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Topological Remodeling of Cultured Endothelial Cells by Characterized Cyclic Strains

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Abstract: Evaluation of mechanical environment on cellular function is a major field of study in cellular engineering. Endothelial cells lining the entire vascular lumen are subjected to pulsatile blood pressure and flow. Mechanical stresses caused by such forces determine function of arteries and their remodeling. Critical values of mechanical stresses contribute to endothelial damage, plaque formation and atherosclerosis. A device to impose cyclic strain on cultured cells inside an incubator was designed and manufactured operating with different load amplitudes, frequencies, numbers of cycles and ratios of extension to relaxation. Endothelial cells cultured on collagen coated silicon scaffolds were subjected to cyclic loading. Effects of mechanical loading on cell morphology were quantified using image processing methods. Results showed change in cell orientation from a randomly oriented before the test up to 80° alignment from load axis after loading. Endothelial cells were elongated with shape index reductions up to 47% after cyclic stretch. By increase of strain amplitude, loading frequency and number of cycles, significant decrease in shape index and significant increase in orientation angle were observed. Change of load waveform similar to arterial pulse pressure waveform resulted in alteration of cell alignment with 9.7% decrease in shape index, and 10.8% increase in orientation angle. Results of cyclic loading tests in a disturbed environment with elevated PH showed lack of remodeling. It was concluded that tensile loading of endothelial cells influences cell morphology and alignment, a mechanism for structural regulation, functional adaptation and remodeling. Disturbed environment results in endothelial dysfunction and injury.

Keyword: Cell Morphology, Cyclic Strain, Endothelial Cells, Remodeling.

1 Introduction

Cardiovascular diseases are among the major causes of world mortality. Atherosclerosis and most of cardiovascular diseases are related to endothelial injuries. Endothelial cell layer located in the inner most part of arteries prone to the blood flow is a natural barrier to cholesterol and other large protein molecules. It is also a membrane for transferring nutrients to the arterial wall media. Endothelial damage results in deposition of lipids such as cholesterol in the arterial wall, and eventually plaque formation leading to luminal stenosis. In many cases plaque rupture is an outcome of progression of the disease, followed by clinical consequences such as stroke [1-3].

Hemodynamic forces have long been implicated in the initiation and localization of endothelial injury and consequently atherosclerosis [2, 4-6]. The endothelial layer as a cover of entire vasculature prone to blood is exposed to mechanical loadings such as hydrostatic pressure, cyclic strain, and wall shear stress [2, 7-9]. The resultant mechanical stresses regulate the structure and function of blood vessels [10-13]; however, their critical values contribute to endothelial damage [14, 15]. Such stresses include shear stress generated by pulsatile blood flow, and circumferential

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stress caused by pulsatile blood pressure. When exposed to mechanical loading, endothelial cells act like mechanical sensors. Their morphology, structure, and functions change with applications of tensile or shear loadings [15-19].

The pulsatile blood pressure causes a circumferential tensile stress on the arterial wall and consequently on the endothelial layer. Elevation of circumferential tensile stress in arterial wall may result in endothelial damage. The degree of cell morphology in response to tensile loading is a determinant of endothelial remodeling and damage. Morphological remodeling of endothelial cells is considered as an adaptive response to mechanical stimuli.

Cellular responses to mechanical stimuli have been studied extensively by exposure of cultured cells or arterial segments to cyclic loading The cellular responses include various parameters such as morphology, structure, mechanical properties, protein synthesize, gene expression, angiogenesis, cell migration, activation of ionic channels, cell proliferation and vessel tone [2, 10, 20-25].

Alteration of endothelial cell morphology as an adaptive response to tensile cyclic loading has been an important field of study of endothelial function [2, 10, 26]. Morphological changes are evaluated by parameters such as degree of cell elongation under mechanical tension (shape index), direction of cell reorientation, cell nucleus shape, and alteration of structural filaments.

Studies have shown that with application of uniaxial stretch on cultured cells, cells are aligned nearly 70^o (oblique) from the load direction in which the minimal deformation of membrane occurs [2, 27]. With uniaxial cyclic strain, cells are oriented perpendicular to the direction of loading [28, 29]. The degree of orientation is a descriptor of morphology which depends on loading parameters such as strain amplitude and rate [27, 30]. Experiments have shown reduction of shape index after stretching referring to cell elongation [22, 31]. Such morphological changes lead to differing cell to cell contact, and consequently altered cellular functions [2, 22, 32].

The objective of this study is to investigate morphological changes of cultured endothelial cells under cyclic tensile loading in a wide range of load amplitudes, frequencies and number of cycles by developing a system with improved design to impose uniform cyclic tensile force on cultured cells in physiological conditions. The analysis is extended to altered load waveform and disturbed environment indexed by elevated PH. The results can be applied in study of cell adaptation to physical and mechanical environments and mechanisms of cell remodeling. A threshold for cell damage can be obtained based on the disability of cells for remodeling in harsh environment or excess of mechanical loading.

