EFFECT OF FLUID FORCES ON VASCULAR ENDOTHELIUM

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

Coronary Artery Disease is one of the most important resaons of the death in the world and Turkey. Endothelial cell dysfunction has been linked to atherosclerosis through their response to fluid forces. Endothelial cells, change their morphology when exposed to mechanical stimuli. The morphological responses include reorientation, elongation, and adhesion molecules rearrangement. Atherosclerotic lesions form at specific arterial regions, where low and oscillatory endothelial shear stress (ESS) occur. In this thesis, the effects of steady and oscillatory flow on Human Umbilical Vein Endothelial Cells (HUVECs) at different hours were determined. Also this thesis is the first study to investigates the effects of fluid forces on living cells in Turkey. The interaction between flow types and time period was studied. Steady flow experiments were performed 500, 1000, and 1500 ml/min at 24 hr. Oscillatory flow experiments were performed 100, 250, 500, and 1000 ml/min at 24 hr. When results were examined, morphological changes easily observed. Immunofluorescent staining methods were performed to determine the localization of VE-cadherin and α5/β1 integrin adhesion proteins.

Keywords: Atherosclerosis, hemodynamic forces, steady and oscillatory flow, VE-cadherin, α5/β1 integrin.

AKIŞKAN KUVVETLERİN VASKULER ENDOTEL HÜCRELERİN ÜZERİNDEKİ ETKİLERİ

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ÖZ

Koroner arter hastalıkları dünyada ve Türkiye'de ölümlerin en önemli sebeplerinden biri olarak önemli rol oynamaktadır. Akışkan kuvvetlerin etkileri ile oluşan endotel hücre fonksiyon bozuklukları damar tıkanıklığı (aterosklerozis) ile bağlantılıdır. Endotel hücreleri mekanik uyarıcı etkilere maruz kaldığında morfolojik olarak yapılarında değişiklikler gözlemlenir. Morpholojik değişiklikler yeniden yapılanmaları, akış yönünde uzamaları ve adezyon moleküllerindeki lokalizasyon farklılıkları içerir. Damar tıkanıklığını oluşturan lezyonların düşük ve salınımlı akış kayma gerilimlerinin oluştuğu arteriyel bölgelerde görüldüğü bilinmektedir. Bu tez çalışmasında, düz ve salınımlı akışların endotel hücreleri üzerindeki etkileri incelenmiştir. Bu tür çalışmalar açısından Türkiye'de yapılan ilk çalışmadır. Bu çalışma ile akışkanların canlı hücreler üzerindeki etkilerini inceleyebilmek için gereken zemin hazırlanmış olup, beraberinde farklı etkilerinin de incelenebileceği bir sistem oluşturulmuştur. Farklı iki akış türünün zamana bağlı etkilerini gözlemlemek hedeflenmiştir. Bu kapsamda düz (steady) akış türünde farklı akış şartlarında, 500, 1000 ve 1500 ml/min olmak üzere 24 saatlik akış sonrası hücreler üzerindeki etkileri incelenmiştir. Deneyler salınımlı (oscillatory) akış türünde 100, 250, 500 ve 1000 ml/min akış debilerinde 24 saatlik etkileri incelenmiştir. Sonuçlar incelendiğinde

morfolojik değişiklikle kolaylıkla gözlemlenebilmektedir. VE-cadherin (vasküler endotel cadherin) ve alfa5/beta1 integrin moleküllerinin akış sonrası hücre içerisindeki lokalizasyonları immunofluorasan boyama teknikleriyle gösterilmiştir.

Anahtar kelimeler: Ateroskleroz, hemodinamic kuvvetler, düz ve salınımlı akış, VE-cadherin, alfa5/beta1 integrin

DEDICATION

To my dear **Mother**, **Father**, and **Sister**

Thanks for their strongest LOVE, SUPPORT, and PATIENCE

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AC	Adherens Junctions
BAECs	Bovine Aortic Endothelial Cells
Ca	Calcium
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMEM LG	Dulbecco's Modified Eagle's Medium Low Glucose
DMSO	Dimethyl Sulfoxide
EC	Endothelial Cell
ESS	Endothelial Shear Stress
FBS	Fetal Bovine Serum
HUVECs	Human Umbilical Vein Endothelial Cells
LDL	Low Density Lipoprotein
NGS	Normal Goat Serum
NHS	Normal Horse Serum
PBS	Phosphate Buffered Saline
RT	Room Temprature
TJ	Tight Junctions
VE-cadherin	Vascular Endothelial Cadherin

CHAPTER 1

INTRODUCTION

Coronary Artery Disease is one of the most important reasons of the death in the world and Turkey. Turkey, a developing country, has a high cardiovascular morbidity and mortality. The designation of the 290 instances of death in adults aged 20 years or older comprised 42% coronary deaths, far exceeding those due to cancer (20%), cerebrovascular accident (11%) and other suspected causes. (Onat A., 2001). Coronary artery disease (CAD) is arteriosclerosis of the inner lining of the blood vessels that supply blood to the heart. Atherosclerosis occurs preferentially at vascular curvature and branch sites where the vessel walls are exposed to fluctuating shear stress and have high endothelial permeability (Shu Chien et al, 2005). Although the entire vasculature is exposed to the atherogenic effects of the systemic risk factors (e.g., hyperlipidemia, cigarette smoking, hypertension, diabetes mellitus, chronic infections, and genetic predisposition), atherosclerotic lesions form at specific regions of the arterial tree, such as in the vicinity of branch points, the outer wall of bifurcations, and the inner wall of curvatures, where disturbed flow occurs (VanderLaan et al., 2004).

Vascular endothelial cells (ECs) which form the interface between the circulating blood and the arterial wall are constantly exposed to hemodynamic forces, including fluid shear stress and mechanical stretch. Shear stress, the tangential component of hemodynamic forces acting on the ECs, is an important modulator of vascular cellular functions (Shu Chien et al, 2005).

Endothelial cells are held together by various types of intercellular adhesion molecules: 1) Ca^{2+}-dependent transmembrane cadherins comprising vascular endothelial (VE) cadherin, P-cadherin, and N-cadherin; 2) platelet endothelial cell adhesion molecule 1 (PECAM-1), a transmembrane adhesion molecule belonging to the

Ca^{2+}-independent immunoglobulin superfamily; and, finally, 3) the integrins α2/β1 and α5/β1 (Drenckhan et al- 1997).

In the carotid artery, for instance, there is a well-known predisposition for atheroma plaques to develop in the outer wall of the vessel at the level of the bifurcation of the common carotid artery, and in the coronary arteries it has also been shown that there is a preferential progression of plaques in segments with bifurcations, as well as along the inner wall of curves in the coronary arteries (Mongain et al, 2006).

CHAPTER 2

LITERATURE REVIEW

2.1 Endothelial Cells and Blood Vessels

Endothelial cells are 0.2-0.5 µm thick, 10-15 µm wide and 25-50 µm long and have a centrally located oval or round nucleus slightly raised compared to the rest of the cell. Endothelial cells are usually flat and elongated in the direction of blood flow (Sumpio et al., 2002)

Vascular endothelial cells line the inner surface of blood vessels and serve as a selective barrier between the blood and other tissues and organs. It is a metabolically active monolayer, which is constantly exposed to both biochemical and biomechanical stimuli. It is well established now that the transduction of these stimuli alone or in combination by the endothelium determines the physiology or pathology of the cardiovascular system (Resnick et al., 2003).

Framework of the cells provides structure and tensile strength to withstand mechanical forces imposed by the body. This structure gives the cell ability to assume different shapes and movement. Morphological changes depend on complex network of protein filaments. The structure of the cytoskeleton is very dynamic and, unlike a rigid skeleton comprised of bone, is changing as cells alter shape, move or divide in response to their environment (Chouquet, et al 2001).

2.2 Structure of the Cell–Cell Junctions

Of all the social interactions between cells in a multicellular organism, the most fundamental are those that hold the cells together. Cells may cling to one another through the direct cell-cell junctions, or they may be bound together by extracelluar materials that they secrete; but by one means or another, they must cohere if they are to form an organized multicellular structure.

The mechanism of cohesion govern the architecture of the body-its shape, its strength and the arrangement of its different cell types. The junctions between cells create pathways for communication, allowing the cells to exchange the signals that coordinate their behavior and regulate their patterns of gene expression. Attachments to other cells and to extracellular matrix control the orientation of each cell's internal structure. The govern the way cells move within the organism, guiding them as the body grows, develops, and repairs itself. Thus, the apparatus of cells junctions, cells adhesion mechanism, and extracellular matrix is critical for every aspect of the organization, function, and dynamics of multicellular structures. Defects in this apparatus underlie an enormous variety of diseases.

Physical attachment is critical, both in epithelia and in nonepithelial tissues, but between cell to cell junctions or between cell to matrix junctions are diverse in structure and do more than just transmit physical forces. Four main functions can be distinguished, each with a different molecular basis:

1. **Anchoring junctions,** including both c*ell-cell adhesions* and *cell-matrix adhesions,* transmit stresses and a retethered to cytoskeletal filaments inside the cell.
2. **Occuluding junctions** seal the gaps between cells in epithelia so as to make the cell sheet into an impermeable (or selectively permeable) barrier.
3. **Channel-forming junctions** create passageways linking the cytoplasms of adjacent cells.
4. **Signal-relaying junctions** allow signals to be relayed from cell to cell across their plasma membranes at sites of cell-to-cell contact.

At each of the four types of anchoring junctions, the central role is played by **transmembrane adhesion proteins** that span the membrane, with one end linking to the cytoskeleton inside the cell and the other end linking to other structures outside it. These cytoskeleton-linked transmembrane molecules fall neatly into two superfamilies, corresponding to the two basic kinds of external attachment. Proteins of the **cadherin** superfamily chiefly mediate attachment of cell to cell. Proteins of the integrin superfamily chiefly mediate attachment of cells of matrix. Within each family, there is specialization: some cadherins link to actin and from adherens junctions, while others link to intermediate filaments and from desmosome junction; likewise some integrins link to actin-linked cell- matrix adhesions, while others link to intermediate filaments and from hemidesmosomes (Molecular Biology of the Cell, 2008).

2.3 Integrins and Cell-Matrix Adhesion

Cells make extracellular matrix, organize it, and degrade it. The matrix in its turn exert powerful influences on the cell. The influeces are exerted chiefly through transmembrane cell adhesion proteins that act as *matrix receptors*. These tie the matrix outside the cell to the cytoskeleton inside it, but their role goes far beyond simple passive mechanical attachment. Through them, components of the matrix can affect almost aspect of a cell's behavior. The matrix receptors have a crucial role in epithelial cells, mediating their interactions with tha basal lamina beneath them; and they are no less important in connective-tissue cells, for their interactions with the matrix that surrounds them (Molecular Biology of the Cell, 2008).

Several types of molecules can functions as matix receptors or co-receptors, including the transmembrane proteoglycans. But the principal receptors on animal cells for binding most extracellular matrix proteins are the **integrins.** Like the cadherins and the key components of the basal lamina, integrins are part of the fundamental architectural toolkit that is characteristic of multicellular animals. The members of this large family of homologous transmembrane adhesion molecules have a remarkable ability to transmit signals in both directions across the cell membrane. The binding of a matrix component to an integrin can send a message into the interior of the cell, and conditions in the cell interior can send a signal outward to control binding of the

integrin or matrix (or, in some cases, to a cell-surface molecule on another cell, as we saw in the case of white blood cells binding to endothelial cells). Tension applied to an integrin can cause it to tighten its grip on intracellular and extracellular structures, and loss of tension can loosen its hold, so that molecular signaling complexes fall apart on either side of the membrane. In this way integrins can also serve not only to transmit mechanical and molecular signals, but also to convert the one type of signal into the other. Studies of the structure of integrin molecules have begun to reveal how they perform these tasks. (Molecular Biology of the Cell, 2008).

2.4 Cadherins Mediate Homophilic Adhesion

Anchoring junctions between cells are usually symmetrical; if the linkage is to actin, for example, in the cells on one side of the junctions, it will be to actin in the cell on the other side also. In fact, the binding between cadherins is generally **homophilic** cadherin molecules of a specific subtype on one cell bind to cadherin molecules of the same or closely related subtype on adjacent cells. According to a current model, the binding occurs at the N-terminal tips of the cadherin molecules-the ends that ends lie furthest from the membrane. The protein chain here forms a terminal knob and a nearby pocket, and the cadherin molecules protruding from opposite cell membranes bind by insertion of the knob of each one in the pocket of the other (Molecular Biology of the Cell, 2008).

The spacing between the cell membranes at an anchoring junction is precisely defined and depends on structure of the participating cadherin molecules. All the members of the superfamily, by definition, have an extracellular portion consisting of several copies of a motif called the *cadherin domain.* In the classical cadherins of vertebrates there are 5 of these repeats, and in desmogleins and desmocollins there are 4 or 5, but some nonclassical cadhereins have more than 30. Each cadherin domain forms a more or less rigid unit, joined to the next cadherin domain by a hinge. Ca^{2+} ions bind to sites near each hinge and prevent it from flexing, so that the whole string of cadherin domains behaves as a rigid, slightly curved, rod structures. When Ca^{2+} is removed, the hingis can flex, and the structure becomes floopy. At the same time, the confirmation at the N-terminus is thought to change slightly, weakening the binding affinity for the

matching cadherin molecule on the opposite cell. Cadherin molecules destabilized in this way by loss of Ca^{2+} are rapidly degraded by proteolytic enzymes.

