was compared using Bonferroni least significant difference procedure. Differences among the means were considered significant at $P < 0.05$.

2.4. Results and Discussion

2.4.1. Design and construction of a novel double-layer parallel-plate flow chamber (PPFC)

Flow through a PPFC is a steady and laminar stream of an incompressible and viscous Newtonian fluid. A schematic of parallel plates of a novel double-layer PPFC is shown in Figure 4 with conventional Cartesian coordinate system (x, y, z) defined within. The fluid enters the parallel plates where x is at the origin, flows through a channel with a constant cross-section, and exits where x equals the length of the chamber, l. The ratio between the height and the width of the channel or the lateral channel aspect ratio, β, is a non-dimensional parameter analytically described as

$$\beta = \frac{h}{w}, \tag{1}$$

where h is the height and w is the width of the channel (Munson et al., 2006).

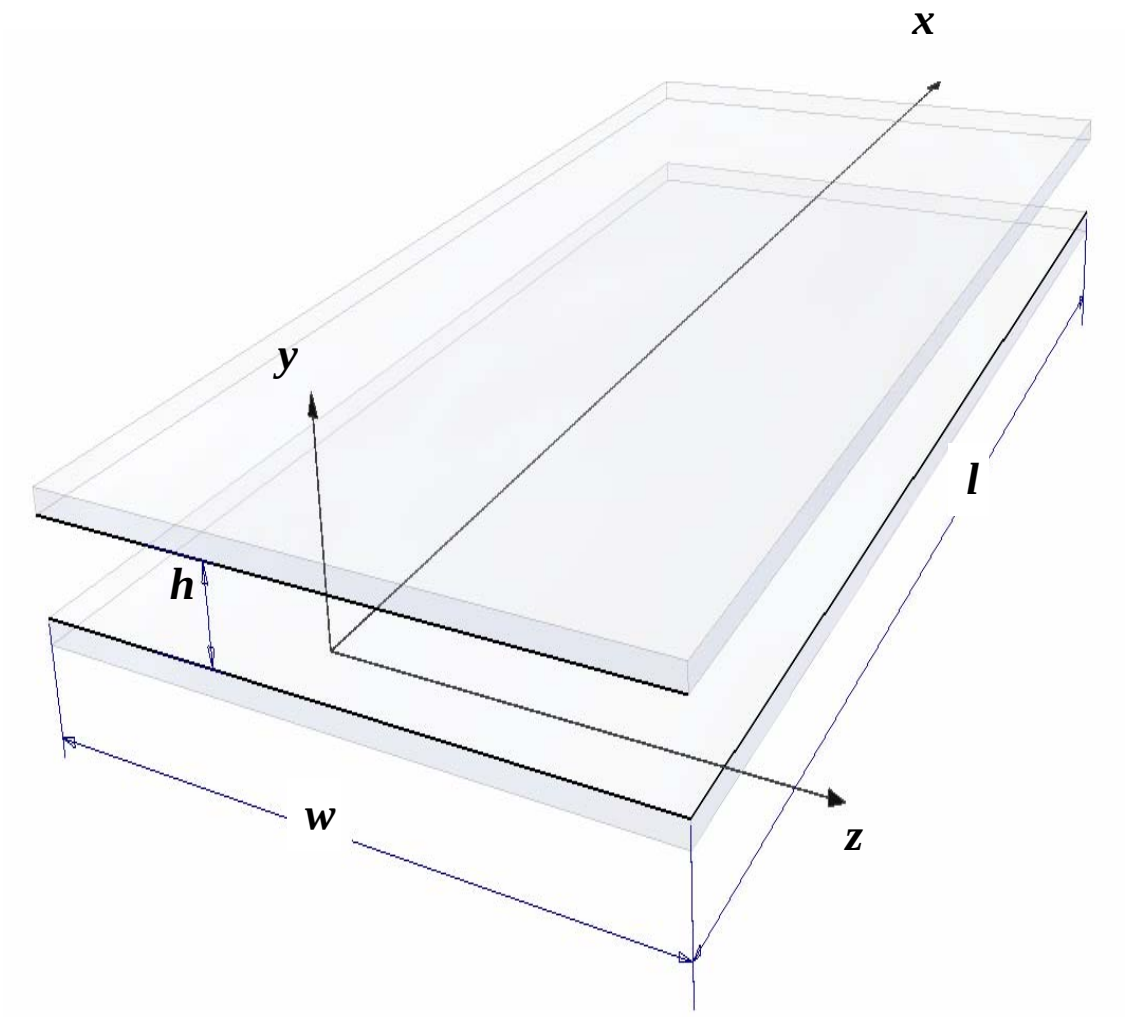


Figure 4. Schematic diagram of the three-dimensional Cartesian coordinate system (*x*, *y*, *z*) of a novel double-layer parallel-plate flow chamber (PPFC). The fluid enters the gap of height *h* between the parallel plates where *x* is at the origin, flows through a channel with a constant cross-section, and exits where *x* equals the length of the chamber, *l*.

The value of β typically ranges from 0.0033 (Chung et al., 2003) to 0.014 (Ruel et al., 1995). This parameter is especially important in determining the validity of the infinite parallel plate assumption. When this assumption is considered reasonable, the fluid particles in such geometry can be assumed to move in a single direction parallel to the two plates of the channel. If we additionally assume that the plates are rigid enough so that its geometry is unaffected by the flow, then the Navier-Stokes equations are greatly reduced to yield an exact solution (Fung, 1997). The solution eventually leads us to a well known analytical estimation of the wall shear stress (WSS), τ_{wall}, of a fully developed laminar flow through the flow channel to be

$$\tau_{wall} = \frac{6Q\mu}{wh^2}, \tag{2}$$

where μ is the dynamic fluid viscosity and Q is the volume flow rate (Nauman et al., 1999).

Equation (2) implies that the WSS varies with accordance to the volumetric flow rate or the average velocity of the fluid with other constants such as the fluid viscosity, the width and the height of the channel. Since the fluid viscosity in typical flow experiments is not usually controllable for the researcher confined with specific media, the width and height of the channel as well as the volume flow rate are to be carefully chosen for the design of the experiment. Within a confined space there is a limitation of how much of hydrostatic pressure can be applied to the cross-sectional area of the channel, which the width and the height as well as the flow speed must be changed with respect to each other to achieve a certain magnitude of shear stress. The velocity profile between two parallel plates can be analytically described as

$$v(x,y) = \frac{1}{2\mu}\left(\frac{\partial p}{\partial x}\right)\left(y^2 - h^2\right), \tag{3}$$

where $\partial p/\partial x$ is the pressure loss throughout the x axis (Fung, 1997). If we assume that there are no forces acting in y and z directions, the pressure drop throughout the length of the channel can be solved with simple force balance to have (Munson et al., 2006).

$$\frac{\partial p}{\partial x} = -\frac{2\tau(w+h)}{hw}. \tag{4}$$

The velocity profile for a pressure-driven flow, however, can also be solved and visualized in a three dimensional space with a Fourier series expansion as

$$v(z^*, y^*) = \frac{1}{2}\left(\beta^2 - y^{*2}\right) + \frac{2}{\beta}\sum_{n=1}^{N}\frac{(-1)^n}{\alpha_n^3}\cos\alpha_n y^* \frac{\exp\left[\alpha_n\left(z^*-1\right)\right] + \exp\left[-\alpha_n\left(z^*+1\right)\right]}{1+\exp\left[-2\alpha_n\right]} \tag{5}$$

where $\alpha_n = (2n-1)\pi/2\beta$ and z* and y* are the normalized width and height of the channel that range from -1 to 1 and $-\beta/2$ to $\beta/2$ accordingly (Panton, 2005).

In fact, the PPFC do not have infinitely long parallel plates but rather have lateral walls as well as inlets and outlets that disturb the flow. The lateral walls, though they are thin and straight through the channel, disturbs the flow profile near the side edges so that some of surfaces of the parallel plates near these walls can be affected with a magnitude of shear stress different from the intended value. The fluid going through the inlets, including the bended passages to enter the channel, is also disturbed to some extent so that a certain length of the channel needs to be dissipated for the flow to become fully developed. The Reynolds number, a non-dimensional parameter, can be useful to determine some of these characteristics of the flow and it is defined as

$$\mathrm{Re} = \frac{\rho \bar{V} h}{\mu}, \tag{6}$$

where $\bar{V}$ is the average velocity of the flow (Munson et al., 2006). A flow is typically considered as laminar if the Reynolds number remains below about 1,400. The Reynolds

number can be used to estimate the entrance length, L_{en}, which is defined as (Nauman et al., 1999)

$$L_{en} = 0.04 \,\mathrm{Re}\, h \,. \tag{7}$$

As mentioned earlier, the lateral walls create boundary layers so that the shear stress exerted on the surfaces of the two parallel plates is varied. The thickness of boundary layer created by the lateral walls can be described as

$$\delta = \frac{5l}{\sqrt{\mathrm{Re}_l}}, \tag{8}$$

where Re_l is the Reynolds number with the length l in the place of the height h in the equation (6) (Ruel et al., 1995). The effects of the lateral walls can be visualized with the three dimensional flow profile given by equation (5) as well as a non-dimensional shear stress ratio, $\tilde{\tau}$, between the three-dimensional shear stress to the two-dimensional shear stress provided by Chung et al. (2003) as

$$\tilde{\tau}(\tilde{z}) = \frac{\tau(\tilde{z})_{3D}}{\tau_{2D}} = \left[1 - \frac{8}{\pi^2} \sum_{n=1,3,5,\ldots}^{\infty} \frac{1}{n^2} \frac{\cosh(n\pi\tilde{z}/2\beta)}{\cosh(n\pi z/2\beta)}\right] \Bigg/ \left[1 - \frac{192}{\pi^5}\beta \sum_{n=1,3,5,\ldots}^{\infty} \frac{1}{n^5} \tanh\left(\frac{n\pi}{2\beta}\right)\right], \tag{9}$$

Equation (9) as well as equation (5) will provide us with a new perspective in the flow profile on a plane viewing from the top of the plates as a function of β and independent to the Reynolds number as opposed to equation (8) (Chung et al., 2003).

