VOL. 18, NO. 23, DECEMBER 2023 ISSN 1819-6608
ARPN Journal of Engineering and Applied Sciences

www.arpnjournals.com

EFFECT OF SHEAR STRESS ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS: A STUDY USING BIOREACTOR

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ABSTRACT

Shear stress has a significant impact on human umbilical vein endothelial cells (HUVECs). Endothelial cells line the inner surface of blood vessels, and they play a crucial role in regulating vascular function and maintaining blood vessel integrity. Shear stress is the force exerted by flowing blood on the endothelial cells, and it is an essential biomechanical factor that influences endothelial cell behavior. Understanding the effects of shear stress on HUVECs is essential for studying vascular physiology, identifying potential therapeutic targets for cardiovascular diseases, and designing better vascular implants and devices. The present study provides compelling evidence of the significant impact of shear stress on the maintenance of endothelial cell (EC) phenotype. By investigating the effects of shear stress on human umbilical vein endothelial cells (HUVECs), we have unveiled its role in regulating the expression of pro-thrombotic and anti-thrombotic genes. Our findings demonstrate that shear stress influences key molecular players, such as eNOS (endothelial nitric oxide synthase) and vWF (von Willebrand factor), which are critical for vascular homeostasis and thrombotic balance.

Keywords: sheer stress, human umbilical vein endothelial cells (HUVEC), endothelial cells.

Manuscript Received 1 September 2023; Revised 18 January 2024; Published 10 February 2024

1. INTRODUCTION

Endothelial cells are polarized cells. Blood constituents and circulating cells directly interact with the EC luminal membrane. However, glycoprotein basement membranes, secreted and anchored to EC cell membranes, distinctly delineate them from surrounding tissues. Endothelial cells (ECs) find their applications in various clinical segments like promoting angiogenesis [1] osteogenic differentiation of mesenchymal stem cells [2], reendothelialization of tissue-engineered grafts, etc., [3]. These cells are derived from donors or engineered invitro. In current-day research, there have been multiple studies reported on human umbilical vein endothelial cells (HUVEC) as a research model for human endothelium. Although this model does not represent all endothelial cell types found in an organism, HUVEC is an excellent model for the study of vascular endothelium properties and the main biological pathways involved in endothelium function.[4]. Over the past 40 years, a breadth of studies have investigated the influence of fluid shear stress on the behavior and function of endothelial cells and demonstrated the importance of the level and pattern of shear stress on, e.g., EC morphology, proliferation, gene expression, or the development of vascular pathologies.[5-8]. Shear stress is a prominent candidate for induction of endothelial dysfunction which leads to cardiovascular disease. Bioreactor creates an environment that enables cells to proliferate and differentiate in vivo, establish spatially uniform cell distributions on 3D scaffolds, maintain desired concentrations of nutrients, provide efficient mass transfer to tissue, and expose tissues to physical stimuli [9]. Bioreactors also provide a controlled and reproducible culture condition. The variables that are controlled include temperature; pH, gas concentration, media flow rate, shear stress, hydrodynamics, and mechanical forces. Bioreactors recreate the in vivo environment by regulating physiological factors and thereby provide a valuable system for studying complex cellular processes. In the present studies, endothelial cell cultures were cultured and characterized under static conditions, and varying shear stress using pulsatile bioreactors. The endothelial function at the molecular level during sheer stress is studied and the comparison of endothelial cell characters and functions is discussed.

2. MATERIALS AND METHODS

2.1 In Vitro Culture of HUVEC

Fibrin composite-coated polystyrene culture dishes were prepared according to the established procedure [10]. Human thrombin and fibrinogen were prepared in sterile conditions. Human thrombin was reconstituted in 0.035M calcium chloride at a concentration of 2 IU/ml. The tissue culture polystyrene (TCPS) (NUNC, Denmark) was coated with thrombin and fibrinogen along with growth factors. The cocktail was allowed to polymerize at 37^0C for 30 minutes. Culture plates were then frozen at -80^0C, lyophilized, and stored at 4^0C until use. Endothelial cells were isolated from human umbilical cord veins according to the method described by Jaffe *et al* (1973) with minor modifications. The human umbilical cord, collected in sterile, ice-cold Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS) containing antibiotics (Pen/strep) and 0.1% w/v D-glucose, was digested with 0.025% collagenase (Gibco, USA) and HUVEC was collected and suspended in complete IMDM (GIBCO, USA) medium (10% serum, 20µg/ml growth factor mixture, 1mg/ml sodium bicarbonate and 1X

antibiotics (Gibco BRL, USA) and seeded on matrix coated TCPS The veins for the study were collected from a maternity hospital, Trivandrum with required consent.

2.2 Characterization of HUVEC

Routine evaluation of the quality and growth pattern of the cultured cells was done using an inverted phase contrast microscope at 10X, and 20X magnifications. Endothelial cells were characterized by the typical cobblestone morphology exhibited by the confluent monolayer.

To evaluate the cell density, the near confluent third passage cells were treated with 1, 1-dioctadecyl-3, 3, 3, 3-tetramethyl Indocarbocyanine perchlorate (Dil) labeled AcLDL. The cells were fixed after incubation and washing for further microscopical analysis under an inverted fluorescent microscope using a Rhodamine filter.

To evaluate the ULEX uptake of the cells, the Fresh medium containing 1:250 dilution of ULEX was added. The cells were fixed after incubation and washing for further microscopical analysis under an inverted fluorescent microscope ULEX stained cells were observed under a fluorescence microscope.