Unlike receptors for soluble signal molecules, which bind their specific ligand with high affinity, cadherins (and most other cell-cell adhesion proteins) typically bind to their partners with relatively low affinity. Strong attachments result from the formation of many such weak bonds in parallel. When binding to oppositely oriented partners on another cell, cadherin molecules are often clustered side-to-side with many other cadherin molecules on the same cell. Many cadherin molecules packed side by side in this way collaborate to from an anchoring juction. The strength of this junction is far greater than that of any individual intermolecular bond, and yet it can be easily disassamled by separating the molecules sequentially, just as two pieces of fabric can be strongly joined by Velcro and yet easily peeled apart. A similar “Velcro principle” also operates at cell-cell and cell- matrix adhesions formed by other types of transmembrane adhesion proteins. The making and breaking of anchoring junctions plays a vital part in development and in the constant turnover of tissues in many parts of the mature body. (Molecular Biology of the Cell, 2008).

2.5 Vascular Permeability and Cell Adhesion

The endothelium controls vascular permeability to plasma proteins and circulating cells. This specific function is regulated by transcellular and intercellular permeability pathways (Dejana et al., 1999). Intercellular permeability is controlled by specific adhesive molecules at cell-to-cell junctions. As it occurs in many other cell types such as in epithelial cells, endothelial junctions are complex structures formed by different transmembrane adhesive proteins linked inside the cells to a network of cytoskeletal and signaling partners (Dejana et al., 2002). Several adhesive molecules are clustered at intercellular junctions in the endothelium. Despite the differences between the endothelia of different types of vessels, most frequently it is possible to distinguish two types adherens junctions (AJ) and tight junctions (TJ) (Dejana et al., 1999).

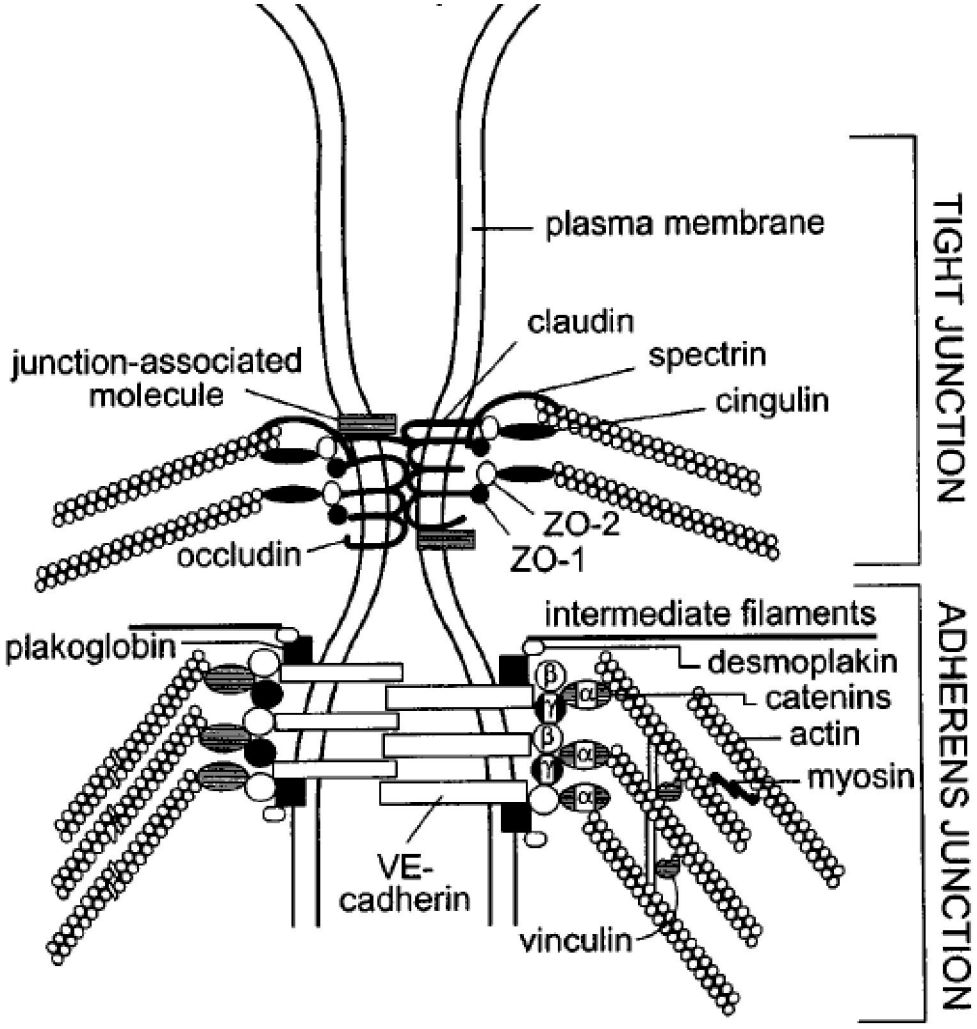


Figure 2.1: The structure of the tight junction and adherens junction (Tarbell, 2003).

The figure 2.1 shows that details of the molecular structure of the tight junction and adherens junction. The adherens junction is a cell-cell adhesive junction that binds cells together and connects the cytoskeleton (actin) to the plasma membrane. Adherens junctions may be considered epicenters for signal reception, transduction, and response to local patterning cues. The adhesive components of adherens junctions are formed by type I cadherins (calcium-dependent adhesive proteins) that belong to the larger cadherin superfamily. Type I cadherins, of which VE-cadherin is the prototype, consist of five tandem extracellular 110–amino acid repeats, a transmembrane domain, and a highly conserved cytoplasmic region. Cadherins mediate highly specific homophilic adhesion with cadherins from apposing cells. Although adhesive associations of the ectodomain occur spontaneously, the cytoplasmic domain is required to support

sustained adhesion. The cytoplasmic domain governs the clustering of cadherins into a cooperative zipperlike arrangement and the tethering of cadherins to the cytoskeleton. The cytoplasmic domain of VE-cadherin forms a stable complex with major proteins: the catenins (alpha, beta, gamma) and plakoglobin. The cadherin-catenin complexes are bound to alpha-actinin, vinculin, radixin, and the actin cytoskeleton (Tarbell, 2003). Also, Dejana et al investigated the endothelial cell functions and VE-cadherin relationships.

2.6 Effects of Fluid Mechanics on Endothelial Transport

Cell-cell junctions are the principal pathway for the transport of water and hydrophilic solutes below the size of albumin. Albumin itself likely traverses the endothelium through a variety of pathways, including intercellular junctions, leaky junctions, and vesicles. Low density lipoprotein (LDL) and high-molecular-weight materials have limited access to the normal intercellular junction and must utilize leaky junctions or vesicles to cross the endothelium. Each of these transport pathways can be influenced by fluid mechanical forces, particularly shear stress, acting on endothelial cells (Tarbell, 2003)

2.7 Definitions of Endothelial Shear Stress

Endothelial shear stress is the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall and is expressed in units of force/ unit area (N/m^2 or Pascal [Pa] or $dyne/cm^2$; 1 N/m^2 =1; Pa =10 $dyne/cm^2$). Endothelial shear stress is proportional to the product of the blood viscosity (μ) and the spatial gradient of blood velocity at the wall (ESS = μ x dv/dy) (Fig. 1.2) (Chatzizisis et al., 2007).

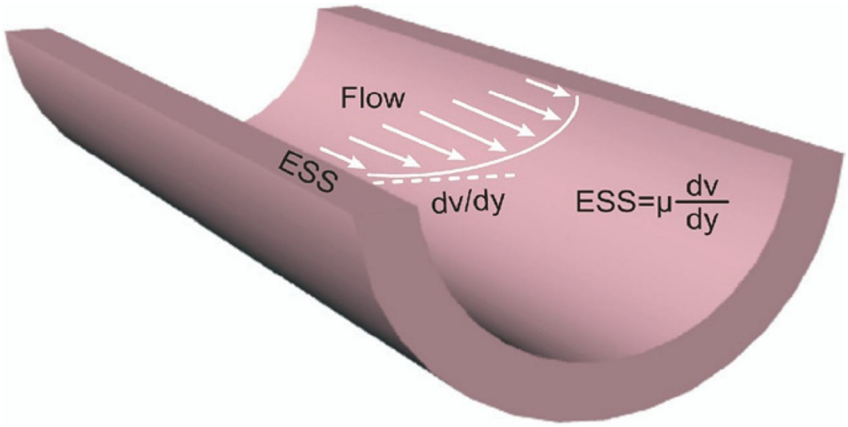


Figure 2.2: Definition of Endothelial Shear Stres (Chatzizisis et al., 2007)

Endothelial shear stress appears to be an important hemodynamic force because it modulates vascular structure and function by regulating gene and protein expression. Shear stress is also important in the pathogenesis of atherosclerosis because atherosclerotic plaques occur preferentially in areas which experience low shear stress and flow reversal (Tai, 2005)

Different forces are imposed on the vascular tree. Fluctuations in blood pressure, vessel motion and fluid shear impose forces on vascular cells. The level of stress is influenced by factors such as vessel geometry, blood pulsatility and tissue mechanical properties (Rouleau, 2006).

Fluid flow might be either laminar or turbulent. Laminar flow refers to a streamlined flow and can be further divided into undisturbed laminar flow, characterized by smooth streamlines , and disturbed laminar flow, characterized by areas with reversed flow (i.e., flow separation, recirculation, and reattachment to forward flow) or circumferential swirling. In turbulent flow the velocity at any given point varies continuously over time, even though the overall flow is steady. For a given geometry, whether the flow will be laminar or turbulent is determined by its Reynolds

number (Re); for low Re values, flow is laminar, whereas for high Re values (typically, above 2.000), flow is turbulent. The pulsatile (unsteady) nature of the arterial blood flow in combination with the complex geometric configuration of the coronaries determines the endothelial shear stress (ESS) patterns, which are characterized by direction and magnitude. In relatively straight arterial segments, ESS is pulsatile and unidirectional with a magnitude that varies within a range of 15 to 70 dyne/cm2 over the cardiac cycle and yields a positive time-average (Fig.1.3). In contrast, in geometrically irregular regions, where disturbed laminar flow occurs, pulsatile flow generates low and/or oscillatory ESS (Chatzizisis et al., 2007).

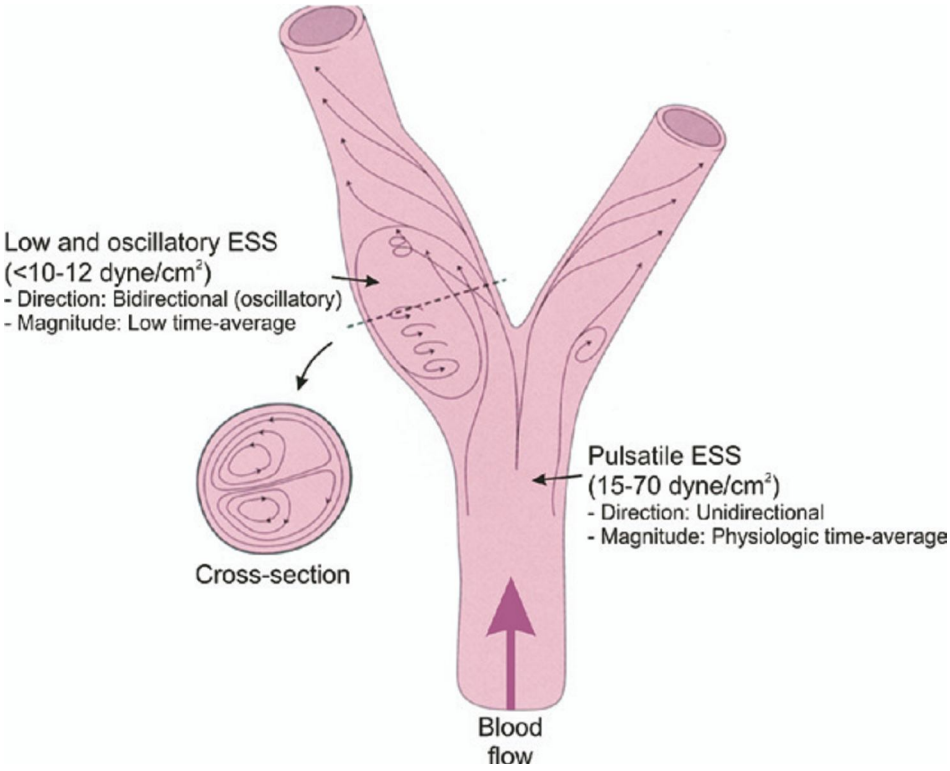


Figure 2.3: Definition and example of pulsatile, low, and oscillatory ESS (Chatzizisis et al., 2007).