With the introduced parameters and equations, an *active test region* (ATR) can be defined where it will assure that a shear stress of a uniform magnitude is applied to the monolayer of endothelial cells (Ruel et al., 1995). As Figure 5 depicts the region of ATR, it excludes the parts where affected by the entrance, L_{en}, and exit length, L_{ex}, as well as the thickness of the boundary layer, L_w, generated by the lateral walls. Since the exit length is typically shorter than the entrance length, we assume that it has the same length

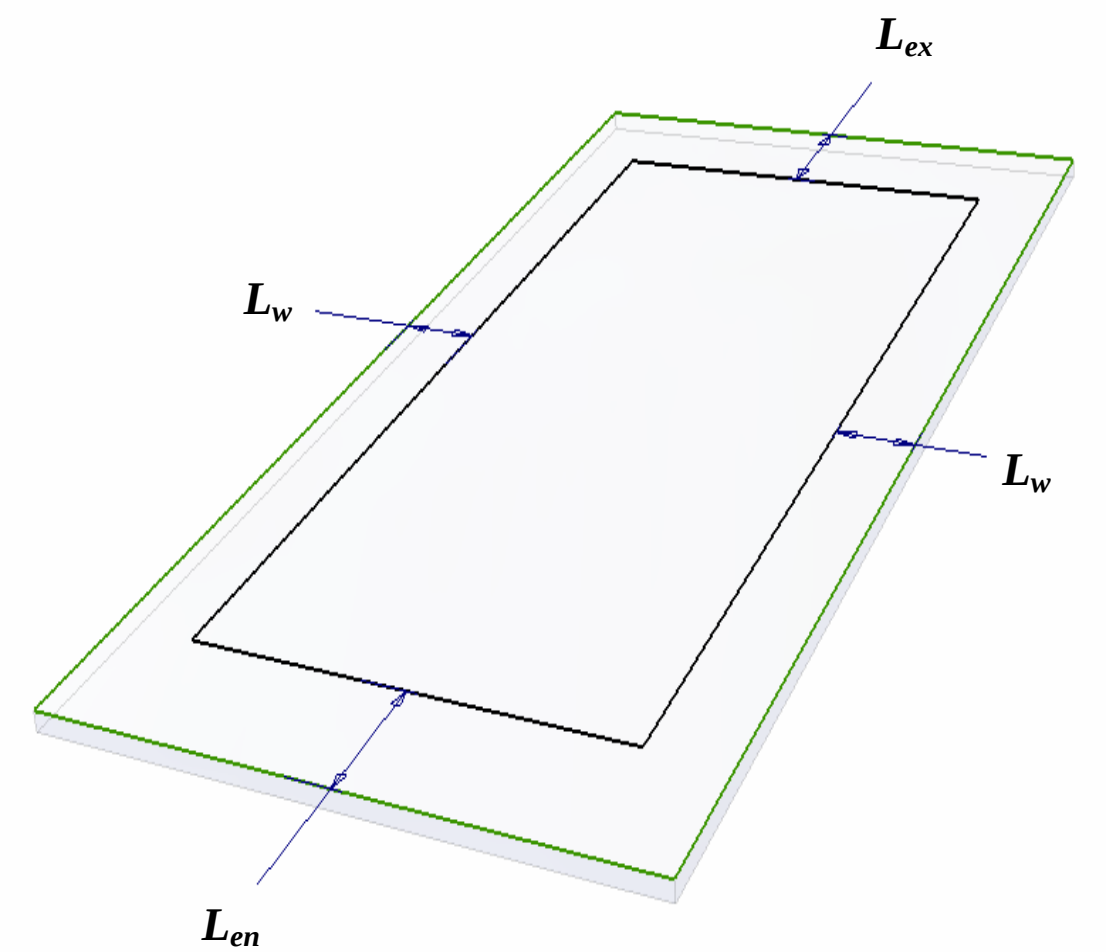


Figure 5. Schematic diagram of an *active test region* (ATR) of a novel double-layer parallel-plate flow chamber (PPFC). The ATR excludes the regions where affected by the entrance length (L_{en}) and exit length (L_{ex}), as well as the thickness of the boundary layer (L_w) generated by the lateral walls. The ATR defined the boundaries where the shear stress drops below 95% of the maximum value, which typically keep almost constant in the middle section. Most of the area of endothelial cell monolayer is to be placed within these limits defined by ATR unless either of the lengths is considered to be insignificant or negligible.

as L_{en} for the symmetry of the design of the chamber. The outlines of ATR will define the boundaries where the shear stress drops below 95% of the maximum value, which will typically keep almost constant in the middle section. Most of the area of endothelial cell monolayer is to be placed within these limits defined by ATR unless either of the lengths is considered to be insignificant or negligible.

The design of new PPFC was aimed to have flexibility in adjusting the basic geometries of the chamber for different purposes of biomedical research as well as versatility for which will provide a wide span of possible variations of applications. The chamber design was primarily intended to be easily made and duplicated without complex manufacturing processes. In addition, special emphasis was placed on the design of new PPFC to increase success rate of the experiments by freeing from leakages, bubbles, broken glass slides, and unreasonably long duration of procedures. Careful thoughts were also taken into account to maximize ATR with minimal space waste, ensure applying a uniform magnitude of shear stress throughout the entire endothelial cell monolayer, and thus avoid erroneous and misleading results. Moreover, another important feature of new PPFC is that it consists of separate layers of different materials and thicknesses so that any new layer can easily be added, replaced or removed. It is also capable of accepting from two to four glass slides in series facing each other where the velocity profile of the flow is exclusively formed by the monolayers of endothelial cells. This is not only beneficial to the fact that the chamber accepts a sufficient amount of cells providing more opportunities for gene and protein expression analyses up to four groups of a total 96 cm^2 but also critical to minimize the pressure loss throughout the channel when glass slides are placed on the top and bottom. Placing glass slides in series also reduces complexity of the web of tubing that connects through the reservoirs, chambers, flow meters, syringes, valves, and peristaltic pump where all except the pump are placed in a confined space of CO_2 incubator.

Each of the successive layers of new PPFC has different roles such as inlet and outlet of the flow, formation of the channel, flushing surfaces between disconnected parts, and a firm grip of glass slides. As depicted in Figure 6A, the chamber has a total of 7 layers of

clear cast acrylic sheets and silicone gaskets with glass slides (38 mm × 75 mm) that can fit into the slots of two of the layers. Layers (1) and (9) are the top and bottom of the chamber cut off from half inch thick cast acrylic sheets. The three ports on the right side of layer (1) serve as inlets where the middle one is for syringe access and the other two are connected to let the flow through from a constant hydrostatic pressure head. The ports on the left side are two outlets. It should be noted that the inlets and outlets where each side has two ports symmetrically placed to provide a more uniform flow through a wide width of the chamber. Layers (2) and (8) are silicone gaskets of 0.03-inch thickness that serve as a firm grip for the glass slides that fit into the holes in the middle of the layers (4) and (7). These silicone gaskets also help render a flush surface for the disconnected gaps between the glass slides, and the layers (4) and (7) by the edges of the two holes in the middle being compressed when the chamber is tightly closed. The smaller holes on each side of layer (2) serves as small reservoirs for the fluid that stay before entering the thin slits of layer (4). Layers (4) and (7) are cast acrylic sheets of 0.08-inch thickness which provide slots for the glass slides (3) and (6). The thin slits on each side of layer (4) more evenly distribute the flow coming in and out from the inlet and outlet reservoirs. Layer (5) is a silicone gasket of 0.01-inch thickness that constitutes the gap between the glass slides (3) and (6) making the channel height h (Figure 4). The width of the middle hole of layer (5) is the width of the channel w (Figure 4) which is slightly shorter than the width of the glass slides to prevent glass slides (3) from falling onto glass slides (6). Thus the flow channel is constituted between glass slides at layer (3) and (6) with the gasket at layer (5) in between as illustrated in Figure 6B. Outer four of the eight glass slides (3) and (6) that form the top and bottom surfaces of the flow channel are plain glass slides that will not have endothelial cells seeded but is placed to compensate the height difference from the thickness of the acrylic sheets (4) and (7). The chamber is closed with twelve stainless wing nuts to provide a tight seal from leakage (Figure 6C). To set up the chamber bubble free, the layers are installed in the orders from (9) to (1) where the flow channel, reservoirs, and the thin slits are filled up with media by means of syringe and pipette as each layer is piled up. The final design uses channel-like reservoirs and two symmetrically placed inlets and outlets to create more uniform flow in the chamber. The thin slit on the next layer where the fluid in the

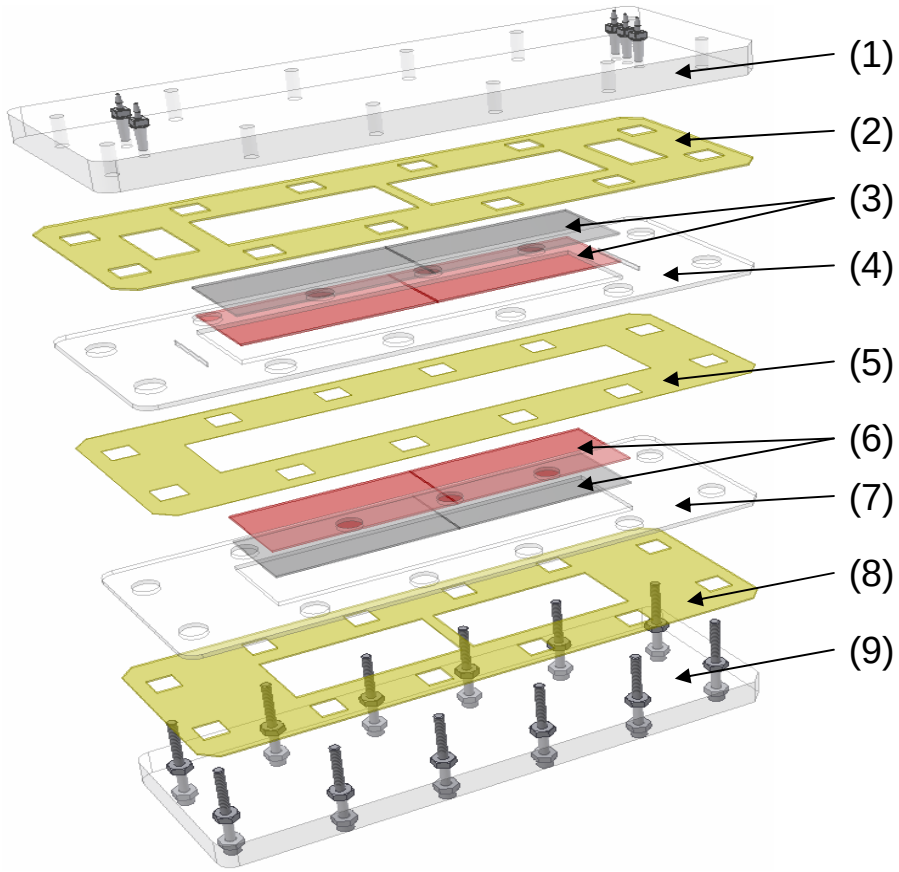


Figure 6A. The basic design of a novel double-layer parallel-plate flow chamber (PPFC). The chamber has a total of seven layers including five clear cast acrylic sheets and two silicone gaskets. Layers (1) and (9) are the top and bottom of the chamber cut off from 0.5”-thick cast acrylic sheets. The three ports on the right side of layer (1) serve as inlets where the middle one is for syringe access and the other two are connected to let the flow through from a constant hydrostatic pressure head. The ports on the left side of layer (1) are two outlets. Two ports of inlets and outlets are designed symmetrically to provide a more uniform flow through a wide width of the chamber. Layers (2) and (8) are silicone gaskets of 0.03” thickness and serve as a firm grip for the glass slides (3) and (6). Layers (4) and (7) are cast acrylic sheets of 0.08” thickness which provide slots for the glass slides. Layer (5) is a silicone gasket of 0.01” thickness that constitutes the gap between the glass slides.