2.3 mRNA Studies

Endothelial cells were grown on fibrin-coated TCPS. The RNA was isolated using TRIzol Reagent (Invitrogen, USA) as per the manufacturer's direction. The yield of RNA was quantified using a spectrophotometer. The purity of the RNA preparation was assessed from the ratio A260/A280. The cDNA was collected through reverse transcription. Real-time PCR for the genes endothelial nitric oxide synthase (eNOS) and von Willibrand factor (VWF) was performed using a Chromo4 system. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. All reactions were carried out in a total volume of 20µl containing 8µl of cDNA, 10µl SYBR Green master mix (Full velocity SYBR Green qPCR mastermix, Stratagene, USA),1 µl forward primer, and 1 µl reverse primer (annealing temperature 55ºC). Primer sequences used were

GAPDH- F: 5'attggctttggtccgagtcc3',
R:5'gggggttctttggcttttac3'
eNOS- F: 5'agctgtgctggcatacagga3'
R: 5'atggtaacatcgccgcagac3'
vWF- F: 5'caccattcagctaagaggagg3'
R: 5'gccctggcagtagtggata3'.

2.4 HUVEC Seeding onto Tubular Polymer Scaffolds

The porous PCL scaffold was coated with fibrin composite and was saturated with thrombin (5 IU/ml.). The scaffolds were lyophilized and dipped into a fibrinogen solution containing gelatin and growth factors. Post incubation, the scaffolds were washed off the excess solution. Fibrin-coated scaffolds were saturated with serum-free medium (SFM). The sides of 2 scaffolds were sealed using connectors and the lumen was filled with EC suspension in a complete medium to get a seeding density of $1X10^5$cells/cm^2. After 2h incubation at 37ºC in a CO_2 incubator, unattached cells were removed. Cell-seeded scaffolds were transferred to a fresh complete medium in a 10 ml polystyrene tube (nunc, Denmark) and incubated for overnight under static culture conditions for further experimentation.

2.5 HUVEC under Static and Dynamic Conditions

One scaffold was connected to medium flow initially (2ml/minute) using a peristaltic pump and the medium flow rate was increased to 5ml/min in 4 hours, to 10ml/min in 24 hours. The other cell-seeded scaffold was kept under static culture conditions in a CO_2 incubator. After every 24 hours medium has been changed for both scaffolds under static and dynamic conditions Visualization of HUVEC in static and dynamic conditions.

2.6 Exposure of Seeded Tubular Polymer Scaffolds onto Shear Stress using Pulsatile Flow Bioreactor

Three scaffolds were connected to medium flow using a custom-made bioreactor for 5 days in a CO_2 incubator and an equal number of scaffolds were continued under static culture conditions in a CO_2 incubator. Initially, a medium flow of 2 ml/min was given subject to a shear stress of 0.04dyne/cm^2. Throughout 4 days flow rate was increased from 2 to 30 ml/min. Then the medium flow was continued for the next 24 hours and therefore subjected to a shear stress of 1.2dyne/cm^2. Medium change was done on alternate days.

2.7 Analysis of HUVEC

To analyze the cell retention in static and dynamic conditions, the cell-grown scaffolds were washed with HBSS and PBs, and fixed. The scaffolds were stained with stained using Maygrunwald-Giemsa stain (MGG). These samples were further studied to understand the morphology and distribution of cells under static and dynamic culture conditions. Acetylated human LDL-uptake assay was performed as mentioned above.

Endothelial cells seeded scaffold kept in dynamic and static conditions was used to evaluate the effect of shear stress upon endothelial cells in terms of antithrombotic and prothrombotic factor expression. One prothrombotic factor Willebrand factor (vWF) and one thrombotic factor-endothelial nitric oxide synthase (eNOS) were analyzed.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of Endothelial Cells

Endothelial cells were attached to the fibrin-coated TCPS. Within 48 hours, ~90% confluence was obtained. Endothelial cells obtained cobblestone morphology on 3rd passage (Figure 1). It is also reported by Ryan [11] that the long-term cultures of endothelial cells maintain cobblestone monolayer morphology, post 24 hours of isolation. Ravishankar *et al*., [12] have reported the isolation of endothelial cells isolation from the human umbilical cord and adherent endothelial cells to possess cobblestone morphology.