2.8 Role of Shear Stress in Atherosclerosis

Hemodynamic forces have been implicated in the initiation, localization, and development of atherosclerotic vascular diseases. The effects of such forces upon the endothelial cell lining of blood vessels, the integrity of which is essential for normal

vascular function. In certain areas of the aorta and its main branches, blood flow characteristics are both variable and complex. In locations such as the descending thoracic aorta and distal carotid arteries, pulsatile laminar flow is prevalent , whereas in other regions, such as coronary arteries and the carotid bifurcation, secondary flows, vortices, and intermittently changing flow directions are encountered . The distribution of atherosclerotic lesions in susceptible species, including humans, is closely correlated with the location of disturbed flow in the major vessels. Time-dependent flow separation and unsteady secondary flow typically occur in localized regions that are usually well defined and of limited size. Furthermore, turbulence will occur in the largest arteries under conditions of increased flow velocity and cardiac output. Thus, shear stresses, which are the direct tractive forces acting on the endothelial cell surface as a result of blood flow, are highly variable in magnitude, frequency, and direction in such regions (Davies et al, 1986) .

Intimal lipid accumulation, hyperplasia, and scarring are stigmata of atherosclerotic vascular disease, whose major complications (myocardial and cerebral ischemia and infarction) continue to be major health problems in developed nations (Topper et al 1999). Differences in flow parameters within various vascular beds account for the localization of atherosclerosis, which tends to occur at sites of low shear stress, turbulence, and oscillating flow. In contrast, areas of laminar flow are relatively resistant to the development of early atherosclerosis. Caro et al in 1969 pointed to the important correlation between low shear stress and atherosclerosis in the vasculature. This concept was further elaborated by Glagov et al elaborated. By reproducing flow conditions in scale human carotid bifurcation models, they showed that the localization of atherosclerosis found at autopsy correlated to areas of flow disruption, which led to increased particle residence time. Similar modeling was performed on the abdominal aorta, in which both oscillating flow and low shear stress were observed along the posterior wall of the infrarenal aorta, where atherosclerotic lesions develop (VanderLaan et al 2003).

Davies et al 1986 compared two different flows. The aim of the their study was determined the effects of hemodynamic forces upon vascular endothelial cell turnover were studied by exposing contact-inhibited confluent cell monolayers to shear stresses

of varying amplitude in either laminar or turbulent flow. Laminar shear stresses (range, 8-15 dynes/cm2; 24 hrs) induced cell alignment in the direction of flow without initiating the cell cycle. In contrast, turbulent shear stresses as low as 1.5 dynes/cm^2 for as short a period as 3 hr stimulated substantial endothelial DNA synthesis in the absence of cell alignment, discernible cell retraction, or cell loss. Laminar flow was generated using a cone angle of 1/2º. Turbulent flow was induced by increasing the flow velocity and using a cone angle of 5º. Time-mean shear stress levels were extrapolated from previous measurements and theory and were estimated to be accurate to ±20%.

Their results included morphological changes when comparing the different flow studies. The shape of confluent cultured endothelial cells normally observed under static culture conditions was altered by exposure to unidirectional shear stresses of 8 dynes/cm^2 in laminar flow within 24 hr. Individual cells became ellipsoidal and the cell population assumed an axial alignment in the direction of flow. In contrast, application of a mean shear stress as low as 1.5 dynes/cm^2 for 16 hr in turbulent flow resulted in random orientation of cells in the monolayer. Endothelial cell turnover was substantially increased in the monolayers exposed to turbulent flow compared to laminar flow over a comparable range of shear stresses.

These results are in marked contrast to those obtained in laminar flow, where they were unable to detect cell loss following 24 hr of laminar flow at comparable mean shear stresses, and the cell density after alignment in laminar flow was unchanged compared with static control monolayers (Davies et al, 1986).

Shu Chien et al investigated the role of different flow patterns in the remodeling of cell junctions in both in vivo and in vitro conditions. The study results showed that VE-cadherin staining at EC borders is much stronger in the descending thoracic aorta and abdominal aorta where simple laminar flow prevails than in the aortic arch and poststenotic dilatation sites where complex flow patterns and fluctuating shear stress dominate. Their in vitro studies using a flow chamber demonstrated that VE-cadherin staining at EC borders became intermittent with numerous gaps after exposing monolayers of bovine aortic endothelial cells (BAECs) to either pulsatile or

reciprocating flow for 6 hr. When the exposure to a pulsatile flow was extended to 24 hr or longer, continuous VE-cadherin staining reappeared around the entire periphery of the cells but not under reciprocating flow.

Both in vivo and in vitro data suggest that pulsatile flow with a strong forward component and complex flow without a significant forward component differentially regulate the cell junction remodeling. This flow pattern-regulated junction remodeling may provide an explanation for the high endothelial permeability and the preferential localization of atherosclerosis in regions where flow patterns are complex, especially where the shear stress exhibits time-fluctuations without a significant net direction (Chien et al, 2005).

2.9 Effects of Shear Stress on Endothelial Cell Junctions

The structure and physiology of the endothelial cells that line the mammalian vasculature are greatly influenced by the shear stresses that are continuously imposed on them by blood flow. The most obvious structural responses of endothelium to shear stress are changes in cell shape and orientation; in areas of low or inconsistent shear stress in vivo, or when cells are maintained in static culture, endothelial cells assume a cuboidal, cobblestone morphology, whereas they elongate and align in the direction of flow when shear stress is moderate or high. These morphological changes are adaptive in that they reduce the spatial fluctuations in shear stress and the maximum shears to which the cells are exposed (Noria, 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1. HUVEC (Human Umbilical Vein Endothelial Cell) cell line

HUVEC cell line was donated by Assoc. Prof. Ali Uğur Ural, GATA and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum

3.2 HUVEC Cell Culture

HUVEC cell line cultured in T-25 culture flask was received from Assoc. Prof. Ali Uğur Ural, GATA The culture flask was then placed in the incubator (humidity, temperature 37°C and gas content 5% CO_2 in air) and the cells were allowed to expand until confluence (approximately 3-4 days) in growth medium (DMEM-Low glucose (DMEM-LG, Applichem) containing 10 % fetal bovine serum (FBS, Applichem), 1 % penicillin streptomycine (Invitrogen)). Then cell line was subsequently expanded to further passes.

3.4 Subculture

Briefly, for each expansion, the culture media was removed and discarded, and the cell surface area was rinsed with PBS (Biochrom) for 30 seconds to remove any traces of serum, a

trypsin inhibitor. The cell layer was then covered with trypsin EDTA solution for 30 seconds, after which some liquid was removed and discarded. After inspection under a light microscope (Nikon) to ensure complete cell detachment, cells were removed by brief washing with growth medium once. The resulting cell suspension was centrifuged at 1500 rpm for 10 minutes, and the supernatant was discarded. The cell pellet was resuspended in growth medium and trasferred into T-75 culture flasks which had been pre-coated with 0.2% gelatin. The cells were incubated until approximately 90-95 % confluency, and the process was repeated. Cells were expanded in this manner up to passage 14 or 15 and cryopreserved cells were thawed for use in later experiments.

3.5 Cryopreservation

Cells were prepared for cryopreservation: following the centrifugation step, the cell pellet was resuspended 10 % sterile DMSO (Applichem) in FBS, and $2x10^6$ cells/cryovial were transferred to cryopreservation vials. When required, cryopreserved cells were removed from liquid nitrogen, thawed, and expanded, as explained previously.

3.6 Shear Stress Experiment

The parallel plate flow chamber used for shear experiment was designed and developed by our laboratory (Figure 3.1). The chamber was designed to generate laminar flow with same magnitude of shear stress. The chamber is equipped with two machined plates which have silicon around the boundary and are able to be fastened by four screws. The distance between upper and bottom chambers have dimension of 1mm x 45mm x 105 mm to allow fluid flow path. The bottom side of chamber is designed to hold glass slide (75 mm x 25 mm). The flow chamber is connected to a resevoir and damper (Figure 3.3 and 3.4). 300 ml of fresh DMEM is added to the reservoir and damper. The medium is circulated by perfusion using a peristaltic pump (Masterflex L/S ; Cole Parmer Instrument Company).