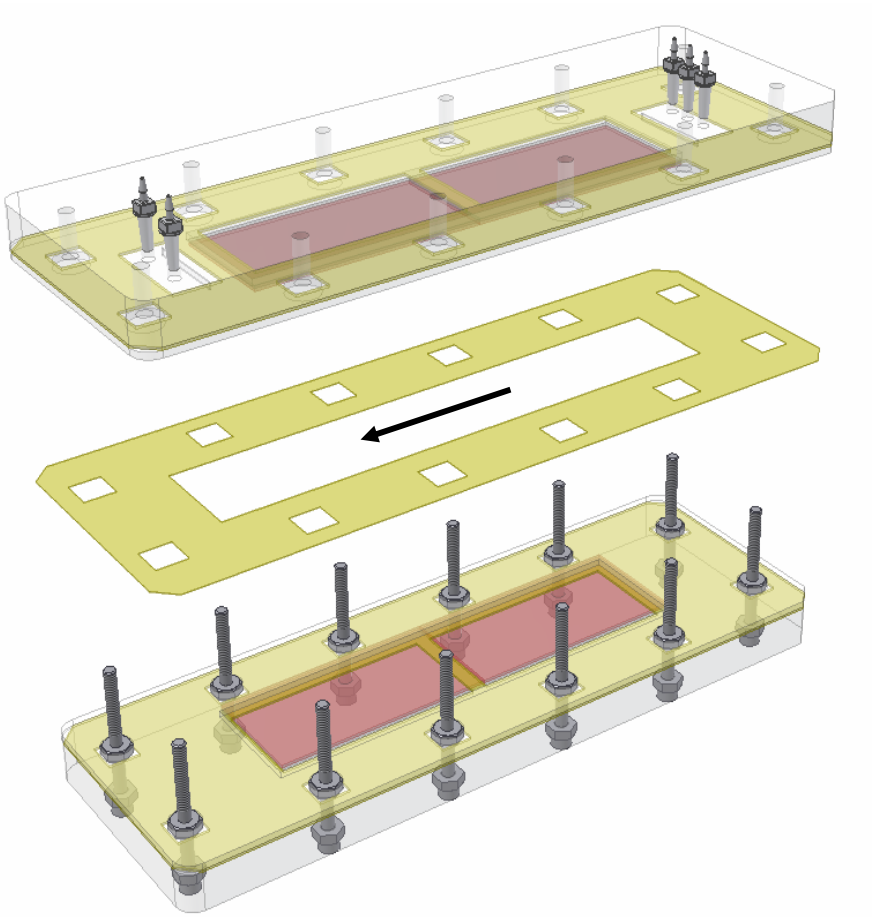


Figure 6B. The flow channel is constituted between upper and lower glass slides with the gasket layer. Arrow indicates the direction of flow.

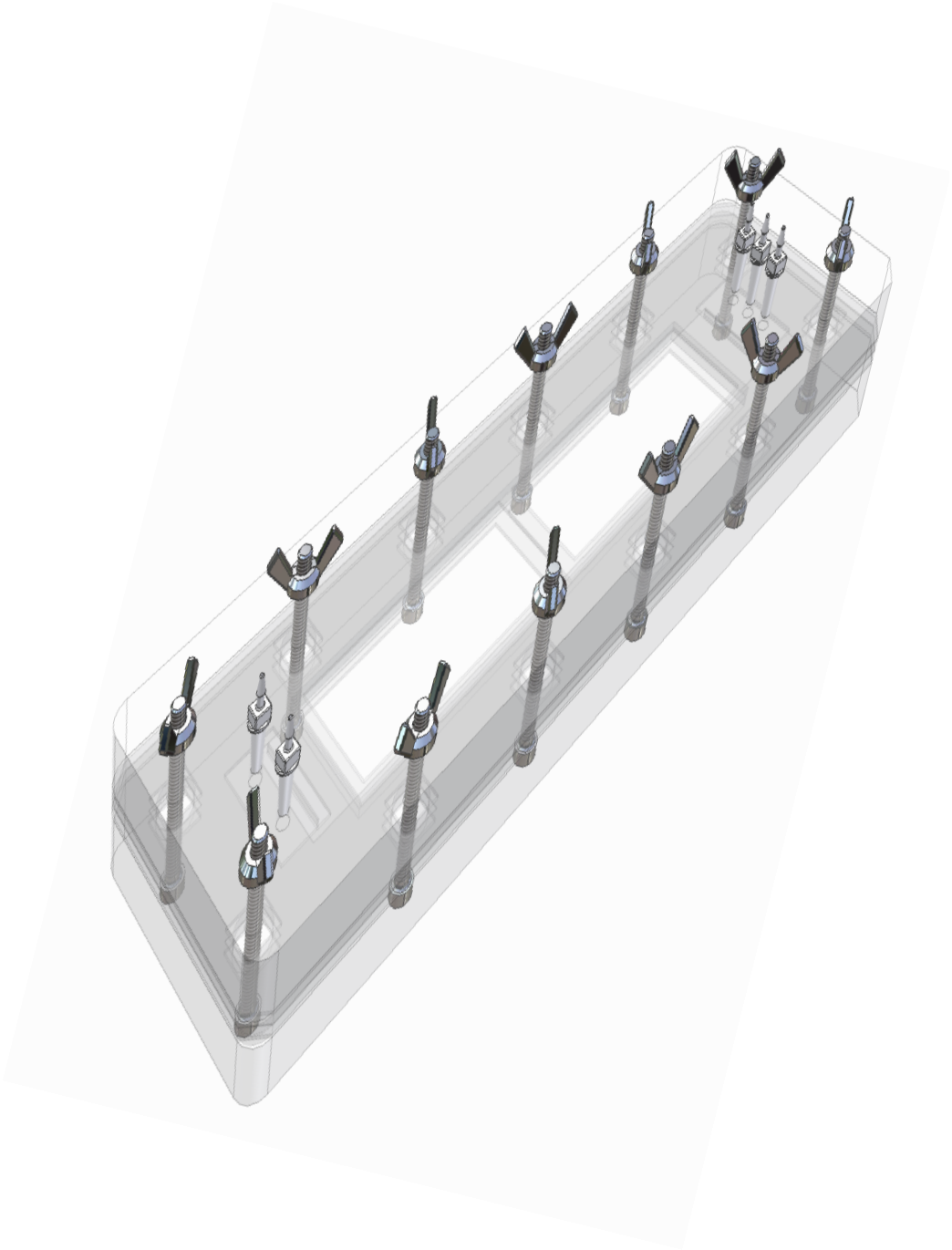


Figure 6C. Schematic diagram of fully assembled novel double-layer parallel-plate flow chamber (PPFC).

reservoir goes through to the channel of the chamber finally distributes the flow evenly throughout the channel. Each layer can also be created by means of machining with less difficulty compared to usual complicated parts with curvatures and grooves. The design does not need any glue to paste separate parts nor groove a recess on any parts where as the pieces are the final products of each layer of the chamber and simply tightened with bolts and nuts. The channel height is determined by the thickness of the gasket or layer (5) so different thicknesses can be chosen for different research purpose. To minimize the effects that would result in different height of the channel when the gasket is compressed tightly with wing nuts, we used a wide area of gasket which is enough to distribute the pressure throughout the chamber. The 1-1/2-inch long of 8-32 bolts are to be firmly tightened down with hex nuts in layer (9). The holes that accept inlet and outlet ports of layer (1) are to be briefly smoothed out by rotating against with a screw driver on the edges to prevent leakage.

The original design with a double-layer of endothelial cells can be simply changed to a monolayer of endothelial cells by removing layers (6), (7), and (8). Layer (9) can have a rectangular hole in the middle with another acrylic sheet of 0.08-inch thickness on top of layer (9) and bolted together to create a window for microscopes. The gasket of layer (5) can be cut differently or pieces of the gasket can be placed within the channel to create bifurcation flow, disturbed flow, and branched flow. Curvature flow, for example, could be applied by cutting each layer of the chamber to accept two glass slides placed in an orientation like a letter L and place gasket with smoothly cut curvature for layer (5). Different sizes and shapes of glass slides can also be fitted by cutting each layer accordingly. With such an easy way to create and use the various types of chamber, a researcher can thus easily manipulate the geometries of the channel based on the concepts how the channel, reservoirs, double layer, and the inlets and outlets are formed to suit their specific research purposes.

In the present study, the geometries of the chamber were decided based upon the boundary layer theory and some other results of numerical analysis introduced previously to fit our specific needs of having a sufficient amount of RNA for gene expression

analysis by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and perfusing an adjustable flow that exerts a moderately low shear stress of 4 dyne/cm^2. The chosen thickness of the silicone gasket for the channel height was 0.0254 cm where the width of the channel was to be 3.2 cm. This height and width of the channel give a lateral channel aspect ratio β of approximately 0.0078 according to equation (1). The dynamic viscosity of the media for the endothelial cells was measured to be 0.72 cP. These values were then used to solve the volume flow rate with equation (2). The flow rate was used to calculate the average velocity through the cross-sectional area of the channel where it was used to find the Reynolds number provided by equation (6). With values of density of water at 37 °C, the Reynolds number is easily calculated to be 8.25. The estimated entrance length of this channel and flow was about 0.0083 cm by equation (7). Although the entrance length for a flow of a Reynolds number lower than 100 is known to be simply 130% of the height of the channel (Fung, 1997) which in our case yields a negligible length of about 0.033 cm, we chose the entrance length to be at 1 cm away from the thin slit on layer (3), which is 0.7 cm away from the inlet ports, providing a total 1.7 cm of entrance length 200 times longer than the estimated value by equation (7). Because the exit lengths are usually expected to be longer than the entrance length, it was chosen to be the same as the entrance length for the symmetry of the design of the chamber.