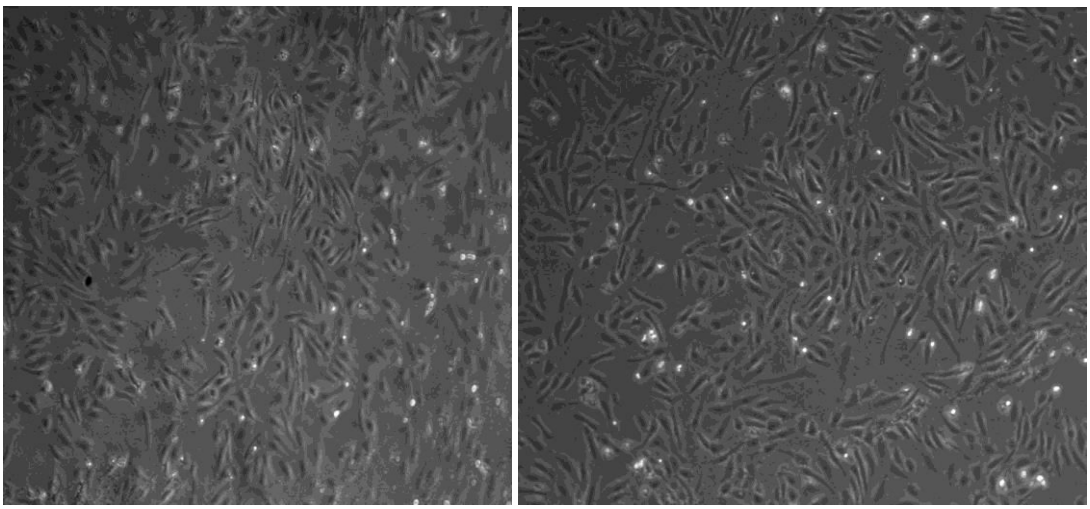

Figure-1. A) Light microscopic image of HUVEC 24 hours after isolation. B) The figure shows the confluence of 80-90% after 48 hours of culturing of endothelial cells onto fibrin-coated TCPS.

3.2 Characterization of HUVECs

The endothelial nature of the isolated cells has been conclusively established based on morphology analysis (Figure 2), AcLDL uptake assay (Figure-3), Lectin binding assay (Figure-4), and molecular analysis.

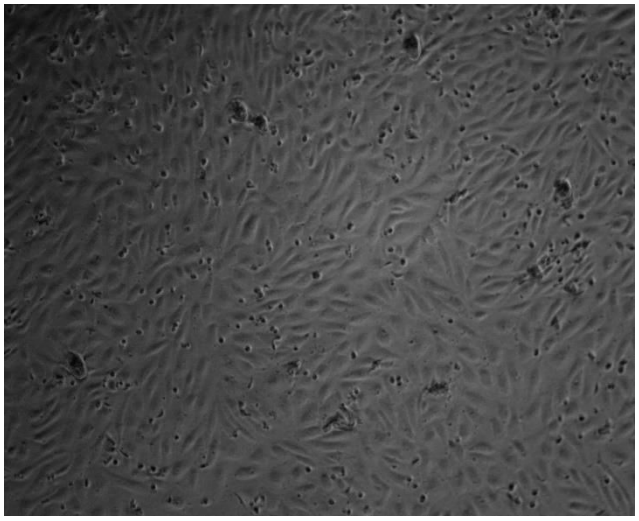

Figure-2. Light microscopic image of HUVEC on 5th passage cultured on fibrin coated TCPS.

The quality & growth pattern of the cultured cells is evaluated using an inverted phase contrast microscope. HUVEC are characterized by typical cobble stone morphology on attaining confluence *in vitro*. The figure shows the typical cobble stone morphology of endothelial cells which could be observed in the confluent monolayer.

Acetylated-low density lipoprotein (Ac-LDL) is taken up by macrophages and endothelial cells via the "scavenger cell pathway" of LDL metabolism [13]. Endothelial cells in the primary culture can be identified by their ability to take up fluorescent Acetylated LDL. Observation under a fluorescence microscope after incubation with Dil-Acetylated LDL revealed the typical pattern of AcLDL uptake by ECs (Figure-3). Akis *et al*., [14] isolated endothelial cells along with visceral epithelial and mesangial cells from mouse and characterized them based on the high uptake of the Acetylated LDL.

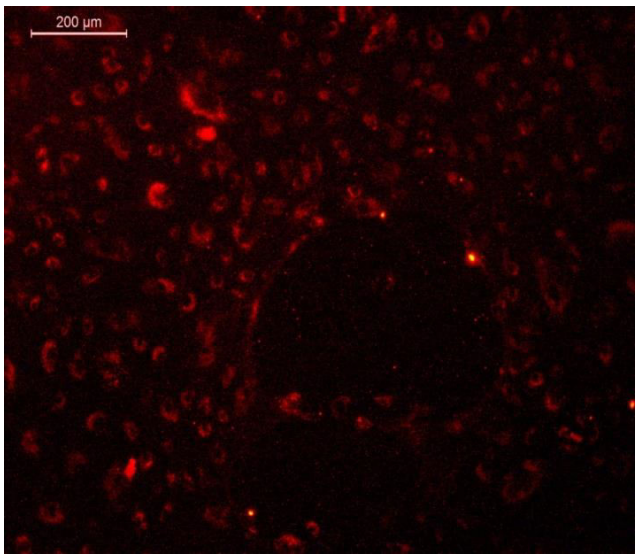

Figure-3. AcLDL uptake by ECs. Endothelial cells have taken up Dil labeled AcLDL and viewed under Fluorescent microscope.

Lectins are proteins or glycoproteins of non-immune origin that agglutinate cells and/or precipitate carbohydrates complex. Ulex europaeus I agglutinin (UEA 1), a lectin specific for some alpha-L-fucose-containing glycol compounds. UEA-1 was found to bind predominantly to endothelial cells within blood vessel structures of all sizes Ulex europaeus I agglutinin can be considered as a marker for human endothelium [15, 16]. FITC-conjugated lectin binding shows the typical pattern of lectin binding on endothelial cells. (Figure-4) Some more recent studies suggested UEA 1 has a binding towards a variety of other cell types including blood-derived cells [17, 18]. Hence, the endothelial phenotype

was confirmed by the following studies at the molecular level.