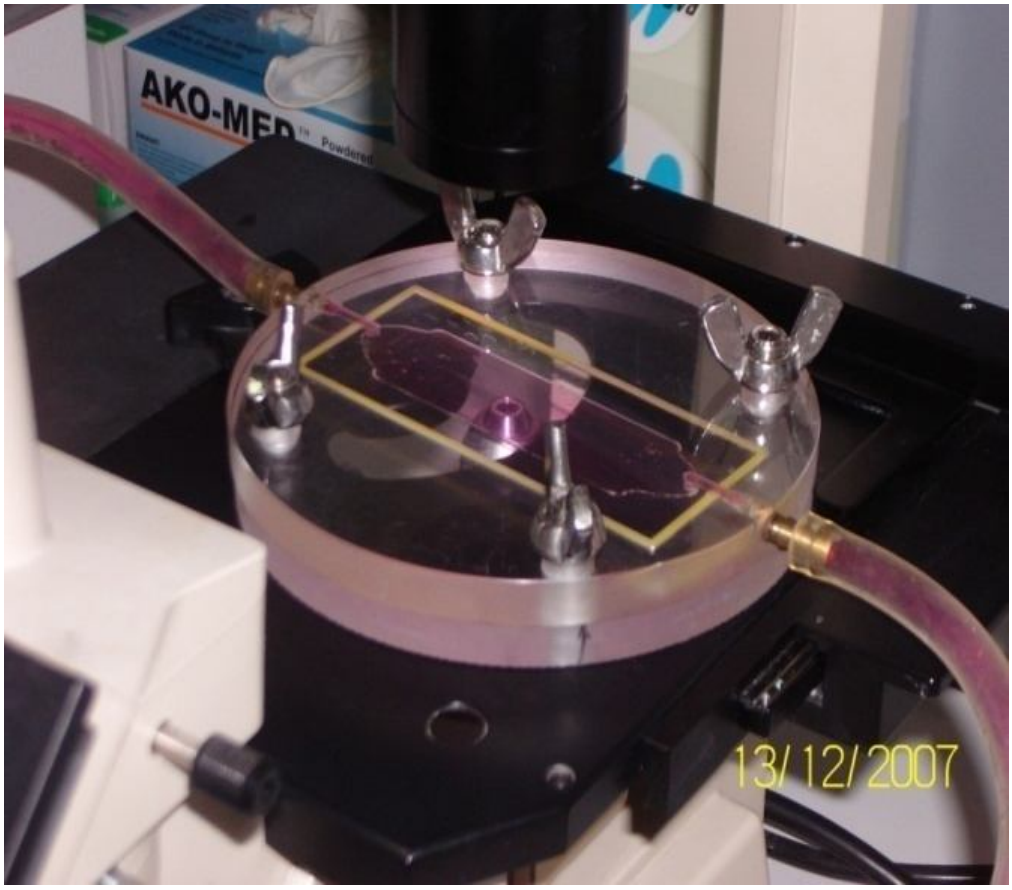


Figure 3.1: Parallel plate flow chamber

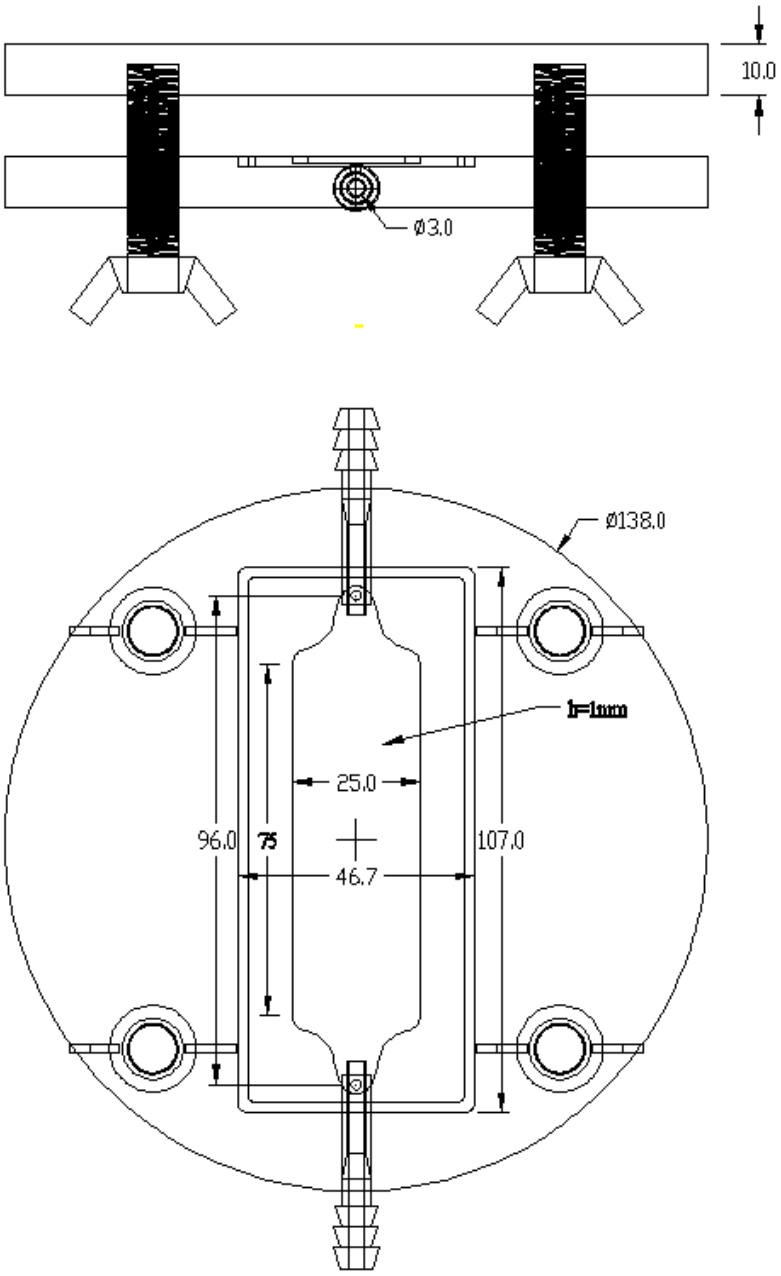


Figure 3.2: Technical drawing of the parallel plate flow chamber

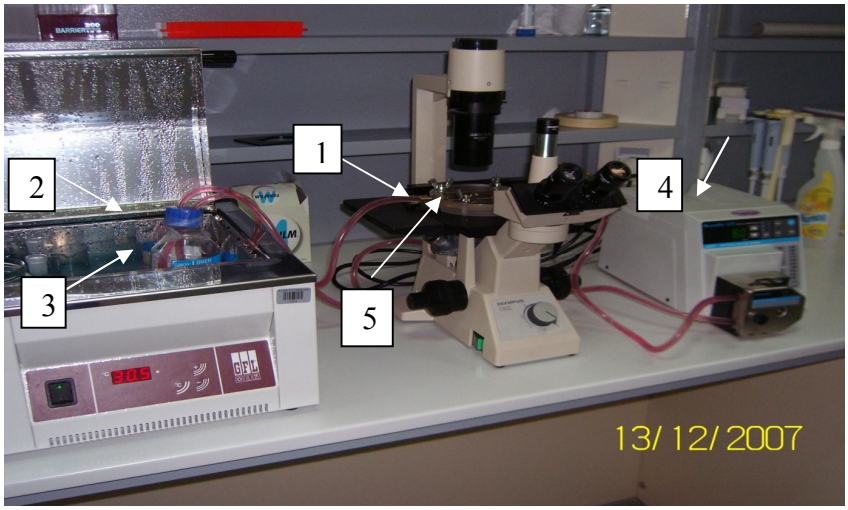


Figure 3.3 : Shear stress experiment equipments.

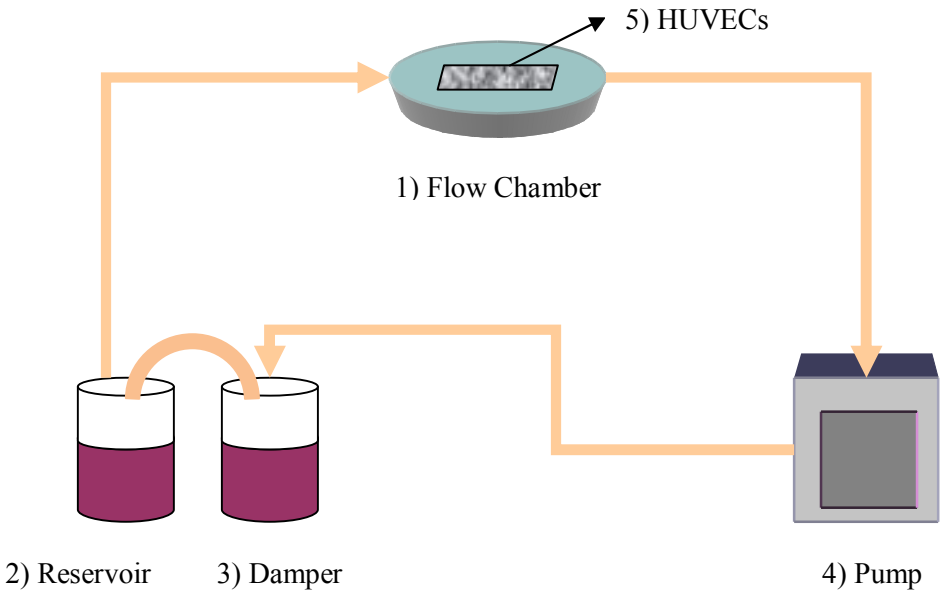


Figure 3.4: Schematic drawing of the shear stress experiment system

The parallel plate flow chamber is composed of following components: 1) parallel plate flow chamber, 2) Reservoir, 3) Damper 4) Peristaltic Pump 5)HUVECs in glass slide.

The peristaltic pump enables the media to flow under controlled speed. Variations in flow rate generated by the peristaltic pump is stabilized and buffered when flow passed through damper by a cushion of air. We used silicon tubing (Masterflex L/S ; Cole Parmer Instrument Company) to connect all the flow chamber components. The arrows in Figure 3.3 indicate the direction of flow for all the shear stress experiments. The parallel plate flow device is designed to generate laminar flow with different shear rate by modulating peristaltic pump. In this study we set the peristaltic pump to generate shear rate. Designed shear rate derived by generated flow is calculated base on the equation as follow:

$\tau = 6\ \mu Q / (Wh^2)$ **(3.1)**

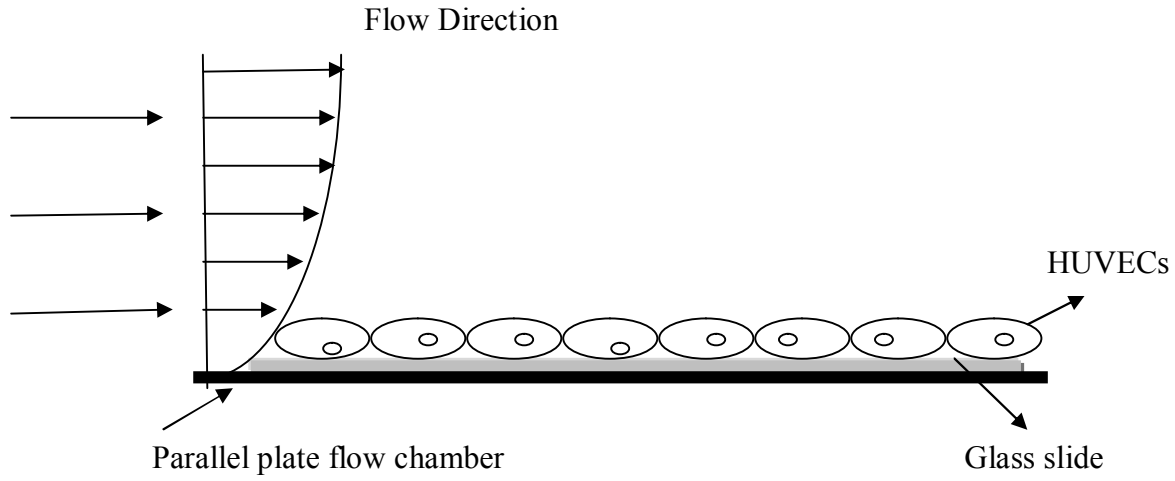


Figure 3.5: Shematic explanation of the flow direction on HUVECs

Shear stress is the force exerted parallel to the vessel, in the direction of blood flow and is thought to elicit significant physiologic and pathologic responses, through an effect on blood components and on endothelial cells, which are in direct contact with flowing blood. For a Newtonian fluid, shear stress (τ) is proportional to the dynamic viscosity (μ) of the fluid and to the speed at wich adjacent layers of fluid slide past each other, i.e. the shear rate ($\frac{dv}{dr}$) as seen in equation:

$$\tau = \mu \frac{dv}{dr} = \mu\gamma \qquad \textbf{(3.2)}$$

The wall shear rate (γ) in a laminar steady flow tubular vessel, assuming an incompressible, rigid and Newtonian fluid, can be calculated as:

$$\gamma = \frac{32Q}{\pi D^3} \quad \textbf{(3.3)}$$

Where Q is the volumetric flow rate and D is the diameter. Combining equations (3.2) and (3.3) gives a reasonable estimate of the mean wall shear stress in straight arteries.

$$\tau = \frac{32\mu Q}{\pi D^3} \quad \textbf{(3.4)}$$

The velocity profiles as well as the shear stress and shear rate patterns are shown in equation (3.2) for laminar flow in straight tubular vessels.

In our experiment the parallel plate flow was used and the shear stress equation is given as (3.1).

3.7 Immunofluorescent Staining

3.7.1 VE-cadherin Detection

VE-cadherin is the major adhesive protein of the adherens junction and is specific for vascular endothelial cells. It can transfer information intracellular by interacting with the cytoskeleton via several anchoring molecules.

HUVEC cell line were used to detect cellular localization of VE-cadherin. 10^4 cells/well were seeded on 0.2 % gelatin coated glass coverslips (12 mm in diameter) in a 24-well plate. After 72 hrs incubation, immunofluorescent experiment is performed. First, cells were permeabilized with TZN buffer (10 Mm pH 7.5 Tris-HCL, 0.5 % Nondet P40, 0.2 mM $ZnCl_2$) for 15 min. then they were fixed with 4% Paraformaldehyde (in PBS) for 10 min. at RT. Blocking was carried out with 10% Normal Goat Serum (NGS, Gibco) and 10% Normal Horse Serum (NHS, Biochrom) in PBS-0.3 % TritonX (PBS-Tx) for 30 min. at RT. After blocking, cells were treated with antibodies for target proteins. For negative controls, normal human serum was used instead of an antibody. Antibodies were diluted in PBS-Tx containing 3% NHS.

First antibody treatment was done for 2 hrs at RT and after washing with PBS & PBS-Tx cells were treated with fluorescently labeled secondary antibodies (GAM-IgG-Alexa Fluor 488 & GAR-IgG-Alexa Fluor 594, Invitrogen) which were diluted 100 times with 0.5% BSA-PBS (final concentration: 20 μg/ml). Secondary antibody treatment was done at RT for 1 hr. After that coverslips were removed from the wells and mounted on glass slides by using Prolong Antifade Kit (Molecular Probes) or Prolong Gold Antifade Reagent (Invitrogen). Slides were observed under fluorescent microscope (Carl-Zeiss) and photographs were taken next day.

3.7.2 Alpha5 / Beta1 (α5/β1) Detection

α5β1 is an integrin that binds to matrix macromolecules and proteinases. Integrins are cell surface receptors that interact with the extracellular matrix (ECM) and mediate various intracellular signals. They define cellular shape, mobility, and regulate the cell cycle. Integrin plays a role in the attachment of cells to other cells, and also plays a role in the attachment of a cell to the material part of a tissue that is not part of any cell. It is the same protocol with detection of VE-Cadherin, but we used anti- α5β1 integrin antibody as 1st antibody.

3.7.3 DAPI Staining

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, blue fluorescent probe that fluoresces brightly when
it is selectively bound to the minor groove of double stranded DNA where its fluorescence is approximately 20-fold greater than in the nonbound state. This selectivity for DNA, along with cell permeability allows staining of nuclei with little background from the cytoplasm, making DAPI the classic nuclear counterstain for immunofluorescence microscopy. Because of this property DAPI is a useful tool in various cytochemical investigations.

Protocol of the DAPI staining similar with VE-cadherin staining. At the last step of the DAPI staining, 1/10000X DAPI (SIGMA D8417) was added. After washing with PBS, coverslips were removed from the wells and mounted on glass slides by using Prolong

Antifade Kit (Molecular Probes) or Prolong Gold Antifade Reagent (Invitrogen). Slides were observed under fluorescent microscope (Carl-Zeiss) and photographs were taken next day.

CHAPTER 4

RESULTS

This chapter represent the results from experiments in which endothelial cells were exposed to shear stress. This chapter divided into three sections. Section 4.1 discusses the steady flow experiment and section 4.2 discusses oscillatory flow experiments. Section 4.3 repressent immunofluorescent staining results.

Steady flow experiments include different flow rates. First experiment was performed 50 mil/min at 6, 12 and 24 hrs. The results of these experiments were evaluated, after that the other experiments were decided how to design. Other steady flow experiments were performed at 24 hr and different flow rates as 500, 1000, 1500 mil/min.

Oscillatory flow experiments were performed, as steady flow experiment, but experiment's flow rate were different from each other. Flow rate were 100, 250, 500 and 1000 mil/min. All experiments were performed at 24 hours.

Section 4.3 includes immunofluorescent staining results. This section has VE-cadherin staining, alpha5/beta1 staining and DAPI staining results.

Test aparatus was designed and developed in our cell culture laboratory to observe cell response. Morphological changes analysis was performed to determine the changes in cell shape and alignment following exposure to ESS and VE-cadherin protein localization.

Morphological changes were observed by using phase-contrast microscopy. Cells grown under the static conditions were randomly oriented as shown in figures of before experiments. When the results of after flow experiments were compared with static culture, reorientation, migration and changes of cell shape were observed.