The velocity profile through the channel of an aspect ratio β of approximately 0.0078 was examined in three dimensions with equation (3) (Figure 7A). In addition, Figure 7B depicts the velocity profile seen from above the chamber, where the velocity is zero near the walls and stay at a constant value throughout the most of the width of the channel. The place where the velocity drops below 95% from its maximum value found to be 0.027 cm away from the lateral walls. Since the length was negligible, we assumed that there were no boundary layers formed by the lateral walls.

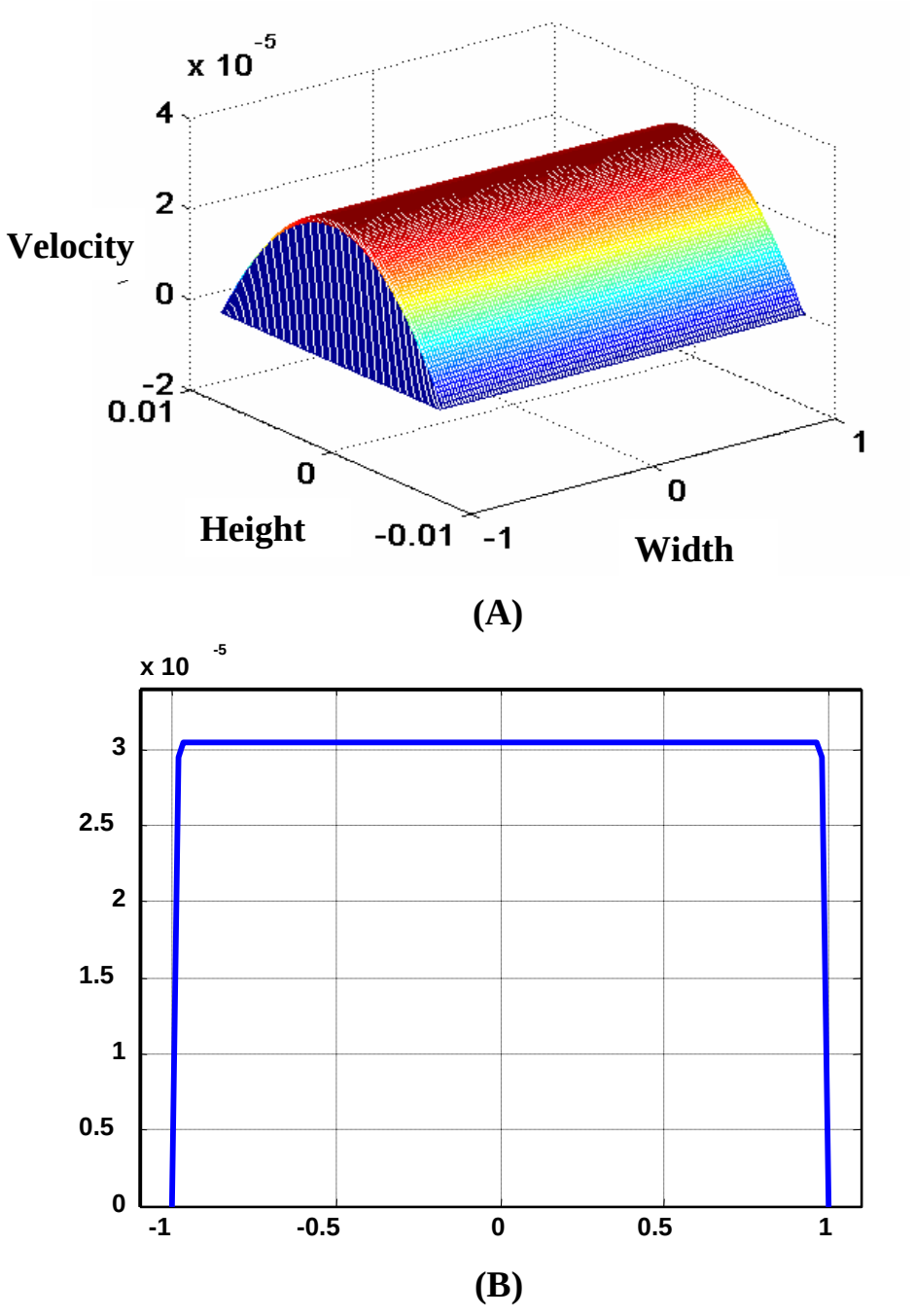


Figure 7. Mathematical analysis of the geometry of a novel double-layer parallel-plate flow chamber (PPFC). (A) The three-dimensional velocity profile. (B) The velocity profile from above or y direction.

The flow inside the channel was visualized by means of dye injection to validate the design of the chamber. Figure 8 was produced by superimposing two separate photos taken with different needle positions in the sides of the chamber. The dye was injected from the channel-like reservoir between the inlets and the thin slits. The streamlines on the top and the bottom were passed against the wall where the middle three lines were placed evenly in the middle of the channel. In addition, the streamlines that flow against the lateral walls were not disturbed throughout the length of the channel ensuring the assumption that the lateral wall effects are negligible. These data from mathematical analysis (Figure 7) and dye injection experiments through the novel PPFC (Figure 8) provide clear evidence that a uniform magnitude of shear stress can be applied throughout the entire surface of endothelial cell monolayer.

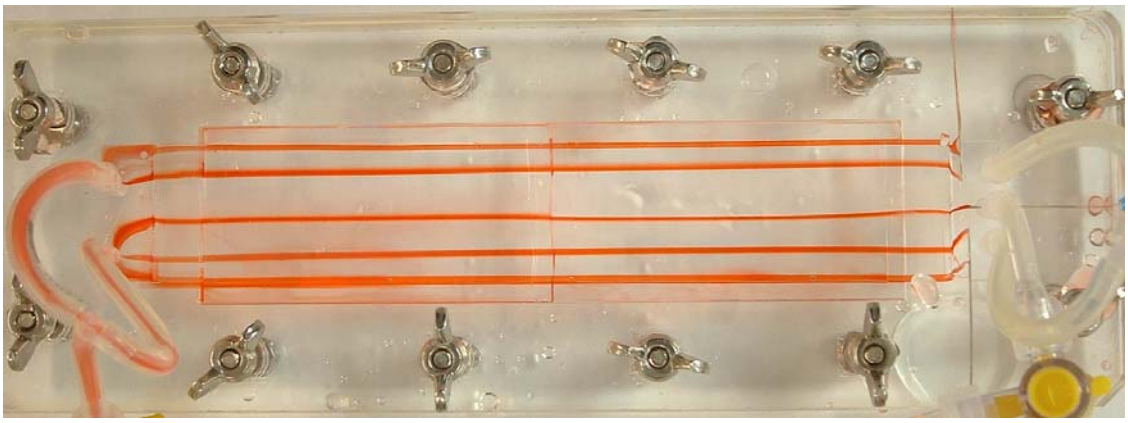


Figure 8. The visualized fluid flow profile through the channel of a novel double-layer parallel-plate flow chamber (PPFC).

2.4.2. Validation of a novel double-layer PPFC for biomedical research applications

To validate whether a novel PPFC can be employed for studies in the areas of biomedical research, the effects of fluid shear stress on the structure and function of endothelial cells were examined. In the present study, we used two different cell types, such as rat brain microvascular endothelial cells (RBE4) and human microvascular endothelial cells (HMEC-1), which are representative of both central and peripheral microvascular systems. RBE4 cells were derived from rat brain microvascular endothelial cells immortalized with the plasmid pE1A-neo containing the E1A region of adenovirus 2 and a neomycin resistance gene. RBE4 cells have been widely used as an experimental model to study the brain microvasculature *in vitro* because they are known to preserve many features of the *in vivo* brain microvascular endothelium (Yang et al., 2001, Yang and Aschner, 2003). HMEC-1 cells are an immortalized cell line obtained by transformation of human dermal microvascular endothelial cells with the SV40 large T antigen. It has been generally believed that these cells retain endothelial cell phenotype and functional characteristics and provide the best physiological cell model in research related to the microvascular endothelium (True et al., 2000, Eum et al., 2004, Eum et al., 2006, Lee et al., 2003b).

Effects of fluid shear stress on morphological responses of endothelial cells.

It is widely believed that the reorganization of the endothelial cell morphology is one of the earliest responses in vascular endothelial cells that are exposed to fluid shear stress. Previous *in vivo* and *in vitro* studies have demonstrated the elongation and alignment of endothelial cells parallel to the direction of flow (Topper and Gimbrone, 1999, Fujiwara et al., 1998, Davies, 1995, Dewey et al., 1981, Isenberg et al., 2006, Li et al., 2005). In the present study, we first examined the morphological changes of endothelial cells associated with exposure to physiologically relevant shear stress using a novel PPFC. Rat brain microvascular endothelial cells (RBE4) and human microvascular endothelial cells (HMEC-1) were either maintained in static condition or exposed to laminar flow at a

shear stress of 4 dyne/cm^2 for 24 h. As shown in Figure 9, fluid shear stress induced morphological changes such as cell elongation and alignment with the direction of flow (*lower panels*). In contrast, endothelial cells in static condition appeared to be round shape and no significant cell orientation was observed (*upper panels*). These results on cell shape and orientation are consistent with those reported previously by other groups (Dewey et al., 1981, Eskin et al., 1984, Levesque and Nerem, 1985, Chiu et al., 1998, Fujiwara et al., 1998, Topper and Gimbrone, 1999, Li et al., 2005, Davies, 1995).

Effects of fluid shear stress on inflammatory gene expression of endothelial cells.

Shear stress-mediated signaling pathways have been known to trigger endothelial cell responses and contribute to the pathophysiology of human vascular diseases. For example, changes in shear stress resulting from altered blood flow play a crucial role in the development of cardiovascular diseases by regulating inflammatory reactions in the vascular endothelium (Li et al., 2005, Davies, 1995). Previous studies have demonstrated that inflammatory responses in the vascular endothelium are primarily regulated through the production of pro-inflammatory mediators (Ross, 1993, Ross, 1999, Lee et al., 2004a, Lee et al., 2004b, Lee and Hirani, 2006). In fact, enhanced expression of pro-inflammatory cytokines (e.g., IL-6) and adhesion molecules (e.g., ICAM-1, VCAM-1 and E-selectin) in vascular endothelial cells and their close interactions facilitate recruiting and adhering inflammatory cells into the vessel wall, and thus stimulate transendothelial migration, which can be considered one of the earliest events in the progression of human vascular diseases. In the present study, expression levels of pro-inflammatory cytokine and adhesion molecules were determined by quantitative real-time RT-PCR to examine the effects of fluid shear stress on inflammatory responses in vascular endothelium. A significant down-regulation of cytokine IL-6 (Figure 10A) and adhesion molecules ICAM-1, VCAM-1 and E-selectin (Figure 10B) was observed in microvascular endothelial cells which were exposed to laminar flow at a shear stress of 4 dyne/cm^2 (*Flow*) for 24 h, compared with those maintained in static condition (*Static*). These findings are in agreement with numerous previous studies suggesting that normal, healthy shear stress can influence the ability of vascular endothelium to carry out many of its

beneficial functions in health including anti-inflammatory effects (Topper and Gimbrone, 1999, Li et al., 2005, Ando et al., 1995, Albuquerque et al., 2000).