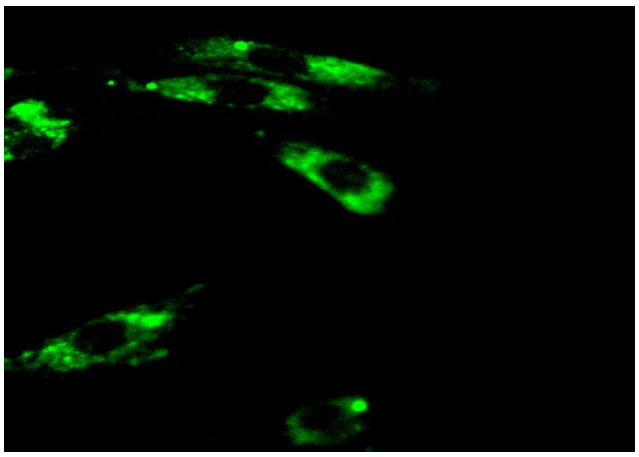

Figure-4. Ulex lectin binding on ECs. EC-specific binding of FITC conjugated ulex lectin was viewed under a fluorescence microscope.

3.3 mRNA Expression Studies

mRNA quantification and purity checking were done before PCR analysis. Spectrophotometric analysis showed the purity of the preparation which is considered to be one of the crucial factors in Real-Time PCR analysis. Absorbance at 260nm is specific for nucleic acids at the same time nil absorbance at 280nm ensures that protein contamination did not occur in the mRNA preparation (Figure-5).

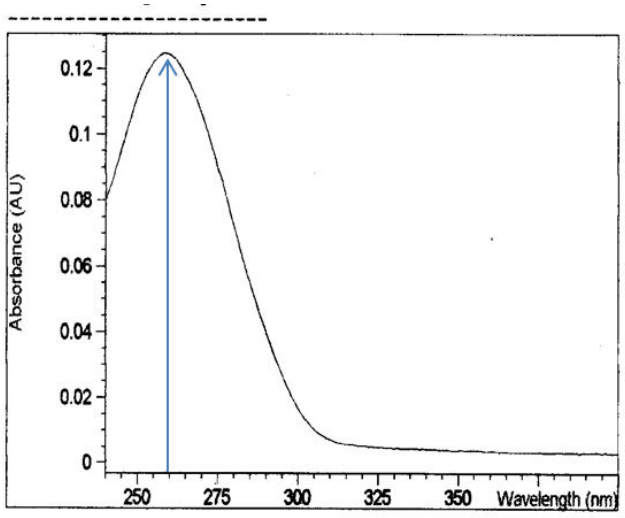

Figure-5. RNA quantification and purity checking using Spectrophotometer. Sharp Absorbance peak at 260nm indicates the purity of mRNA.

Endothelial phenotype was confirmed by PCR analysis of endothelial specific genes. One antithrombotic gene (eNOS) and one prothrombotic gene (vWF) were analyzed and reactions were done in duplicate to ensure the repeatability of the procedure. Early expression of the antithrombotic gene and late expression of the prothrombotic gene ensures the maintenance of healthy phenotype of ECs *in vitro* [19]. In all the reactions amplifications and the melting curve analysis revealed the high specificity of the reactions. Data were calculated following comparative CT (ΔΔCT) mode and listed as relative RNA levels after normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ct values 22, 24, and 28 were obtained for GAPDH, eNOS, and vWF respectively as expected (Figure-6 & Figure-7). Higher expression of eNOS when compared with vWF confirms the maintenance of healthy EC phenotype on fibrin coated surfaces. In latest studies like Yang *et al*., [20], Gimbrone *et al*., [21], and Ragaseema *et al*., [22], it is reported that the combined effect of eNOS andvWF appears synergistic.

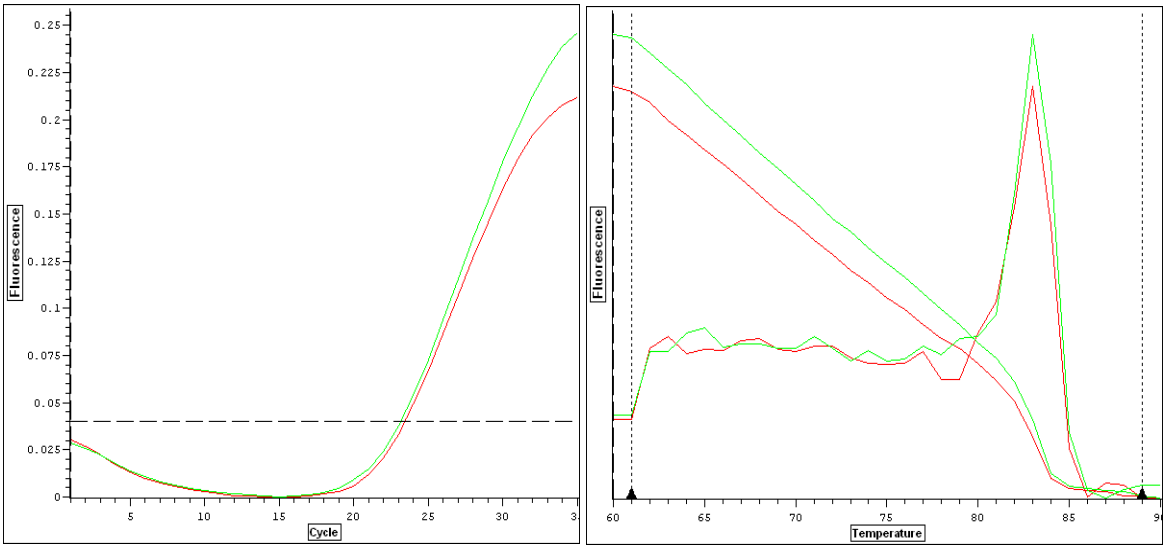

Figure-6. Real-time PCR analysis of GAPDH. (A) Amplification curve for GAPDH, C(t) value=22. (B) Melting curve of the product, Tm= 83^0C.