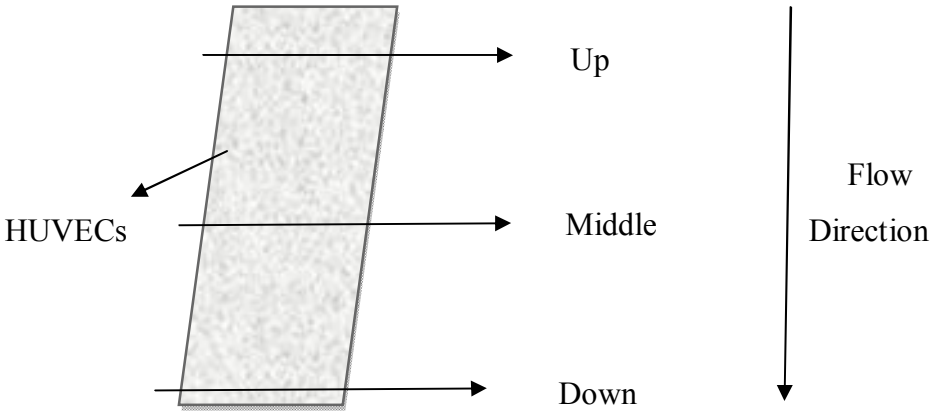


Figure 4.1 : Explanation of the flow direction and up, middle, down regions on glass slide.

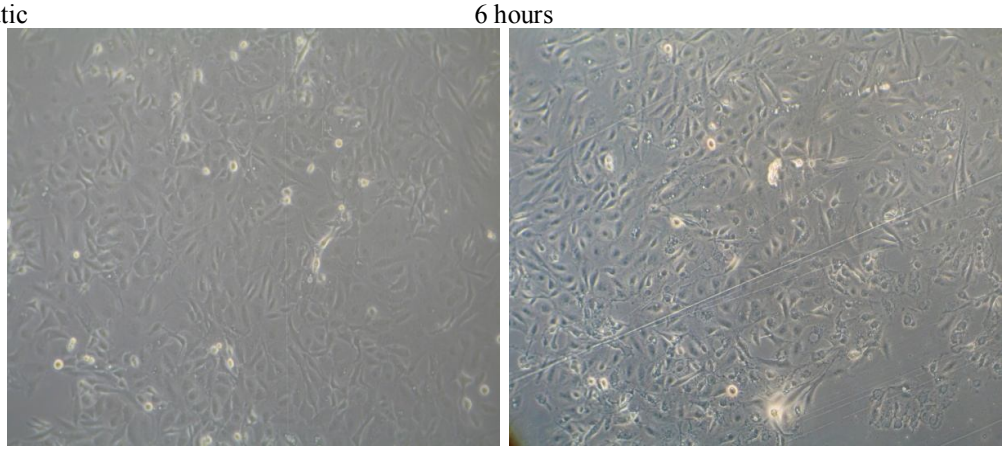
Figure 4.1 explains the meaning of the up, middle and down region. 3 different rate were investigated. Up region was the initiation of the flow. Middle region was investigated to observe cell migration and other effects of fluid forces. Finally down region was investigated.

The result of these studies suggest that steady and oscillatory flow have effects on EC morphology.

4.1 Steady Flow Experiments

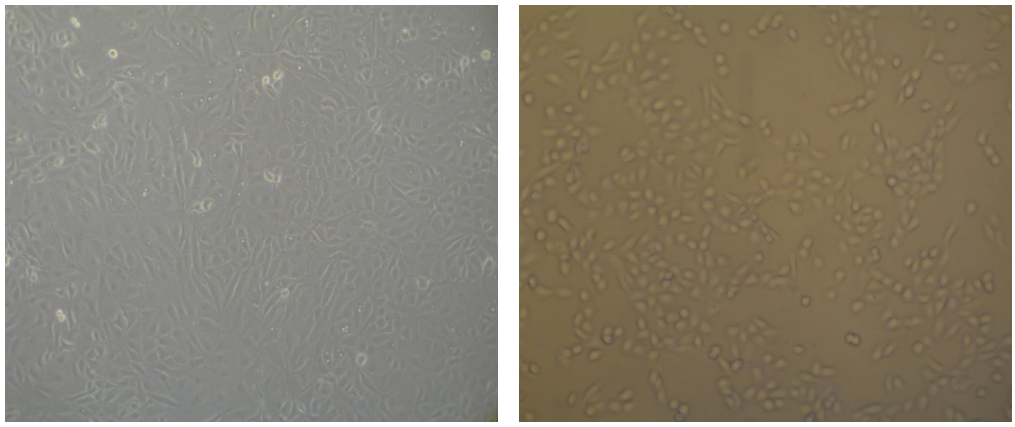
4.1.1 Steady flow (50 ml/min) at 6, 12 and 24 hours

Static 6 hours



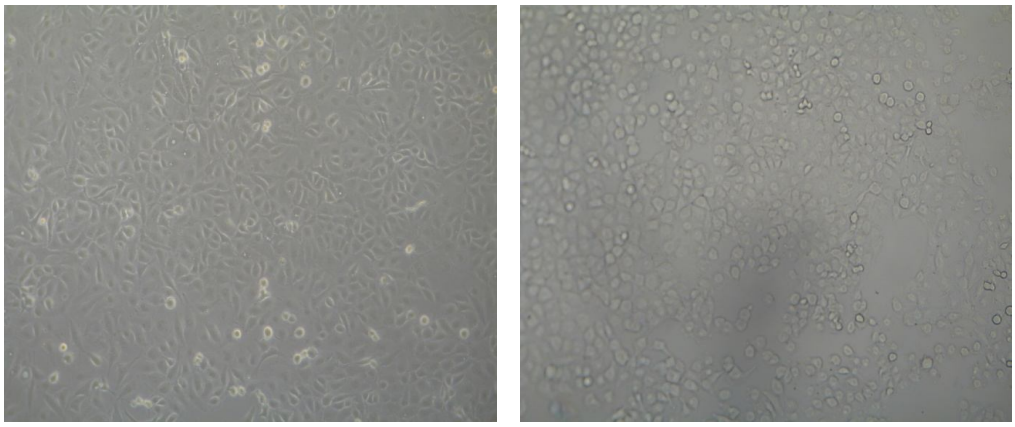
A **D**

Static 12 hours



B **E**

Static 24 hours



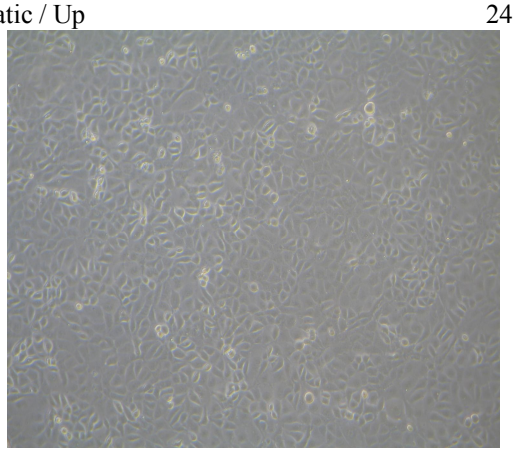
C F

Figure 4.2: HUVECs in static culture before experiment (A,B,C). After 6 hr of exposure at 50 ml/min in steady flow (D). 12 hr of exposure at 50 mil/min in steady flow (E). 24 hr of exposure at 50 ml/min in steady flow (F)

In this experiment, HUVEC's were exposed to steady flow (50 ml/min) at 6, 12, 24 hours. Results were shown Figure 4.2. Morphological changes induced in confluent HUVECs by exposure to shear stress in laminar flow. (A,B,C) Monolayer in static culture. Under static (control) conditions, cells exhibit a polygonal configuration with no preferred orientation. (D) Alignment of cells in a confluent endothelial monolayer after 6 hr of exposure to steady flow at 50 ml/min in laminar flow. Note ellipsoidal shape change. (E) Confluent endothelial monolayer after 12 hr of exposure to shear stress at 50 ml/min in laminar flow. Cell shape in the monolayer is more variable than in A, no alignment is apparent, and significant numbers of rounded cells can be seen dettached to the upper surface of the monolayer. Migration was observed. (F) Similar effects were noted after 24 hr of exposure to 50 ml/min in laminar flow. (Phase-contrast; 10X)

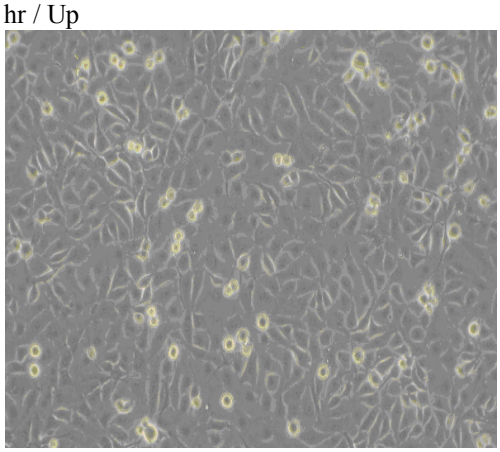
4.1.2 Steady flow (500 ml/min) at 24 hours

Static / Up



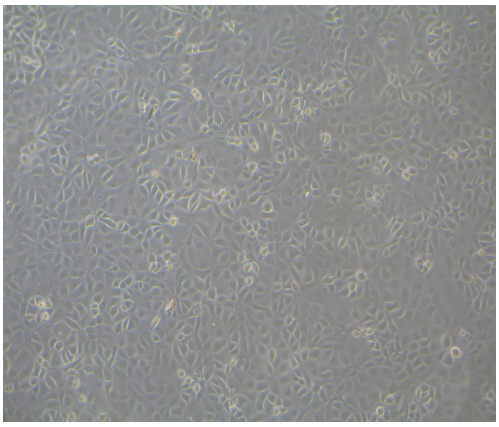
A

24 hr / Up



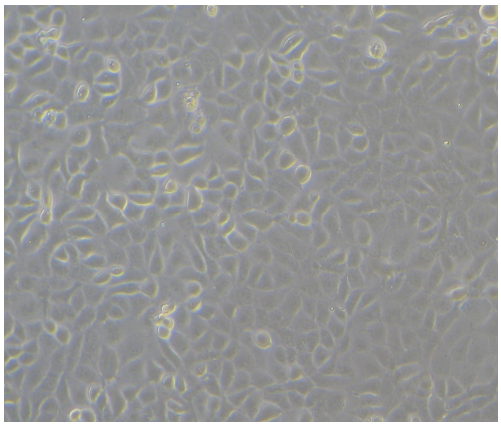
D

Static / Middle



B

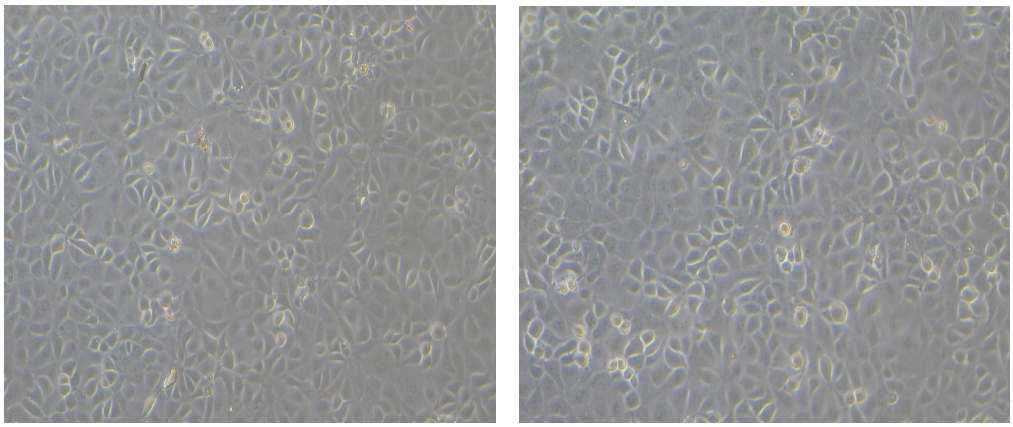
24 hr / Middle



E

Static / Down

24 hr / Down



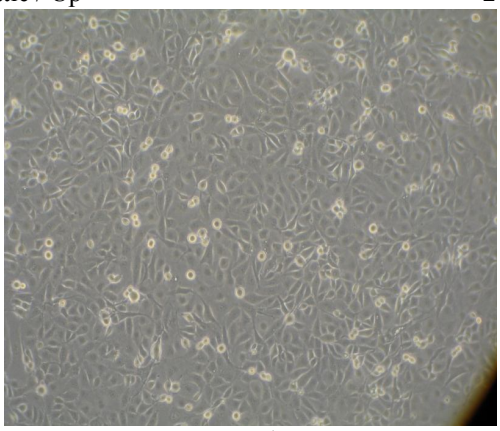
C F

Figure 4.3: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposure at 500 ml/min in steady flow in order of up, middle, down (D,E,F).