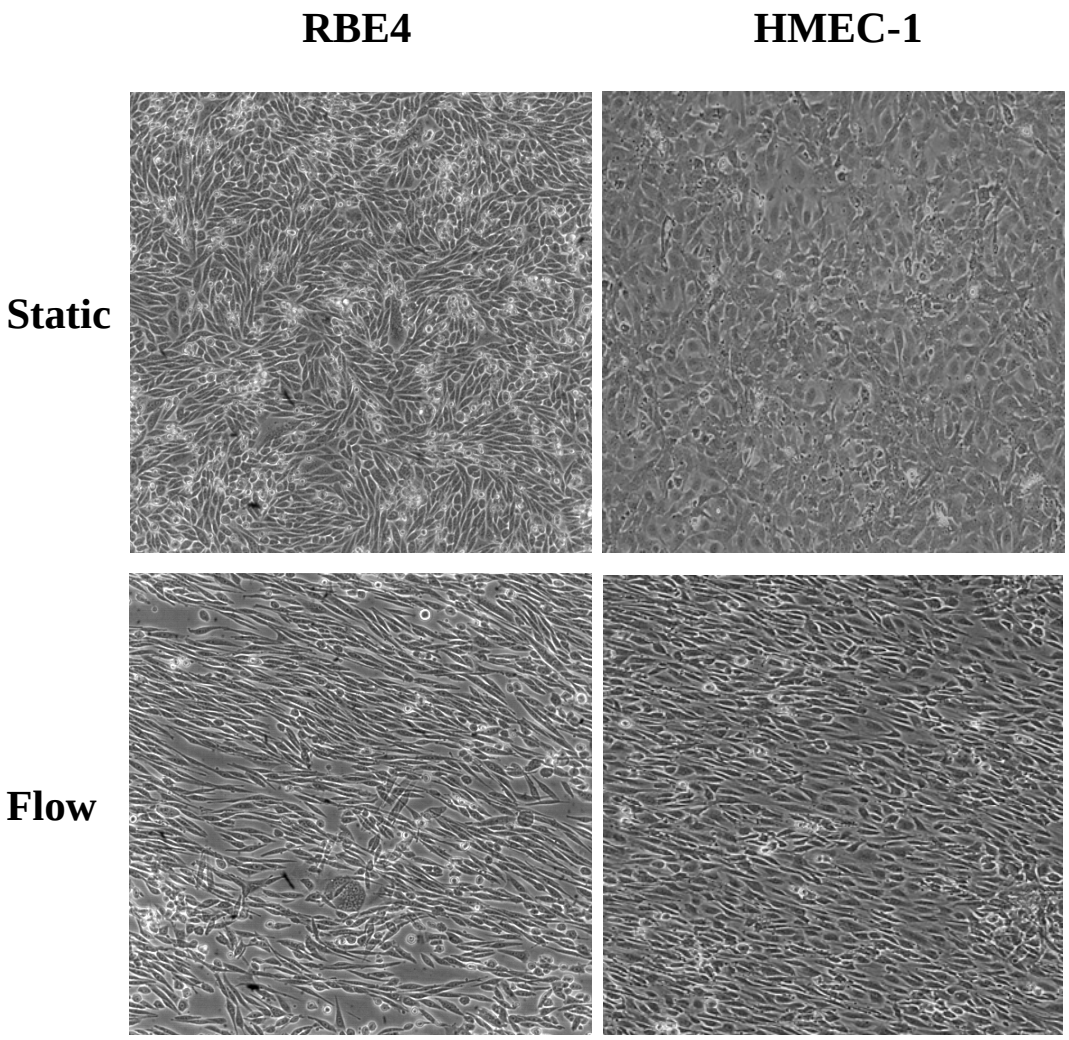


Figure 9. Fluid shear stress causes morphological changes in microvascular endothelial cells. Rat brain microvascular endothelial cells (RBE4; *left panels*) and human microvascular endothelial cells (HMEC-1; *right panels*) were either maintained in static condition (*upper panels*) or exposed to laminar flow at a shear stress of 4 dyne/cm^2 (*lower panels*). Following incubation for 24 h, morphological changes of endothelial cells were visualized as described in *Materials and Methods*.

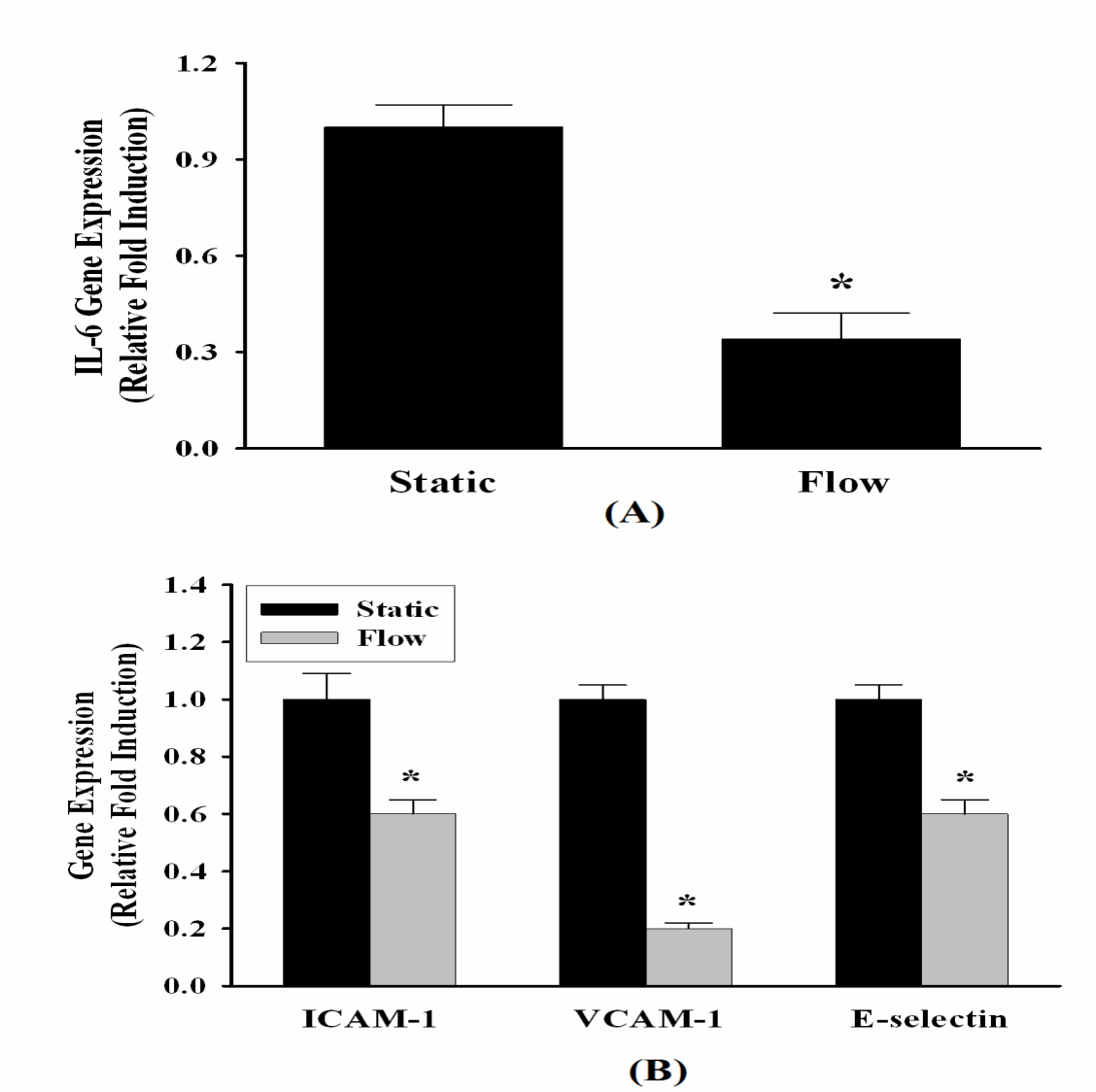


Figure 10. Fluid shear stress attenuates expression of pro-inflammatory mediators in microvascular endothelial cells. Rat brain microvascular endothelial cells (RBE4) and human microvascular endothelial cells (HMEC-1) were either maintained in static condition (*Static*) or exposed to laminar flow at a shear stress of 4 dyne/cm^2 (*Flow*) for 24 h. Expression levels of pro-inflammatory mediators, such as IL-6 in RBE4 (A) and adhesion molecules (ICAM-1, VCAM-1, and E-selectin) in HMEC-1 (B), were determined by quantitative real-time RT-PCR as described in *Materials and Methods*. Data are means ± SEM of 4 determinations. *Statistically significant compared with the static group ($P < 0.05$).

Biomedical Research Applications of PPFC: A Novel *In Vitro* Ischemia/Reperfusion Injury Model

3.1. Abstract

The reperfusion of blood flow occurred in a number of conditions such as stroke and organ transplantation immensely augments tissue injury and can cause more severe damage than prolonged ischemia. The injuries caused by cessation and reperfusion of blood flow are closely related to the inflammatory reactions involving in endothelium-leukocyte cascade responding to a shear stress exerted by the flow. Shear stress is also known to play an important role in human chronic diseases including atherosclerosis, neurological disorders, and cancer metastasis. Therefore, it is important to investigate the transmission of mechanical stimuli such as shear stress to various complex endothelial cell signaling pathways which process as a whole is often referred as mechanotransduction. Shear stress-mediated signaling pathways have been known to trigger endothelial cell responses and contribute to the pathophysiology of human vascular diseases. The present study was designed to apply the novel PPFC to biomedical research, especially ischemia/reperfusion injury. The changes in mRNA and protein expression of inflammatory mediators in endothelial cells were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. RBE4 and HMEC-1 cells were either maintained in continuous laminar flow condition (*Normal Flow*) or subjected to 1 h of flow cessation followed by reperfusion of flow (*Ischemia/Reperfusion*) for 24 h. *Ischemia/Reperfusion* significantly up-regulated expression of inflammatory mediators, such as IL-6, MCP-1, ICAM-1, VCAM-1, and E-selectin, in microvascular endothelial cells. Furthermore, antioxidant pyrrolidine dithiocarbamate (PDTC) significantly attenuated ischemia/reperfusion-induced overexpression of pro-inflammatory mediators. These data indicates that our newly designed PPFC provide a better *in vitro* system for versatile applications of biomedical research.