2 Materials and Methods

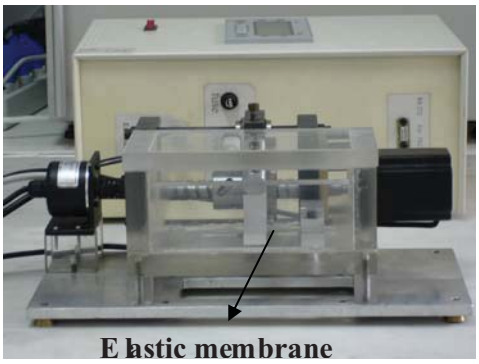
2.1 Design of Cyclic Axial Stretch Device

A device is designed and manufactured to impose a uniform, homogeneous, and axial strain, either dynamic or static, on cells grown on an elastic membrane or soft tissues. The device is able to apply a constant strain in a wide range of test durations (up to 48 hours). The device is required to operate in an incubator limiting the size, weight and structural materials. Since there is a negligible level of shear stress imposed on the cells due to the motion of the fluid on top of the cells, it can be assumed that the loading is pure tension. The ability to control strain profile, magnitude, and frequency independently enables the characterization of the temporal and spatial cell responses to mechanical parameters *in vitro* separately.

Compared to previous devices, the current design has some advantages including: application of tensile cyclic loading on both cultured cells and soft tissues, capability of operation in wide ranges of strain amplitude, loading frequency, and strain rates in stretching and relaxation with precise control, and ability to produce different load waveforms by differing rate of extension to relaxation.

The device consists of electronic and mechanical units (Figure 1(A,B)). The electronic unit consists of PLC + HMI (LG Industrial System, Korea), stepper motor driver (Autonix, Korea), and power supply (Siemens, Germany), enabling the device

to be connected to a computer for data acquisition. A special program is designed operating in manual and automatic operation modes to set initial length of sample, applied strain, frequency of loading, ratio of extension to relaxation and number of cycles.



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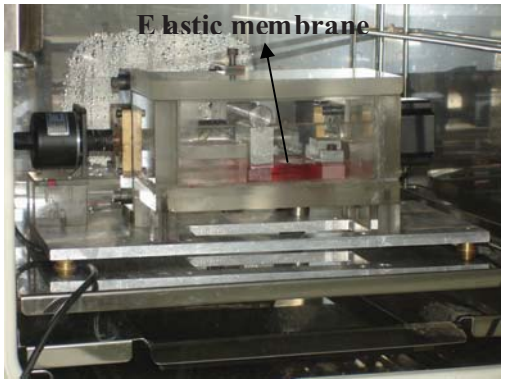

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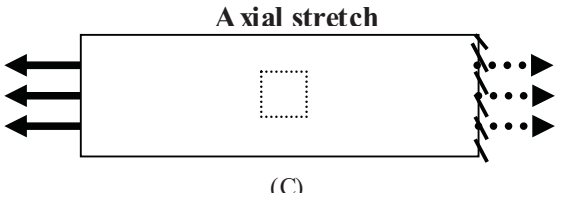


(C)

Figure 1: (A and B) Cyclic axial stretch device with electronic and mechanical units (C) schematic diagram of elastic membrane under loading.

The mechanical unit contains mobile and fixed parts. Mobile part includes step motor (Autonix, Korea), ballscrew (TBI, Taiwan), connector rod, encoder (Autonix, Korea), mobile and fixed grippers inside a tank filled by culture medium before test. The membrane is clamped into grippers. The mobile gripper stretches and releases the membrane by its displacement caused by the stepper motor through the ballscrew and the connector rod.

The uniformity of applied strain along the membrane is justified by measurement of stretch ratios along the collagen coated part of membrane for cell attachment (Figure 1C). Different locations of the membrane are stained and after application of loads with differing amplitude, the displacements of the stained locations are measured. The consequent strains are calculated and compared for different location along the coated region of the membrane. Results show maximum deviation less than 0.5% of applied strain for measured locations. This shows the uniformity of the strain along the membrane length as a proper assumption.

2.2 Cell Culture and Test Preparation

Human umbilical vein endothelial cells (HUVECs) obtained from National Cell Bank of Iran (NCBI-C554) are cultured according to the previously reported cell culture procedures [8, 23, 29]. The cells arecultured in essential basic growth medium DMEM+Ham's F12 (Gibco, USA) containing 20%fetal bovine serum (Seromed, Germany), 2mM L-glutamin (Gibco, USA), 50μg/ml heparin, 50μg/ml ECGS and 1% penicillin/streptomycin (Sigma, USA) [33, 34]. The cell culture procedure is done in a humidified 5% CO_2 incubator at 37°C. Medical grade silicon elastic membrane (0.25mm thick) obtained from Iran Polymer and Petrochemical Institute is used as the scaffold of cell culture. The central 6×6 mm region of a 50×15 mm silicone membrane is coated with 0.1 ml of 0.5 mg/ml collagen type ? (Sigma, USA) for a proper cell attachment [27, 35, 36]. Cells from the 2nd to 8th passages are transferred to the collagen coated region and incubated to allow firm attachment to the membrane [36, 37]. The membrane is then covered with fresh growth medium. After incubation overnight, the membrane is exposed to tensile cyclic loading by stretch of silicone membrane using the de-

signed cyclic axial stretch device.