HUVEC's were exposed to steady flow (500 ml/min) at 24 hours. Results were shown Figure 4.3. As It was explained before, under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation (A,B,C). (D) Alignment of cells in a confluent endothelial monolayer after 24 hr of exposure to steady flow at 500 ml/min in laminar flow. Ellipsoidal morphological change was noted. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 500 ml/min in laminar flow. In the middle of the glass slide cell shape in the monolayer is more variable than in A, no alignment is apparent, and significant numbers of rounded cells can be seen dettached to the upper surface of the monolayer. (F) Similar effects were noted in the down area, after 24 hr of exposure to 500 ml/min in laminar flow. (Phase-contrast; 10X)

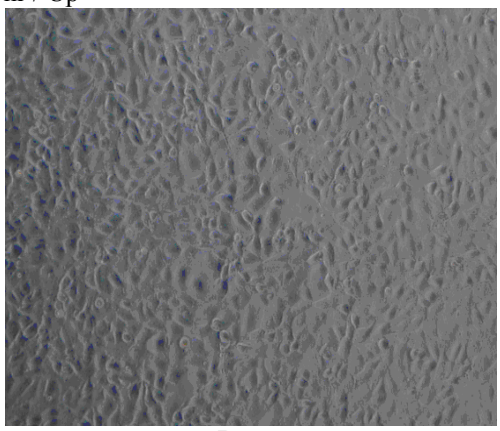
4.1.3 Steady flow (1000 ml/min) at 24 hours

Static / Up



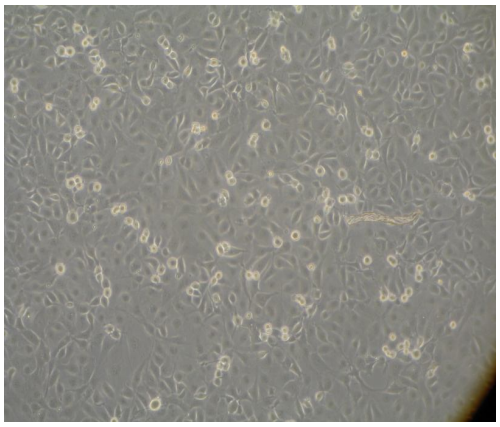
A

24 hr / Up



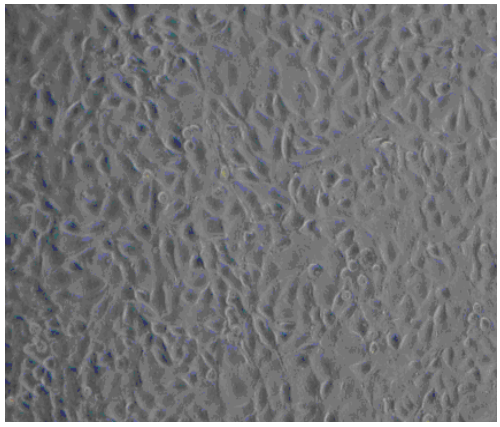
D

Static / Middle



B

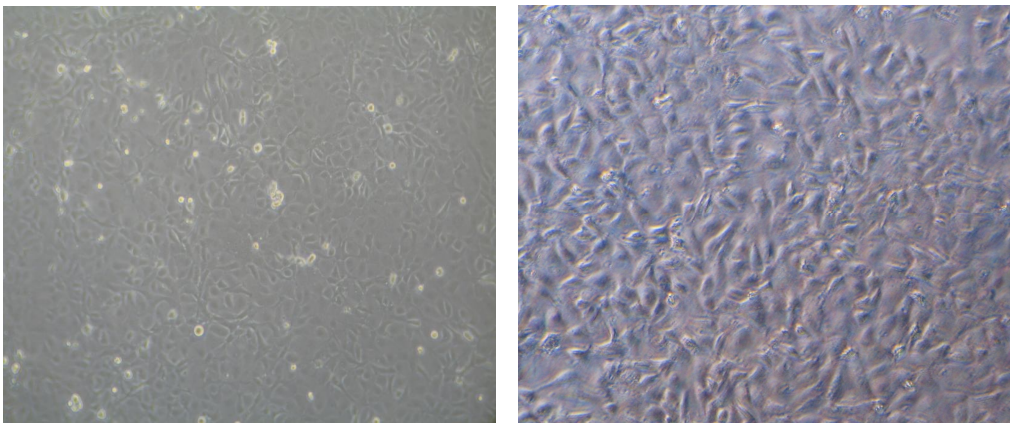
24 hr / Middle



E

Static / Down

24 h / Down



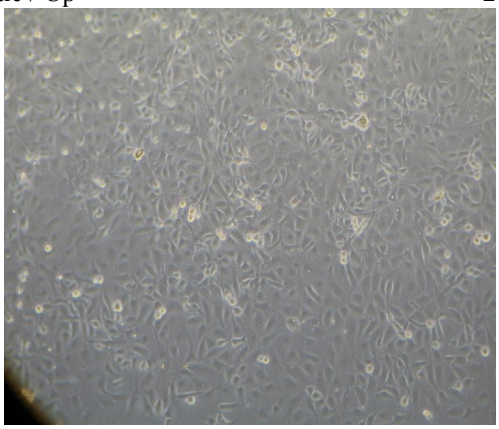
C F

Figure 4.4: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposure at 1000 ml/min in steady flow in order of up, middle, down (D,E,F).

HUVEC's were exposed to steady flow (1000 ml/min) at 24 hours. Results were shown Figure 4.4. (A,B,C) Monolayer in static culture. Under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation. (D) Alignment of cells in a confluent endothelial monolayer after 24 hr of exposure to at 1000 ml/min in steady flow. Elongatinal morphological change was noted in the direciton of flow. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 1000 ml/min in laminar flow. In the middle of the glass slide cells in the monolayer are more variable than in A, no alignment is apparent, and significant numbers of rounded cells can be seen attached to the upper surface of the monolayer. (F) Similar effects were noted in the down area, after 24 hr of exposure to 1000 ml/min in laminar flow. (Phase-contrast; 20X)

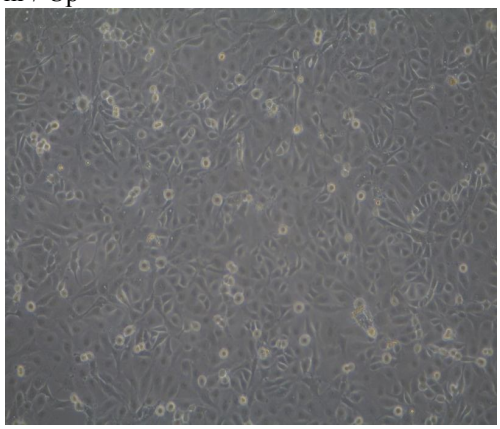
4.1.4 Steady flow (1500 ml/min) at 24 hours

Static / Up



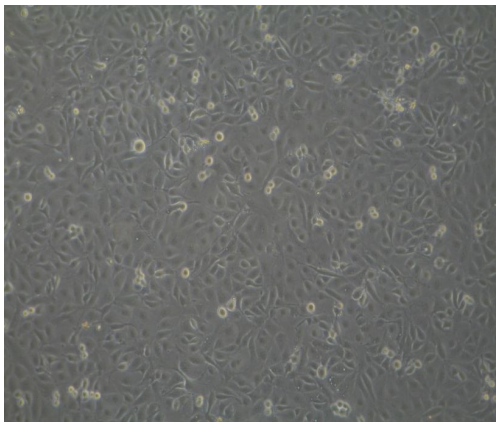
A

24 hr / Up



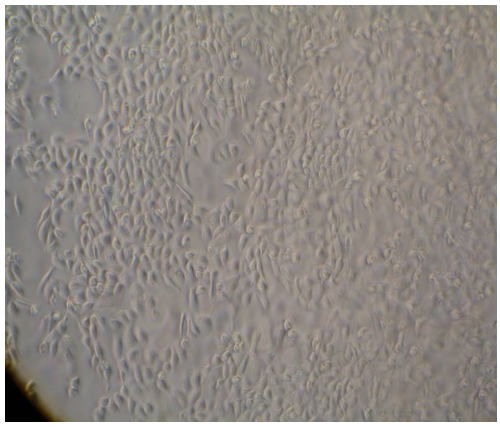
D

Static / Middle



B

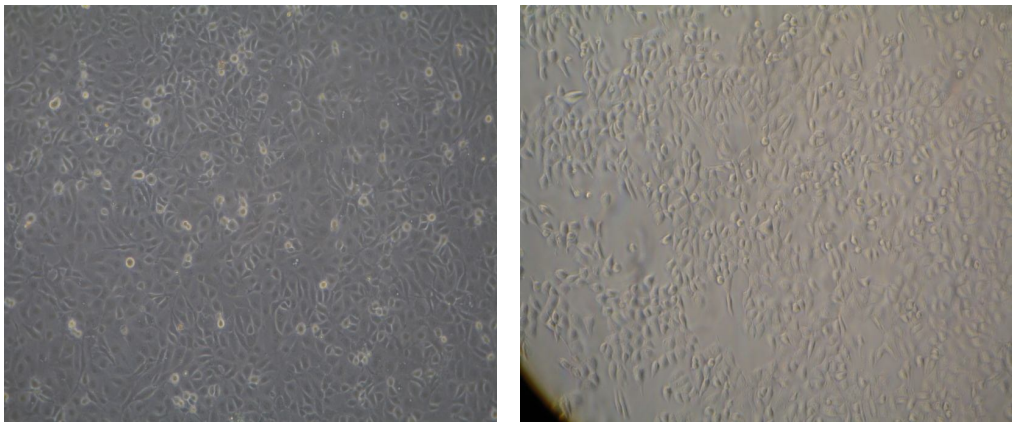
24 hr / Middle



C

Static / Down

24 hr / Down



C F

Figure 4.5: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposure at 1500 ml/min in steady flow in the order of up, middle, and down (D,E,F).

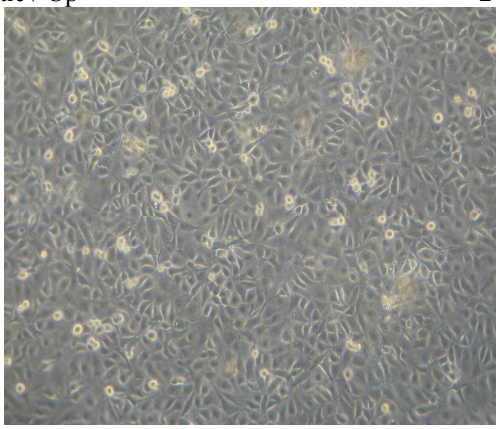
HUVEC's were exposed to steady flow (1500 ml/min) at 24 hours. Results were shown below. (A,B,C) Monolayer in static culture. Under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation. (D) Alignment of cells in a confluent endothelial monolayer after 24 hr of exposure to at 1500 ml/min in steady flow. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 1500 ml/min in laminar flow. 1500 ml/min flow conditions can modulate cell migration elongational changing was very high (F) Similar effects were noted in the down area, after 24 hr of exposure to 1500 ml/min in laminar flow. Cell migration was observed. The empty spaces on the slide explaines the migration of the cell. (Phase-contrast; 10X)

Steady blood flow in which velocity does not vary with time. This type of flow does not occur in vivo; however, it has been largely used in in vitro fluid dynamic studies.

4.2 Oscilatory Flow Experiments

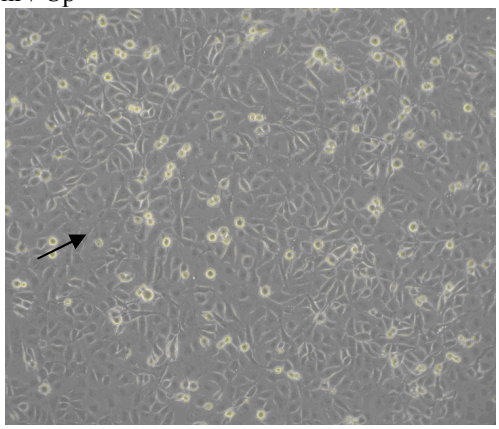
4.2.1. Oscilatory Flow (100 ml/min) at 24 hours