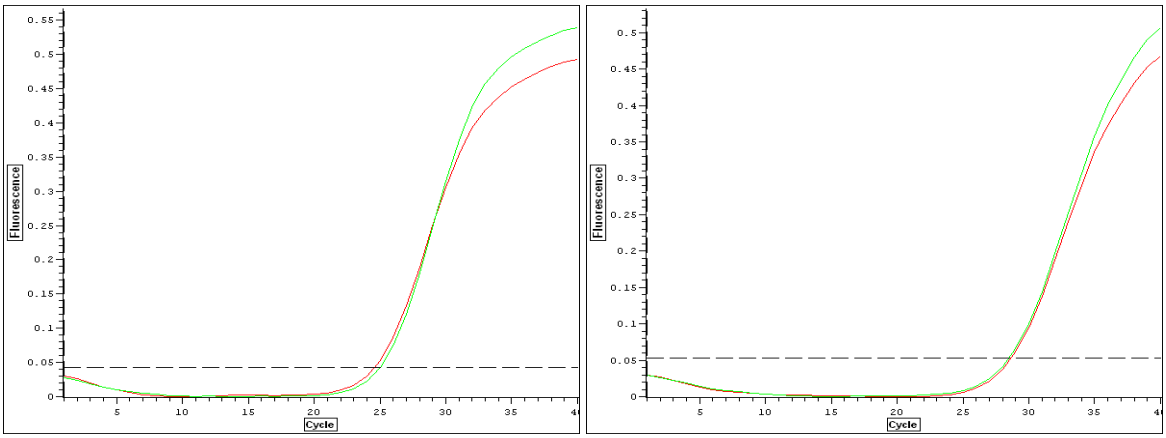

Figure-7. A) Real-time PCR analysis of eNOS (A) Amplification curve for eNOS C(t) value=24. Melting curve of the product, Tm= 80^0C **B)** Real-time PCR analysis of vWF. (A) Amplification curve for vWF, C(t) value=28. Melting curve of the product, Tm= 80^0C.

3.4 HUVEC Seeding onto Tubular Polymer Scaffolds

Biological scaffolds that have been molded into tubes (such as collagen or fibrin gel) can be used for scaffolding human cells. The cells can also be seeded on electrospun scaffolds that are biodegradable or biohybrid [23]. Poly (caprolactone) (PCL) has been used as a scaffold for HUVEC seeding. Tubular porous scaffolds, size 8cm in length & 4mm diameter were used. The efficacy of PCL to support endothelial cell adhesion, spreading, proliferation, and survival and therefore as a biodegradable scaffold for vascular tissue engineering application has already been evaluated [24, 25]. Fibrin was used as a biomimetic matrix on PCL scaffolds. The ability of fibrin to serve as a stable matrix for the generation of tissue-engineered nonthrombogenic vascular grafts has been studied [26-28]. ECs of the third passage were used for the experiment to ensure the normal healthy phenotype of cells. HUVECs were seeded onto the luminal surface of the tubular scaffold to mimic the inner luminal endothelial lining of blood vessels. Seeding may be carried out in a separate operation where the cells are allowed to attach to the scaffold and then the seeded scaffold is inserted into the bioreactor, or the cells are seeded directly onto the scaffold within the bioreactor. In either case, scaffold seeding is a batch operation to ensure maximum cell attachment. A key challenge for seeding is to be able to uniformly distribute high initial cell numbers onto a 3D scaffold [29, 30]. Static seeding is most widely used; however, there are advantages to be found with the use of dynamic seeding [31] leading to higher quality tissue in terms of structure and composition [9, 29]. A variety of dynamic seeding methods have been reported including simple mixing, seeding in spinner flasks, and convective seeding in perfusion reactors [32]. In the present study, static seeding was done and allowed the cells to attach to the polymer surface under static conditions.

3.5 Characterization of HUVEC under Static vs Dynamic Condition

In order to standardize the procedure and to understand the effect of flow on endothelial phenotype an initial experiment was carried out with a perfusion pump. Simultaneously cell seeded scaffolds were kept under static conditions for 48 hours. MGG staining procedure carried out after 48 hours revealed that cell distribution under dynamic conditions was more uniform and cell survival was much higher under dynamic culture conditions when compared with static culture (Figure-8).