3.2. Introduction

It has become evident that the cessation of blood flow (*ischemia*) causes tissue damages and the restoration of blood flow (*reperfusion*) produces even more injuries to a wide variety of tissues. Indeed, ischemia/reperfusion injury occurs in a broad spectrum of pathological outcomes in patients after organ transplantation and cardiovascular surgery as well as myocardial infarction and stroke (Khalil et al., 2006). Compelling body of evidence has indicated that ischemia/reperfusion injury is closely associated with inflammatory reactions in vascular endothelium. Inflammatory responses such as leukocyte activation and recruitment contribute to one of the early mechanisms of ischemia-reperfusion injury. For example, Krizanac-Bengez et al. (2006) showed that exposure of a dynamic *in vitro* blood-brain barrier (BBB) model to flow cessation/reperfusion resulted in an acute release of pro-inflammatory cytokines, such as TNF-α and IL-6, and disruptions of BBB integrity. It was also found that myocardial ischemia/reperfusion injury is related to an inflammatory response though attachment of polymorphonuclear leukocytes (PMNs) to the vascular endothelium with subsequent infiltration into the damaged myocardium (Hansen, 1995, Onai et al., 2003). In the present study, we developed a novel *in vitro* system to mimic ischemia/reperfusion injury *in vivo* in order to examine the effects of ischemia/reperfusion on pro-inflammatory pathways in microvascular endothelial cells.

3.3. Materials and Methods

3.3.1. Cell cultures

Human microvascular endothelial cells (HMEC-1) and rat brain microvascular endothelial cells (RBE4) were cultured as described in "*Section 2.3.1*".

3.3.2. *In vitro* ischemia/reperfusion injury model

Confluent endothelial cells on glass slides were loaded in the PPFC as described in "*Section 2.3.2*". Endothelial cells were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted endothelial cells were then either maintained in continuous laminar flow condition (4 dyne/cm^2) (*Normal Flow*) or subjected to 0.5 h or 1 h of flow cessation followed by reperfusion for 6 h, 12 h, or 24 h with laminar flow (4 dyne/cm^2) (*Ischemia/Reperfusion*). In the present studies, similar experimental conditions were employed to mimic *in vivo* ischemia/reperfusion injury.

In selected experiments, the cells were treated with antioxidant pyrrolidine dithiocarbamate (PDTC; Sigma, St. Louis, MO) to determine involvement of an oxidative stress-related mechanism in ischemia/reperfusion injury.

3.3.3. Real-time RT-PCR

The mRNA expression levels of pro-inflammatory genes were determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) as described in "*Section 2.3.3*".

3.3.4. Enzyme-linked immunosorbent assay (ELISA)

The protein expression levels of human MCP-1 in cell culture supernatants were determined using Human MCP-1 Immunoassay kit (R&D Systems) according to the protocol of the manufacturer. This assay employs the quantitative sandwich enzyme immunoassay technique using a murine monoclonal antibody against human MCP-1 and a polyclonal secondary antibody conjugated with horseradish peroxidase. The minimum detectable concentration was less than 5.0 pg/mL.

3.3.5. Statistical analysis

Statistical analysis of data was completed using SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA). One-way ANOVA was used to compare mean responses among the treatments. For each endpoint, the treatment means was compared using Bonferroni least significant difference procedure. Differences among the means were considered significant at $P < 0.05$.

3.4. Results and Discussion

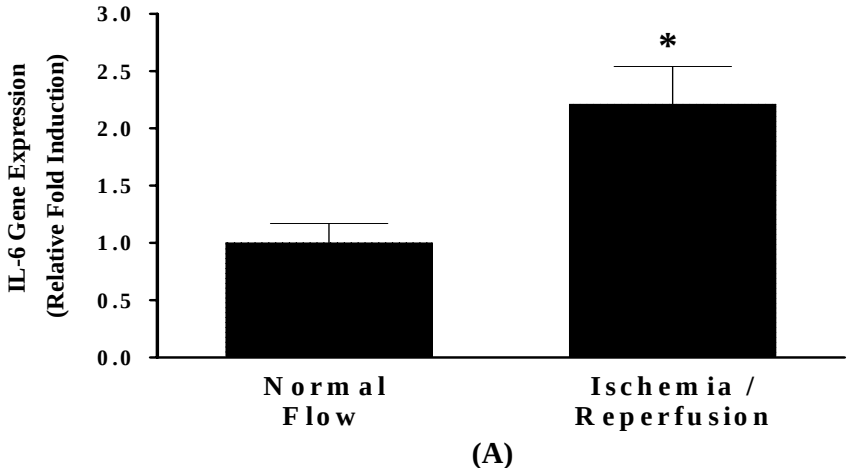
Previous *in vivo* and *in vitro* studies have revealed that transendothelial migration of blood leukocytes into tissues is mediated primarily by adhesion molecules, such as E-selectin, ICAM-1, and VCAM-1, which are overexpressed on the surface of vascular endothelium in response to ischemia/reperfusion injury (Cassie et al., 2004, Billups et al., 1995, Fleming et al., 2003, Masatsugu et al., 2003, Wang et al., 1998). The crucial role for adhesion molecules in ischemia/reperfusion-mediated inflammatory reactions and subsequent tissue injury has been further confirmed in a number of animal models of ischemia/reperfusion by blocking these molecules through antagonist and monoclonal antibody treatments, genetic deletion, and exposure to antisense oligonucleotides (Onai et al., 2003, Kurose et al., 1994, Winn et al., 1993, Wang et al., 1998, Connolly et al., 1996, Toda et al., 2000). In the present study, we developed a novel *in vitro* ischemia/reperfusion injury model using our newly designed PPFC and examined the effects of ischemia/reperfusion on pro-inflammatory pathways in microvascular endothelial cells. As shown in Figure 11, ischemia/reperfusion markedly and significantly up-regulated expression of pro-inflammatory cytokine IL-6 and adhesion molecules ICAM-1, VCAM-1, and E-selectin (Figure 11). In addition, the effects of time exposure on changes in gene expression levels of pro-inflammatory mediators are shown in Figure 12. Significant

and maximum elevation of mRNA expression of IL-6, MCP-1, ICAM-1, E-selectin, and VCAM-1 was observed 12 h after reperfusion. Interestingly, flow cessation (ischemia) alone did not affect gene expressions of pro-inflammatory mediators. These results are in agreement with previous studies from other investigators. For example, a constitutive expression of adhesion molecules was not up-regulated by hypoxia alone but hypoxia-reoxygenation (Ichikawa et al., 1997) and the expression levels were reached maximum at 12 h after reoxygenation (Corcoran et al., 2006).

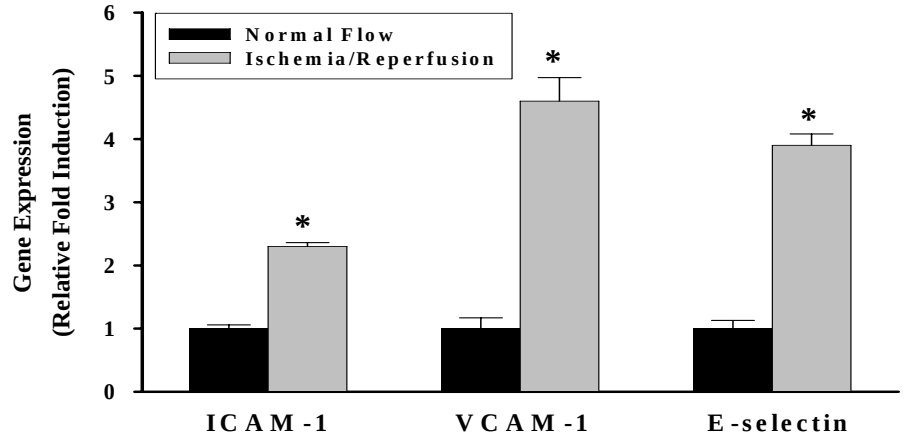
To verify ischemia/reperfusion-induced increases in mRNA levels can be translated to elevated protein expression, concentration of MCP-1 protein was determined in HMEC-1 by ELISA. Consistent with the gene expression analysis, ischemia/reperfusion resulted in significant up-regulation of MCP-1 protein levels (Figure 13). These results further confirm that ischemia/reperfusion can induce pro-inflammatory environments through the up-regulation of mRNA and protein expression of inflammatory mediators in human microvascular endothelial cells.

To determine whether ischemia/reperfusion-induced overexpression of pro-inflammatory genes is mediated by an antioxidant-sensitive mechanism, antioxidant pyrrolidine dithiocarbamate (PDTC) was employed. PDTC has been widely used as an antioxidant compound and specific inhibitor of oxidative stress-related transcription factor NF-κB to study redox regulation of the intracellular signaling pathway and of cell function (Iseki et al., 2000). As shown in Figure 14, pretreatment with PDTC at the concentration of 10 μM markedly and significantly attenuated up-regulation of pro-inflammatory gene expression induced by ischemia/reperfusion. These results are consistent with previous *in vivo* studies demonstrating that antioxidants may prevent ischemia/reperfusion injury through inhibiting the activity of NF-κB (Tian et al., 2006a, Tian et al., 2006b). To examine more detailed mechanisms underlying this process, dose-dependent effects of PDTC on ischemia/reperfusion injury *in vitro* need to be further investigated.

These data provide robust evidence that a novel PPFC can be used as an effective *in vitro* system to mimic ischemia/reperfusion injury *in vivo*.



(A)



(B)

Figure 11. Ischemia/reperfusion up-regulates expression of pro-inflammatory mediators in microvascular endothelial cells. Rat brain microvascular endothelial cells (RBE4) and human microvascular endothelial cells (HMEC-1) were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted microvascular endothelial cells were then either maintained in continuous laminar flow condition (4 dyne/cm^2) for 25 h (A) or 13 h (B) (*Normal Flow*) or subjected to 1 h of flow cessation followed by reperfusion with laminar flow (4 dyne/cm^2) for 24 h (A) or 12 h (B) (*Ischemia/Reperfusion*). Expression levels of pro-inflammatory mediators, such as IL-6 in RBE4 (A) and adhesion molecules (ICAM-1, VCAM-1, and E-selectin) in HMEC-1 (B) were determined by quantitative real-time RT-PCR as described in *Materials and Methods*. Data are means ± SEM of 4 determinations. *Statistically significant compared with the normal flow group ($P < 0.05$).