Static / Up



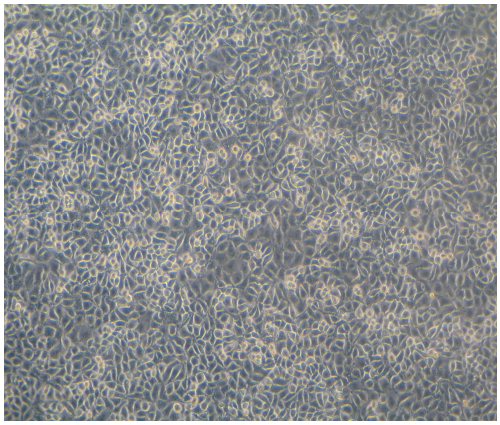
A

24 hr / Up



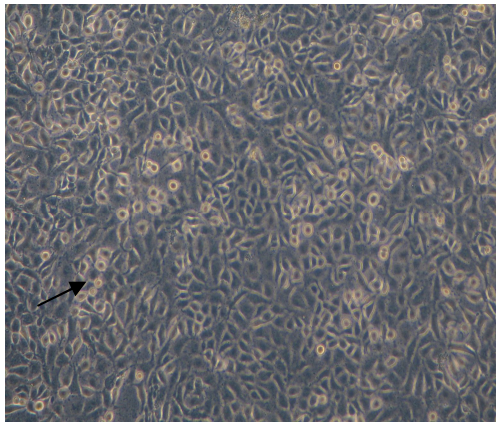
D

Static / Middle



B

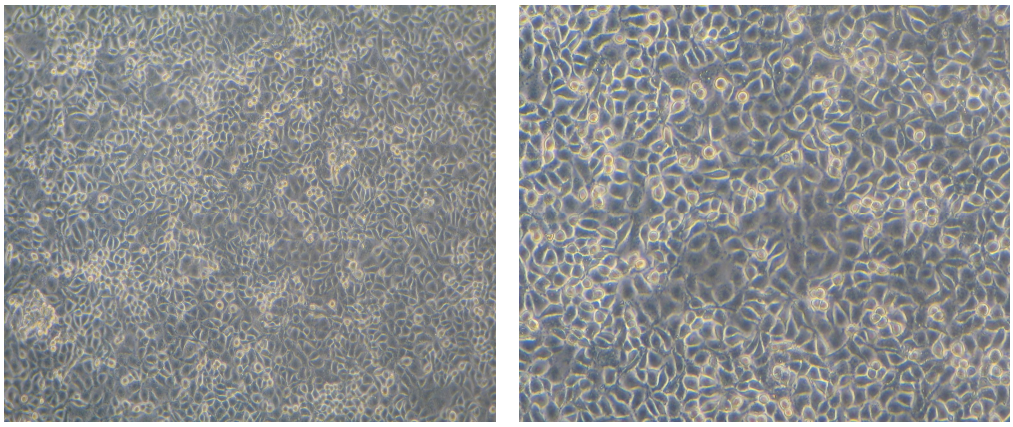
24 hr / Middle



E

Static / Down

24 hr / Down



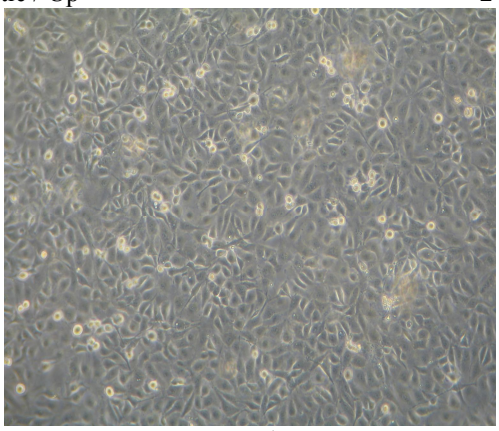
C F

Figure 4.6: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposure at 100 ml/min in oscillatory flow in the order of up, middle, and down (D,E,F).

HUVEC's were exposed to oscillatory flow (100 ml/min) at 24 hours. Results were shown Figure 4.6. (A,B,C) Monolayer in static culture. Under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation. (D) Alignment of cells in a confluent endothelial monolayer after 24 hr of exposure to at 100 ml/min in steady flow. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 100 ml/min in oscillatory flow. 100 ml/min flow conditions can modulate elongational changing. Arrow indicates changing of the cell shape in the direction of flow.(F) Similar effects were noted in the down area, after 24 hr of exposure to 100 ml/min in oscillatory flow. Rounded cells were observed very highly (Phase-contrast; 20X)

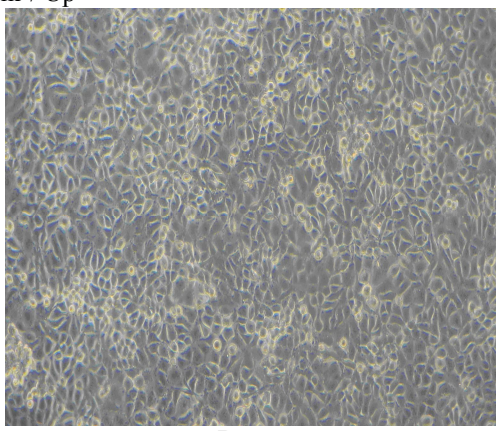
4.2.2. Oscilatory Flow (250 ml/min) at 24 hours

Static / Up



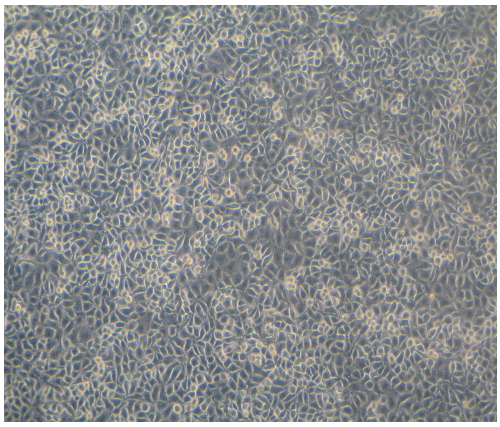
A

24 hr / Up



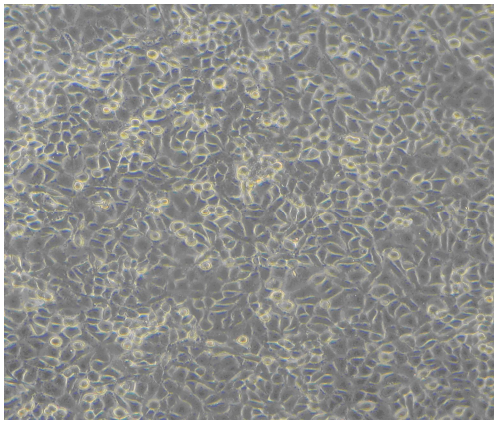
D

Static / Middle



B

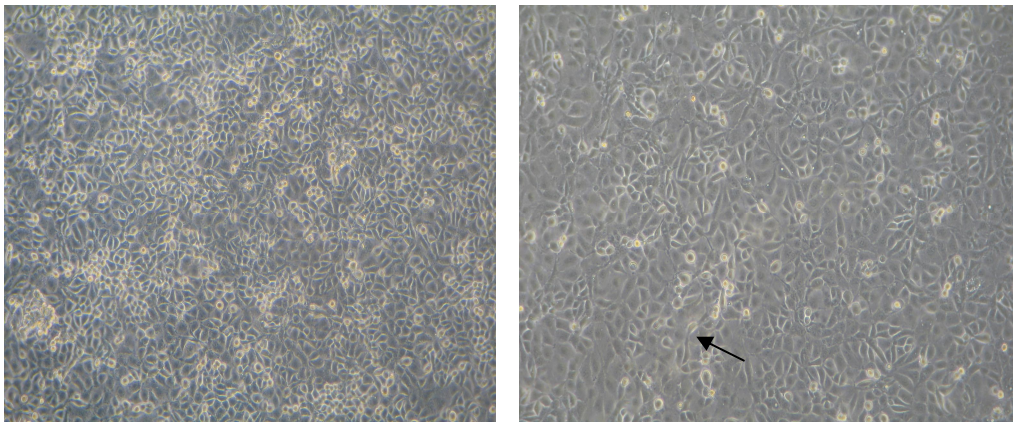
24 hr / Middle



E

Static / Down

24 hr / Down



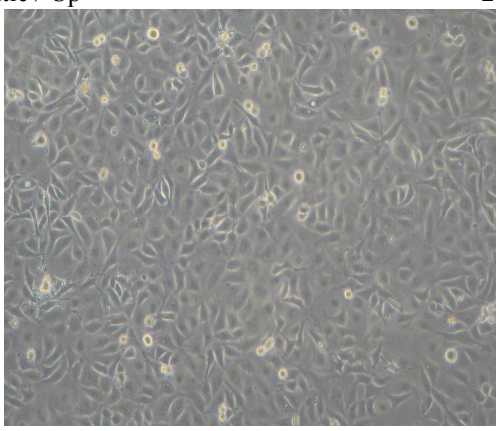
C F

Figure 4.7: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposure at 250 ml/min in oscillatory flow in the order of up, middle, and down (D,E,F).

HUVEC's were exposed to oscilatory flow (250 ml/min) at 24 hours. Results were shown Figure 4.7. HUVEC's were exposed to oscilatory flow (250 ml/min) at 24 hours. Results were shown below. (A,B,C) Monolayer in static culture. Under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation. (D) Rounded cells in a confluent endothelial monolayer after 24 hr of exposure to at 250 ml/min in steady flow. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 250 ml/min in oscillatory flow. 250 ml/min flow conditions can modulate polygonal shape changing.(F) Similar effects were noted in the down area, after 24 hr of exposure to 250 ml/min in oscillatory flow. Rounded cells were observed very highly. Arrow indicates changing of the cell shape in the direction of flow (Phase-contrast; 10X)

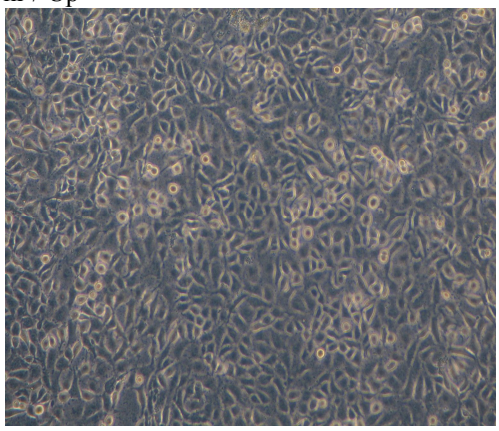
4.2.3. Oscilatory Flow (500 ml/min) at 24 hours

Static / Up



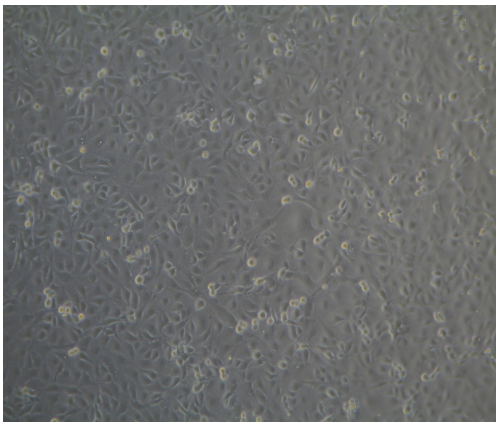
A

24 hr / Up



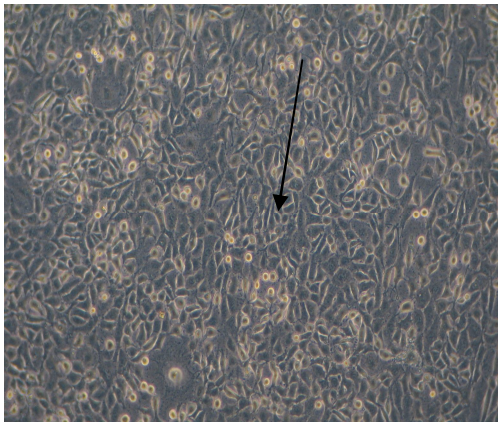
D

Static / Middle



B

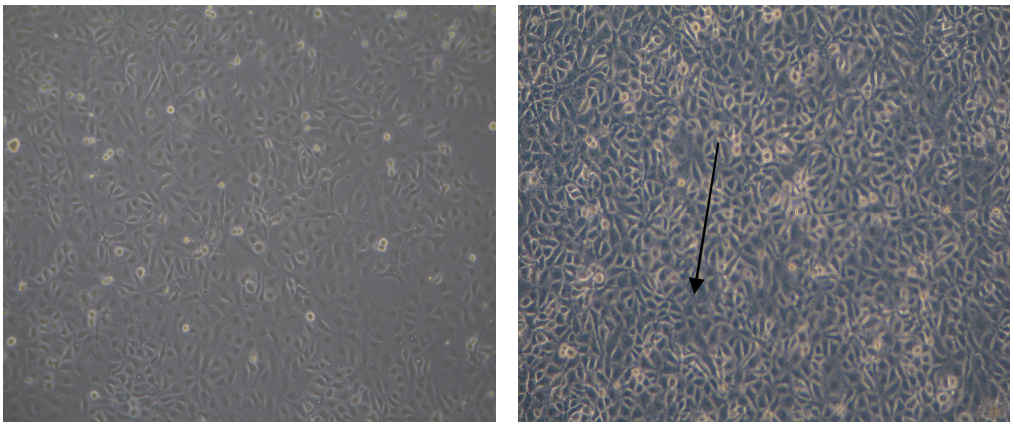
24 hr / Middle



E

Static / Down

24 hr / Down



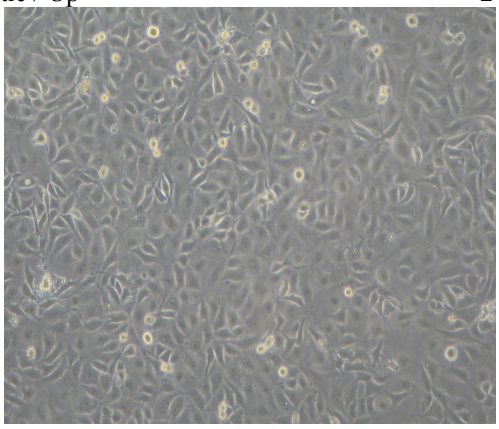
C F

Figure 4.8: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposed to at 500 ml/min in oscillatory flow in the order of up, middle, and down (D,E,F).