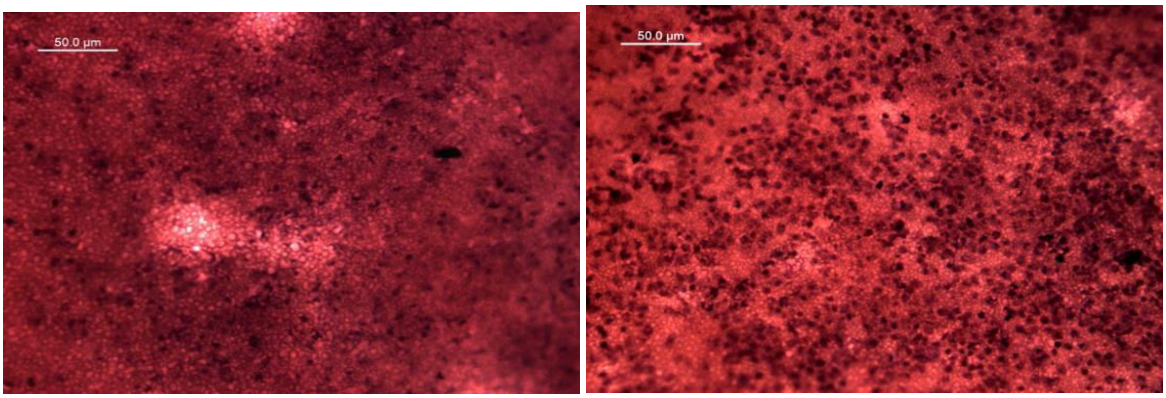

Figure-8 A. Figure-8 B.

Figure-8. Visualization of HUVEC on the scaffold after MGG staining. Figure shows a Light micrograph of HUVEC seeded onto the scaffold A) Static B) After Perfusion. The cells seeded onto the scaffold were cultured for 48 hours, and stained with MGG.

There was a remarkable difference between static and dynamic culture in terms of HUVEC spreading and morphology. Endothelium on the polymeric scaffold was further analyzed by AcLDL uptake and Ulex lectin binding. Both the techniques of Acetylated LDL uptake Assay and the Ulex Lectin binding revealed the same result of MGG binding. From the above observations, it is understood that the spreading of HUVEC on the hybrid scaffold in the dynamic condition is more favorable because of improved focal adhesion [33]. Zaho *et al*., [34] in their studies confirmed that firm focal adhesion was generated at the interface between the HUVECs on a PCL scaffold. Obtaining cells with high regenerative capacity can be achieved through dynamic cultures for all 3D scaffolds [35].

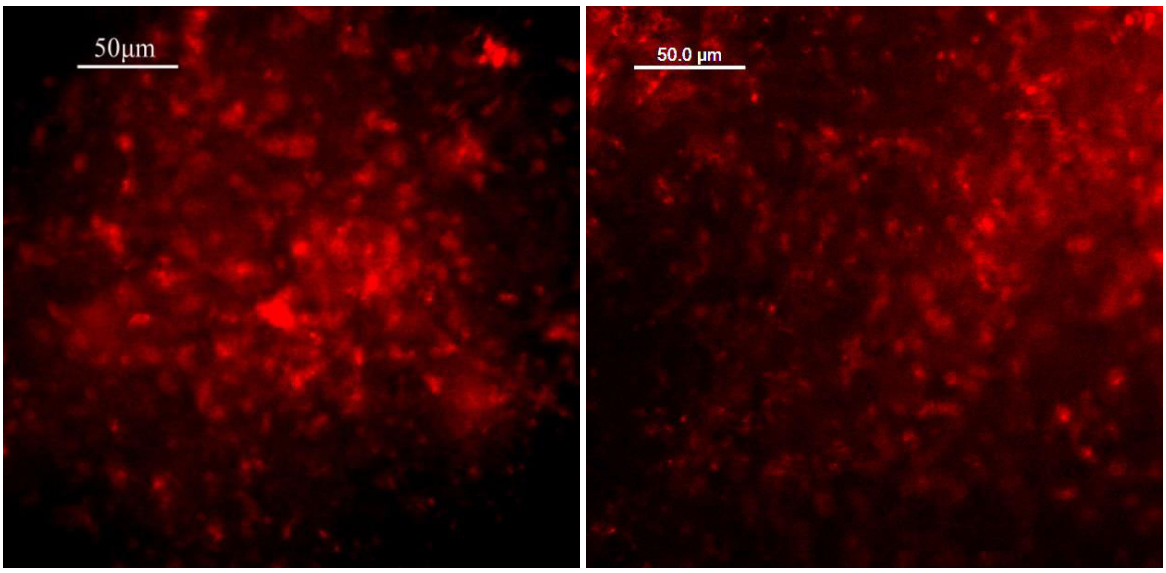

Figure-9. AcLDL uptake by ECs after in vitro culture on polymeric scaffold. A) static B) Dynamic. The cells seeded onto the scaffold were placed in dynamic/static culture conditions for 48 hours and incubated with Dil labeled AcLDL. Red fluorescence indicates AcLDL uptake by endothelial cells.