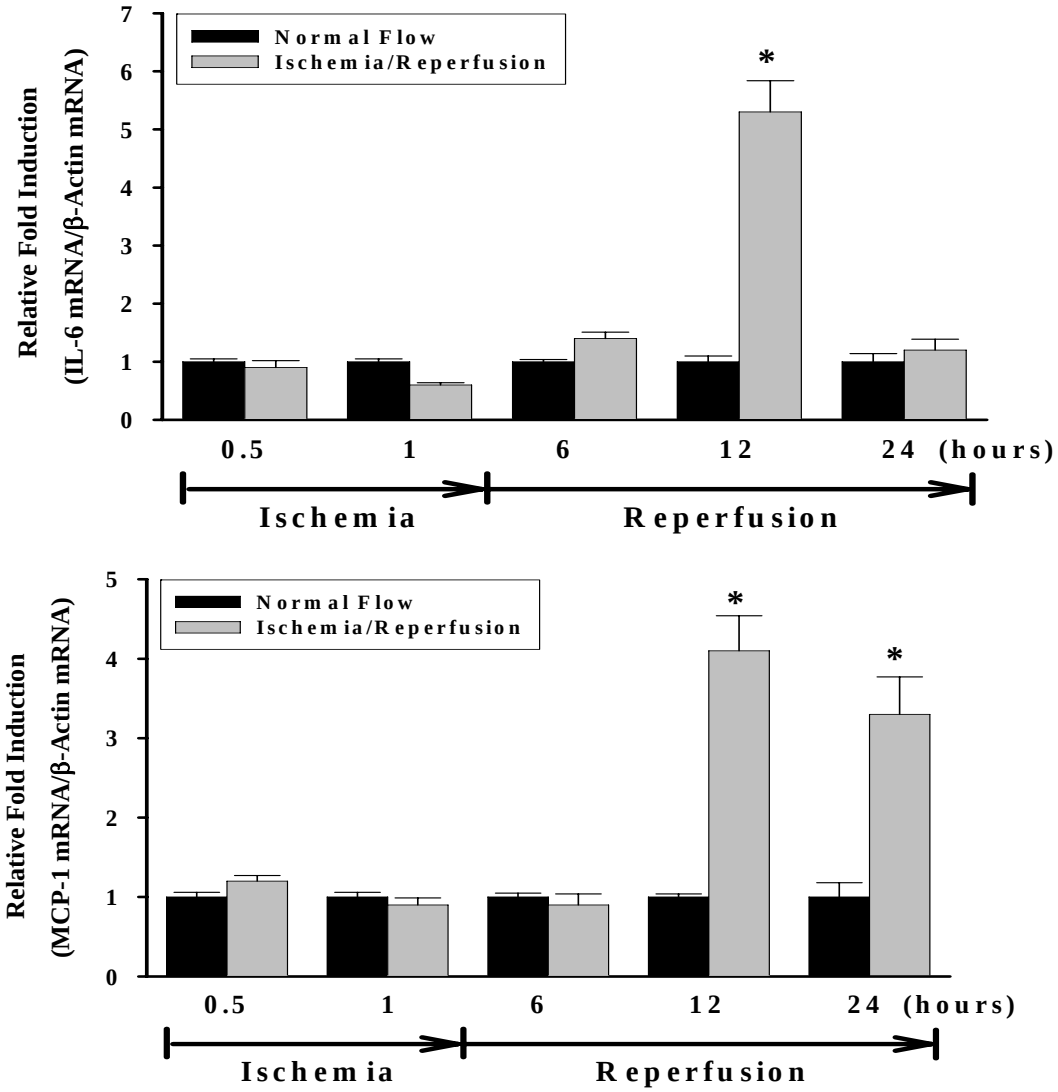


Figure 12A. Time-dependent effects of ischemia/reperfusion on gene expression levels of IL-6 and MCP-1 in microvascular endothelial cells. HMEC-1 were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted microvascular endothelial cells were either maintained 0.5 h and 1 h of flow cessation (*Ischemia*) or subjected to 1 h of flow cessation followed by reperfusion with laminar flow (4 dyne/cm^2) for up to 24 h (*Ischemia/Reperfusion*). Expression levels of pro-inflammatory cytokine IL-6 (*upper panel*) and chemokine MCP-1 (*lower panel*) were determined by quantitative real-time RT-PCR as described in *Materials and Methods.* Data are means ± SEM of 4 determinations. *Statistically significant compared with the normal flow group ($P < 0.05$).

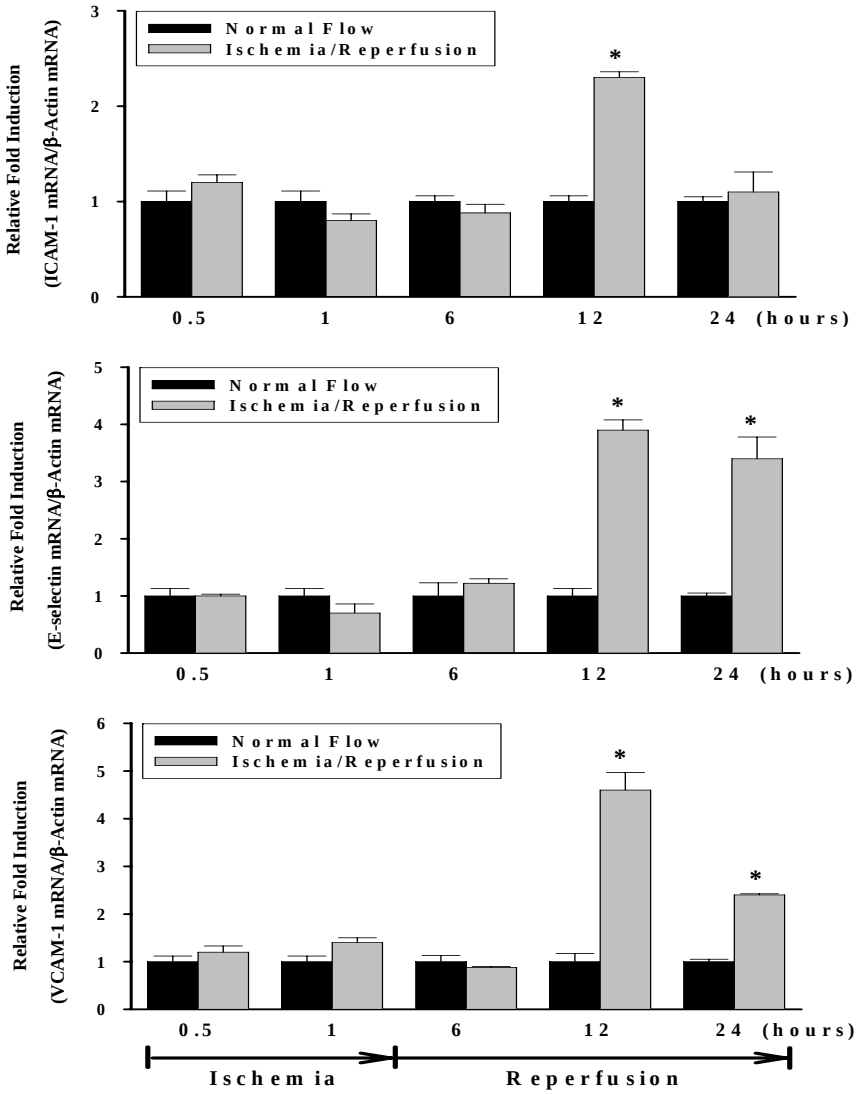


Figure 12B. Time-dependent effects of ischemia/reperfusion on gene expression levels of ICAM-1, E-selectin, and VCAM-1 in microvascular endothelial cells. HMEC-1 were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted microvascular endothelial cells were either maintained 0.5 h and 1 h of flow cessation (*Ischemia*) or subjected to 1 h of flow cessation followed by reperfusion with laminar flow (4 dyne/cm^2) for up to 24 h (*Ischemia/Reperfusion*). Expression levels of adhesion molecules ICAM-1 (*top panel*), E-selectin (*middle panel*), and VCAM-1 (*bottom panel*) were determined by quantitative real-time RT-PCR as described in *Materials and Methods.* Data are means ± SEM of 4 determinations. *Statistically significant compared with the normal flow group ($P < 0.05$).

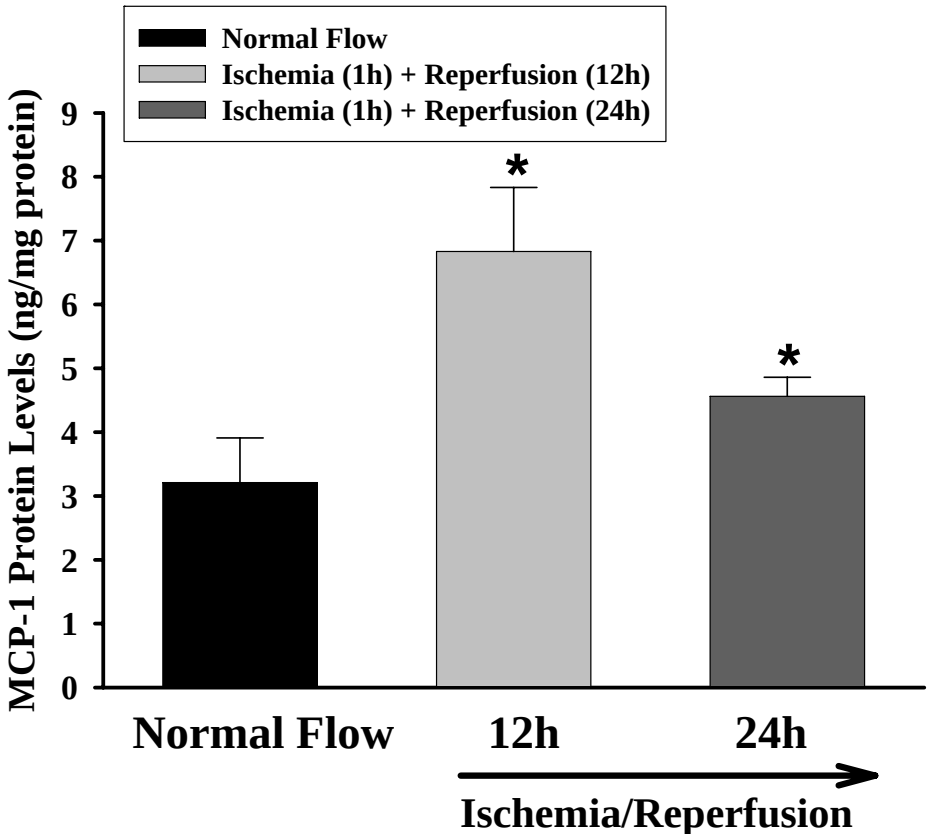


Figure 13. Ischemia/reperfusion increases the protein expression of MCP-1 in microvascular endothelial cells. HMEC-1 were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted microvascular endothelial cells were either maintained in continuous laminar flow condition (4 dyne/cm^2) for 13 h and 25 h (*Normal Flow*) or subjected to 1 h of flow cessation followed by reperfusion with laminar flow (4 dyne/cm^2) for 12 h and 24 h (*Ischemia/Reperfusion*). The protein expression levels of MCP-1 were measured by ELISA as described in *Materials and Methods*. Data are means ± SD of 4 determinations. * Statistically significant compared with the control group ($P < 0.05$).