HUVEC's were exposed to oscillatory flow (500 ml/min) at 24 hours. Results were shown Figure 4.7. In this experiment, different results were observed. Depending on the flow rate, up and middle regions show how the cell shape occurs changes in the direction of flow. (D) Rounded cells in a confluent endothelial monolayer after 24 hr of exposure to at 500 mil/min in steady flow. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 500 ml/min in oscillatory flow. 500 ml/min flow conditions can modulate polygonal morphological change.(F) Similar effects were noted in the down area, after 24 hr of exposure to 500 ml/min in oscillatory flow. Rounded and migrating cells were observed very highly. Arrow indicates the changing of the cell shape in the direction of flow (Phase-contrast; 10X)

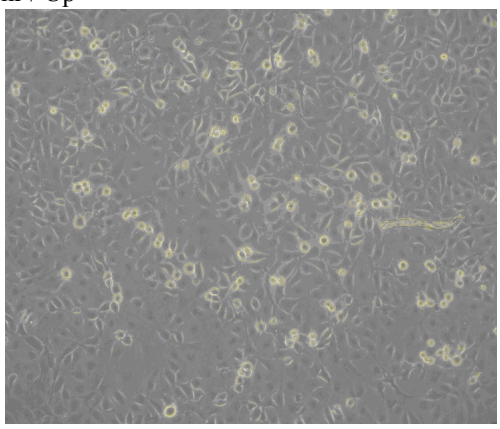
4.2.4 Oscilatory Flow (1000 ml/min) at 24 hours

Static / Up



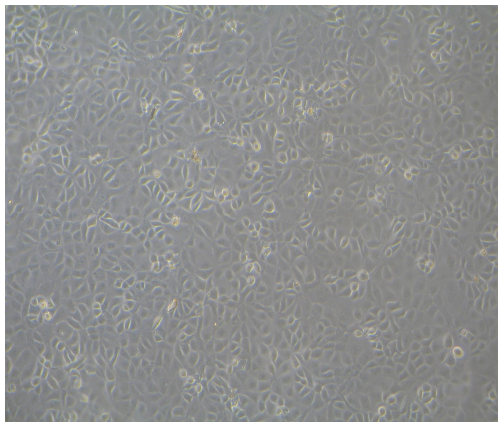
A

24 hr / Up



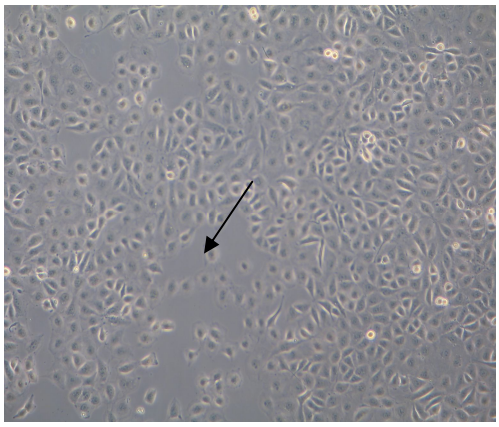
D

Static / Middle



B

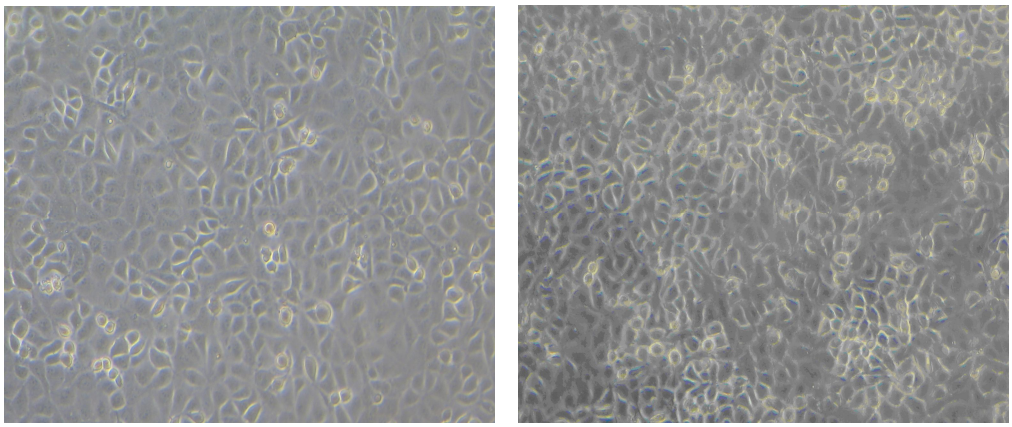
24 hr / Middle



E

Static / Down

24 h / Down



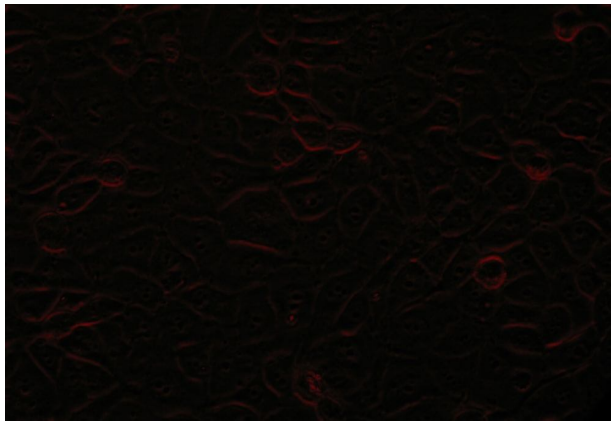
C F

Figure 4.9: HUVECs in static culture before experiment (A,B,C). After 24 hr of exposure at 1000 ml/min in oscillatory flow in the order of up, middle, and down (D,E,F).

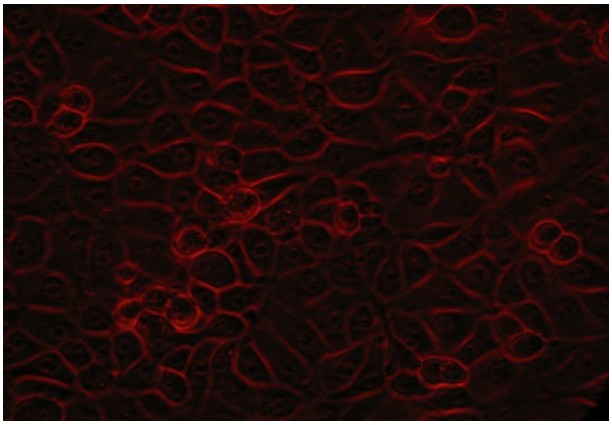
HUVEC's were exposed to oscilatory flow (1000 ml/min) at 24 hours. Results were shown Figure 4.9. (A,B,C) Monolayer in static culture. Under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation. (D) Rounded cells in a confluent endothelial monolayer after 24 hr of exposure to at 1000 ml/min in steady flow. (E) Confluent endothelial monolayer after 24 hr of exposure to shear stress at 1000 ml/min in oscillatory flow. Cell migration was observed. The spaces on the slide explained to migration of the cell. Arrow indicates cell migration in the direction of flow (Phase (F) Similar effects were noted in the down area, after 24 hr of exposure to 1000 ml/min in oscillatory flow. Rounded cells were observed very highly. 1000 ml/min flow conditions can modulate polygonal shape changing (contrast; 10X).

4.3 Immunofluorescent Staining

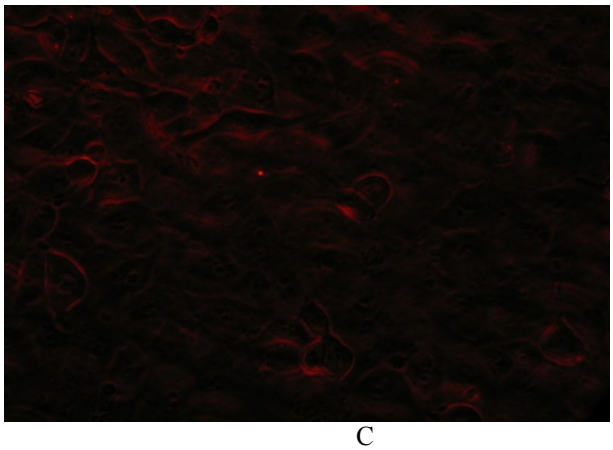
4.3.1 VE-cadherin Determination



A



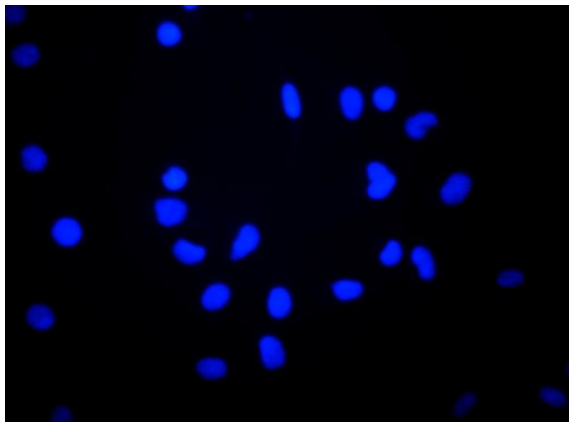
B



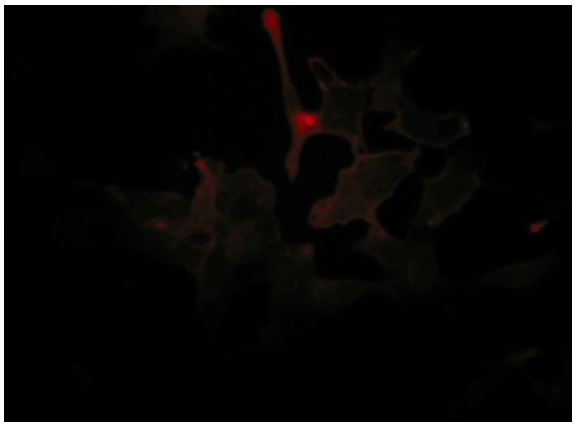
C

Figure 4.10: VE-cadherin determination. Static control (A), steady flow (500 mil/min) at 6 hours (B), oscillatory flow (500 mil/min) at 6 hours.

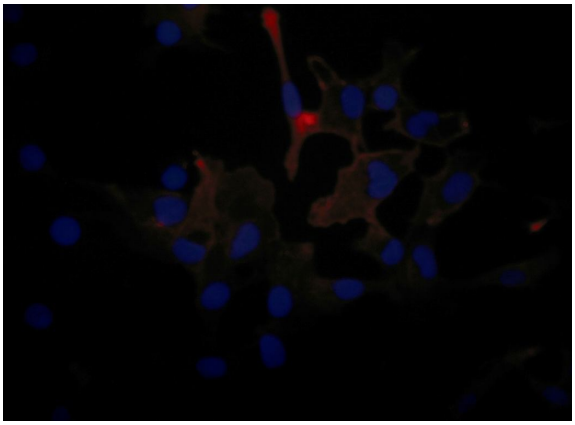
4.3.2 DAPI Staining and Integrin Determination



A



B



C

Figure 4.11: After oscillatory flow (500 mil/min) at 6 hours DAPI Stainig (A), α5β1 staining (B), Overlapping images of the (A and B) with photoshop (C).

CHAPTER 5

DISCUSSION

Blood vessels are lined by a thin layer of cells known as the vascular endothelium. Endothelium is exposed to a number of biomechanical stimuli resulting from the different type of flows of blood within the vascular system. One of the biomechanical stimuli is endothelial shear stresses. Endothelial shear stress, the frictional force from blood acting on the vessel wall, plays a crucial role in regulation of vascular structure. Certain *in vivo* observations suggest that hemodynamic forces such as shear stresses can play a critical role in vascular homeostasis and pathophysiology (Topper et al, 1999).

Firstly, HUVEC's were exposed to steady flow 50 mil/min at 6, 12, and 24 hr. morphological changes were observed at all flow conditions. After 6 hours, lower empty regions and very low cell migration were observed (Figure 4.2-D). The other step of experiment, 12 hr later, significant number of rounded cells can be seen detached to the upper surface of the monolayer (Figure 4.2-E) . Migration was observed. Similar effects were noted after 24 hr of exposure (Figure 4.2-F).

Second experiment was done under the flow rate of 500 mil/min during 24 hours. Cell cultures on glass slide were also examined at three different regions. When flow entered into the channel which is the upper part of the coverslip, initial effects were determined in this region (Figure 4.3-D). Gradual cell shape change was observed in this region and along the middle flow region the cell was elongated more in the direction of the flow (Figure 4.3-E). The accumulation of the cells were seen at the lower part of the coverslip which may be due to the motility of the cells from upper and middle part of the coverslip (Figure 4.3-F).

Cell morphology change to polygonal shape under the oscillatory flow conditions which was obtained by the movement of the coverslips inside the flow channel (Fifure 4.5 and 4.6). The elongated shape was seen in the middle and lower regions of the coverslips under oscillatory flow conditions (Figure 4.7 and 4.8).

The density of the VE-Cadherin along the cell periphery was detected in static and under flow conditions (Figure 4.10-B). The higher dense regions after the steady flow experiment was clearly seen at junctions of the cells. Similar but at lower magnitude results are also seen under oscillatory flow conditions (Figure 4.10-C).

The result of this study suggest that the magnitude of shear stress and exposure time important determinants of EC response. The flow conditions can modulate EC migration in a flow device designed to induce steady and oscillatory flow.

Flow studies will continue to better understanding the adhesion protein localization such as VE-cadherin and actin under steady, oscillatory and disturbed flow conditions.

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