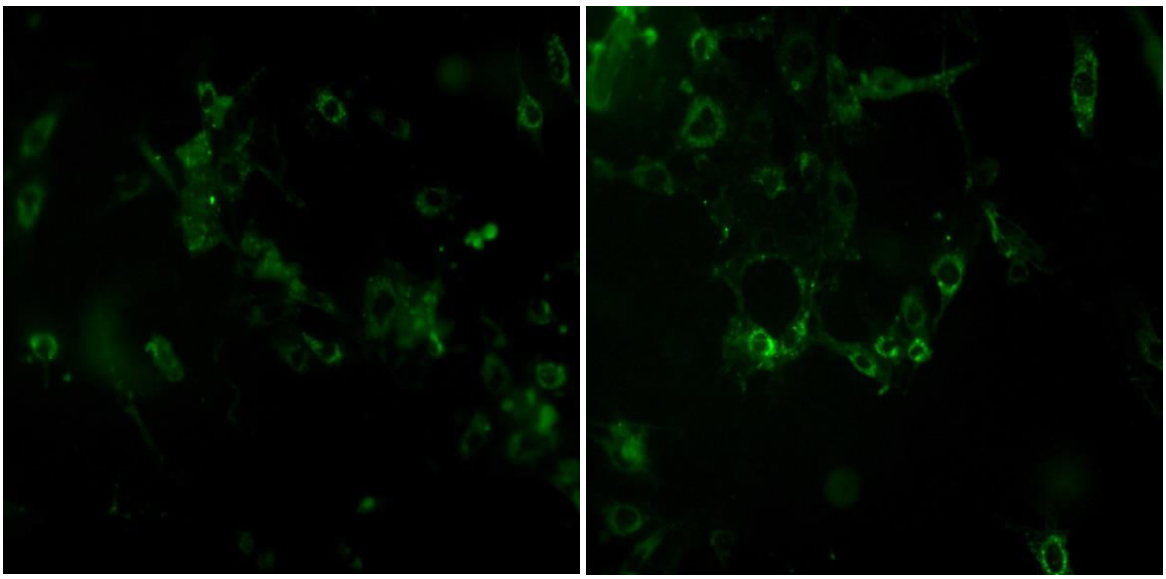

Figure-10. ULEX lectin binding on ECs after in vitro culture on the polymeric scaffold. A) static B) Dynamic. After 48 hours static/ dynamic culture cells were incubated with FITC conjugated Ulex lectin. Green Fluorescence indicates lectin binding on ECs.

3.6 Effect of Shear Stress on HUVEC Seeded Hybrid Scaffold using Bioreactor

A vital aspect of vascular biology is to maintain the phenotype of the endothelium. The endothelial phenotype is influenced by a variety of factors. The biological behavior of the cell depends heavily on hemodynamic forces since endothelium is constantly exposed to blood flow. Blood flow produces shear stress on ECs and causes hyperpolarization of the cell membrane via potassium channels. This activation stimulates the release of nitric oxide (NO) by transcriptional upregulation of endothelial nitric oxide synthase (eNOS) and leads to vasodilation [36, 37]. Among this shear stress is the most important candidate. Shear stress plays a major role in the regulation of antithrombotic and prothrombotic phenotype of endothelium [8]. In the present study, HUVECs have been subjected to shear stress using a pulsatile flow bioreactor (Figure-11) and the phenotype of endothelium has been studied in terms of expression of antithrombotic (eNOS) and prothrombotic (vWF) genes.

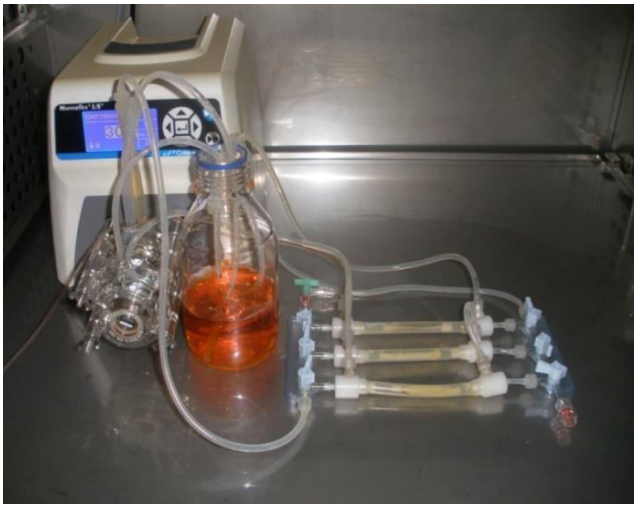

Figure-11. HUVEC seeded PCL scaffolds under dynamic condition in a Pulsetile flow Bioreactor. The figure shows HUVEC seeded PCL scaffolds in a pulsetile flow bioreactor, consisting of a Peristaltic pump, Medium reservoir, pulse detector, controlled by software.

3.7 mRNA Expression Studies

mRNA expression studies revealed that shear stress has a profound impact on the phenotype of endothelium. Amplification of desired genes was confirmed from amplification curve analysis (Figure-12) and further calculations were done.

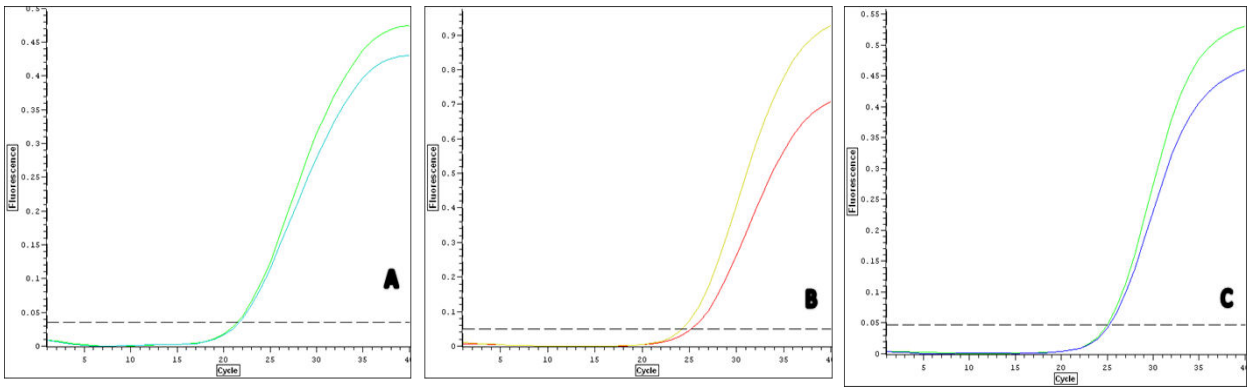

Figure-12. A). Amplification curve for GAPDH Blue line indicates static condition and green line indicates dynamic condition. B) Amplification curve for eNOS Red line indicates static condition and the Yellow line indicates dynamic condition C) Amplification curve for vWF Green line indicates static condition and blue line indicate dynamic condition.