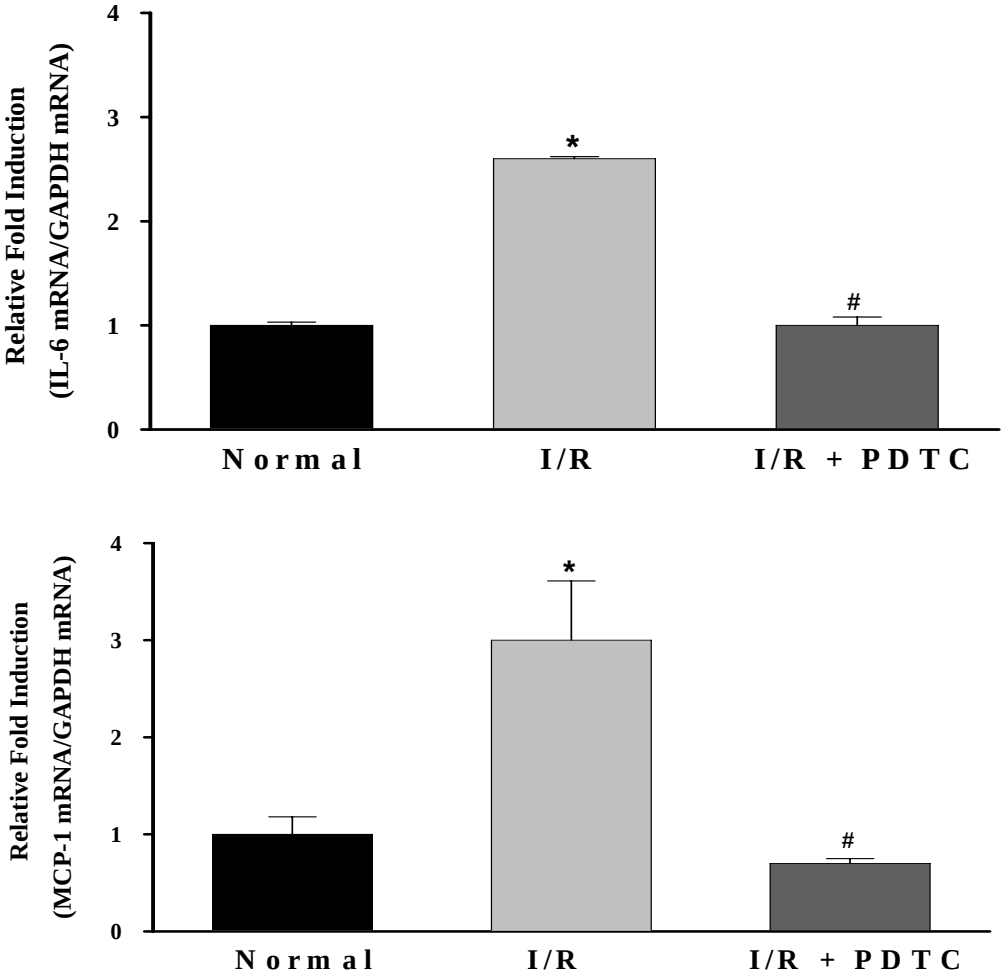


Figure 14A. Antioxidant PDTC attenuates ischemia/reperfusion-induced overexpression of IL-6 and MCP-1 in microvascular endothelial cells. HMEC-1 were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted microvascular endothelial cells were either maintained in continuous laminar flow condition (4 dyne/cm^2) (*Normal*) or subjected to 1 h of flow cessation followed by reperfusion with laminar flow (4 dyne/cm^2) for 12 h (*I/R*) in the presence or absence of 10 μM PDTC. Expression levels of pro-inflammatory cytokine IL-6 and chemokine MCP-1 were determined by quantitative real-time RT-PCR as described in *Materials and Methods*. Data are means ± SEM of 4 determinations. *Statistically significant compared with the normal flow group ($P < 0.05$). # Values in the I/R + PDTC group are significantly different from the I/R group ($P < 0.05$).

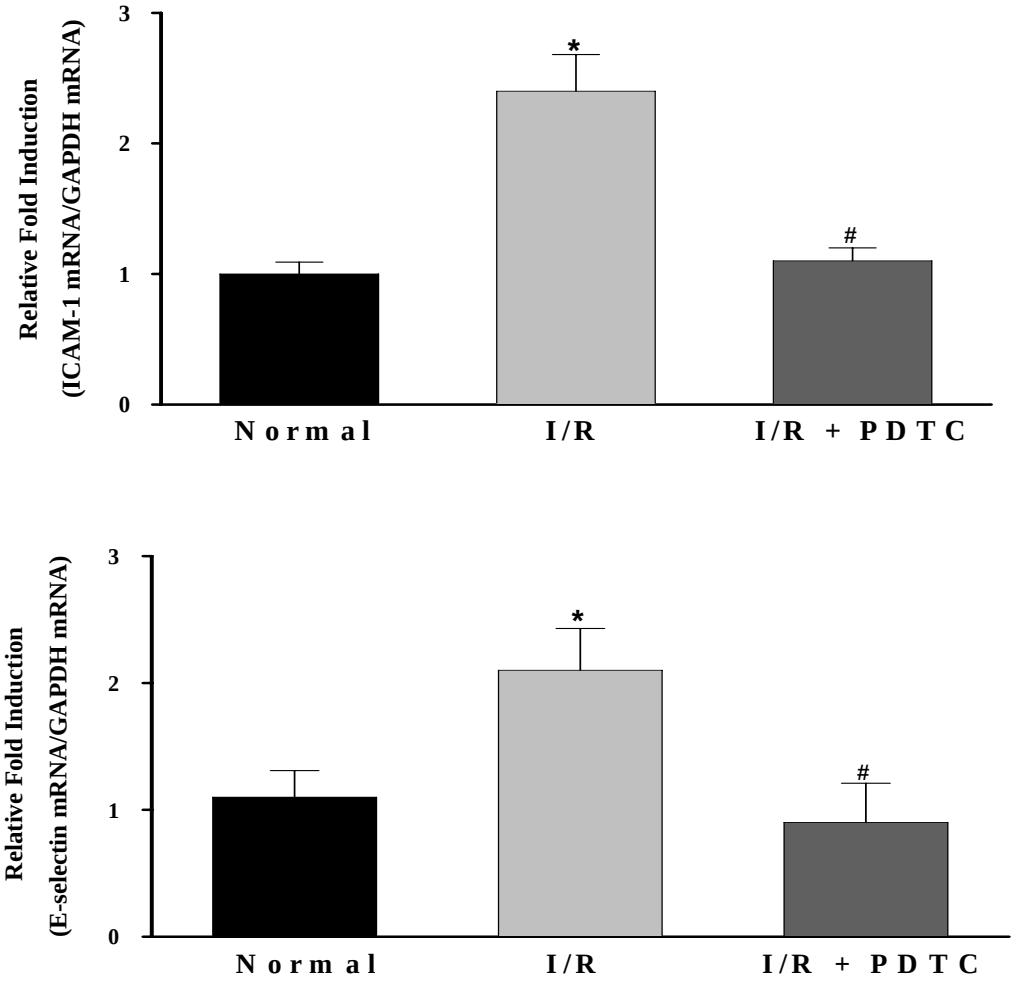


Figure 14B. Antioxidant PDTC attenuates ischemia/reperfusion-induced overexpression of ICAM-1 and E-selectin in microvascular endothelial cells. HMEC-1 were exposed to laminar flow at a shear stress of 4 dyne/cm^2 for 24 h. Flow-adapted microvascular endothelial cells were either maintained in continuous laminar flow condition (4 dyne/cm^2) (*Normal*) or subjected to 1 h of flow cessation followed by reperfusion with laminar flow (4 dyne/cm^2) for 12 h (*I/R*) in the presence or absence of 10 μM PDTC. Expression levels of adhesion molecules ICAM-1 and E-selectin were determined by quantitative real-time RT-PCR as described in *Materials and Methods*. Data are means ± SEM of 4 determinations. *Statistically significant compared with the normal flow group ($P < 0.05$). # Values in the I/R + PDTC group are significantly different from the I/R group ($P < 0.05$).

Conclusion

The vascular endothelial cells form the interface between the blood and vessel wall. These cells are continuously subjected to hemodynamic forces generated by blood flow on the endothelial cell surface. Since wall shear stress is a major hemodynamic factor, it has been widely accepted that integrity and functions of vascular endothelial cells are greatly affected by shear stress.

Since there a number of limitations in animal experiments, the development of *in vitro* cell culture systems under precisely controlled fluid conditions has been required to investigate relationship between shear stress and endothelial cell biology. The present study primarily focused on design and construction of a novel double-layer PPFC. Both mathematical analysis and dye injection experiments clearly demonstrated that a uniform magnitude of shear stress can be applied throughout the entire surface of endothelial cell monolayer within the chamber. Altered morphology as well as attenuated pro-inflammatory gene expression in response to physiologically relevant shear stress was also observed. In addition, we developed a novel *in vitro* ischemia/reperfusion injury model using a novel PPFC to examine the effects of ischemia/reperfusion on pro-inflammatory pathways in microvascular endothelial cells.

These data provide robust evidence that a novel PPFC can be used as an effective *in vitro* system to mimic ischemia/reperfusion injury *in vivo*. Furthermore, our results showing that antioxidant PDTC protected endothelial cells from ischemia/reperfusion injury may offer a potential therapeutic approach toward the prevention and/or treatment of disease progression specifically targeted against the pro-oxidative and pro-inflammatory pathways of ischemia/reperfusion injury.

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