When expression of eNOS was analyzed and fold change was calculated by comparing with 0h sample, cells under dynamic culture showed 2-fold increases in expression. In the case of static culture, eNOS expression was slightly higher but comparable with 0h samples (Figure-13). However, when the statistical analysis was carried out to compare the expression of eNOS under static and dynamic culture conditions, a very significant increase in the expression was obtained in the case of dynamic culture (p-value <0.05).

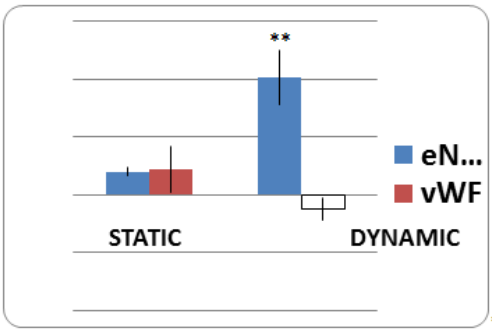

Figure-13. Quantification of mRNA expression: Fold change was calculated for static and dynamic samples by comparing with zero hour sample and represented as Barr diagram.

Various pathophysiological conditions affect the level of expression of eNOS. In cultured cells, eNOS expression is increased by shear stress, cyclic strain, exposure to lysophosphatidylcholine, low concentrations of oxidized low-density lipoprotein, and cyclic GMP analogs. In a study, the endothelial cells 1 to 100 dynes/cm2 when exposed to arterial levels of steady or pulsatile unidirectional shear stress tend to adopt an anti-inflammatory, anti-thrombotic, and anti-proliferative phenotype [38]. Weber *et al.*, [39] demonstrated that HUVECs subjected to prolonged USS (24 hours, 15 dynes/cm2) or static conditions, cause increased expression of eNOS activity and decreased apoptosis. The data by Weber & Searles [40] also demonstrates the importance of the extent of miRNA upregulation on endothelial cell phenotype. Exposure of cultured endothelial cells to TNF*a*, hypoxia, and high concentrations of oxidized low-density lipoprotein decreases eNOS levels. Under these conditions, posttranscriptional changes in mRNA half-life play an important role in the downregulation of eNOS expression [41]. In several other studies, it has been identified that Atherosclerosis lesions exhibit reduced eNOS expression in endothelial cells. Endothelial vasomotion would be affected dramatically by a loss of eNOS expression [42, 43]. Expression of prothrombotic factor (vWF) was analysed and fold change was calculated for static and dynamic samples by comparing them with 0h samples. Expression of vWF was reduced in dynamic samples, but it was comparable with 0h samples. When fold expression was compared statistically between static and dynamic samples, there was no significant change (Figure-13). Fernandez *et al.*, [44] demonstrated that under laminar shear stress causes downregulation of prothrombotic genes such as vWF. Dekker *et al.*, [45] demonstrated that prolonged shear stress can induce various gene factors in endothelial cells including vWF and KLF 2. There are also studies reported at the molecular level that antagomiR-92a can prevent endothelial dysfunction. Wide arrays of processes have been linked to atherosclerosis and cardiovascular diseases due to the newly emerging gene regulators, miRNAs [46].

4. CONCLUSIONS

Shear stress plays a critical role in the function and behavior of human umbilical vein endothelial cells (HUVECs). Endothelial cells line the inner surface of blood vessels and are constantly exposed to blood flow, which generates shear stress on their surfaces. A vital aspect of vascular biology is to maintain the phenotype of the endothelium. It's important to note that the specific effects of shear stress can vary depending on the magnitude, duration, and frequency of the force applied. Research in this area continues to provide valuable insights into vascular biology and potential therapeutic interventions for cardiovascular disorders. Understanding

the effects of shear stress on HUVECs is essential for unraveling vascular biology and the pathophysiology of various cardiovascular diseases. Researchers often study these effects in vitro using specialized flow chambers and in vivo through animal models or human studies. shear stress to HUVECs leading to changes in eNOS andvWF.

In conclusion, this study provides compelling evidence of the significant impact of shear stress on the maintenance of endothelial cell (EC) phenotype. By investigating the effects of shear stress on human umbilical vein endothelial cells (HUVECs), we have unveiled its role in regulating the expression of pro-thrombotic and anti-thrombotic genes. Our findings demonstrate that shear stress influences key molecular players, such as eNOS (endothelial nitric oxide synthase) and vWF (von Willebrand factor), which are critical for vascular homeostasis and thrombotic complications. Further research in this area could open new avenues for therapeutic strategies aimed at preserving vascular health and reducing the risk of cardiovascular disorders. We did not examine the mechanisms behind these changes as part of this study. In future molecular studies, endothelial cells should be investigated for different shear stress levels based on the results of this study.

Funding Information:

This research was not a part of any funded project.

Conflict of Interest:

The author declares no conflict of interest.

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