2.3 Image Processing and Analysis

To compare morphology of endothelial cells before and after tests photomicrographs of the cultured endothelial cells are captured using an invert microscope (Zeiss ID03, Germany) and a digital camera (Sony DSC-W7, Japan). An image processing algorithms is applied to quantify morphological changes by calculation of topological parameters for resultant images from each test. Such algorithm includes design of a specific image analysis code in MATLAB 7.2 image processing toolbox. Firstly, a grayscale format of the captured RBG formatted image is produced. Then a gradient detection algorithm is applied on the grayscale image. To identify cell profiles properly, binary thresholding is performed on the gradient image by selecting its mean gray value as the threshold value. Finally a geometric filter is applied for selection of profiles with area greater than specific number of pixels to remove small remaining artifacts.

Topological analysis is performed to obtain *cell orientation angle* (θ) and *cell shape index (SI)*. The cell orientation is quantified by the angle of the long axis of the cell with the direction of stretching [27, 38]. The shape index (SI) as a measure of the degree of cell elongation, is calculated as $\left[(4\pi \times area)/perimeter^2\right]$, in which area and perimeter represent the total area and total perimeter of the cells in a selected cellular network region respectively [22, 30, 39]. The SI value of a circle is one and that of a straight line is zero.

2.4 Statistical Analysis

The tests are performed for different values of load amplitude, frequency and number of cycles and resultant topological parameters are obtained. For each variable, average and standard deviation values of resultant test parameters are calculated. To verify significance of effects of cyclic loading on cell morphology, multiple measurement analysis of variance (ANOVA) is performed to compare mean values of pre-test and after-test topological parameters for different test variables. The P value for each statistical analysis is calculated. The correlation between shape index (SI) and orientation angle (θ) as indicators of cell morphology is investigated by calculation of correlation coefficients.

2.5 Experimental Protocol

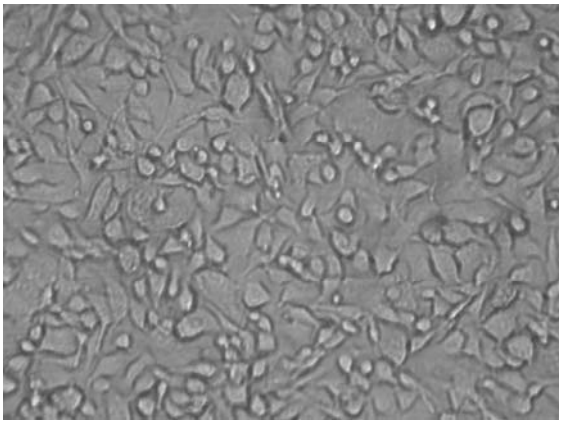
To investigate effects of mechanical loading on cell morphology, different sets of experiments are performed with variation of strain amplitude, frequency and number of cycles (test duration). An additional experiment is performed to investigate a different load waveform on cell morphology. To study effects of cyclic loading on dysfunctioning cells, an experiment is carried out on endothelial cells in a disturbed environment with an elevated PH value.

After cell culture procedure, cells are transferred to the silicon membrane. The membrane is placed into test grippers. The tank is filled with culture medium for maintaining physiological functions of cells, and the loading apparatus is placed into an incubator for the operation. HUVECs seeded on collagen-coated silicon membrane are subjected to cyclic stretching with defined mechanical conditions set by the software. The images of cell network seeded on the membrane before and after the test are captured and processed by the designed image processing code to calculate topological parameters. To obtain validated results four wells of cultured cells are evaluated from each group and for each test, four image zones are processed from the obtained image. For each zone, four randomly chosen fields of the image are analyzed and the average values and standard deviations of topological parameters are calculated [40]. The tests are repeated to obtain statistically validated results. Morphological alterations of endothelial cells are quantified based on the statistically significant change in topological parameters with different test variables.

3 Results

Experiments are performed based on change in three major parameters: strain percentage, loading frequency and number of cycles (test dura-

tion). Figure 2 shows microscopic views of the cells before and after a typical test respectively. The test conditions comprise 10% strain with frequency of 1Hz, number of cycles of 36000 (10h duration), and the ratio of stretch to relaxation of one.



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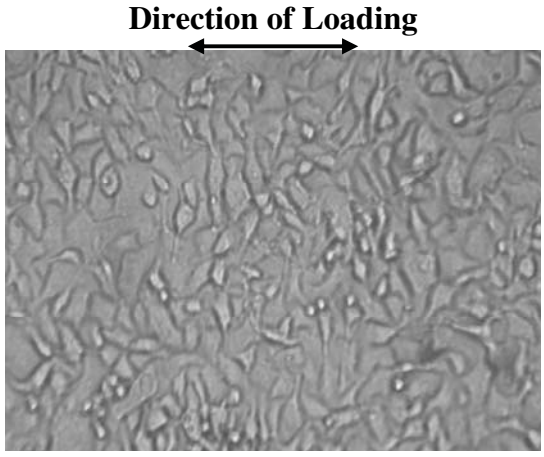
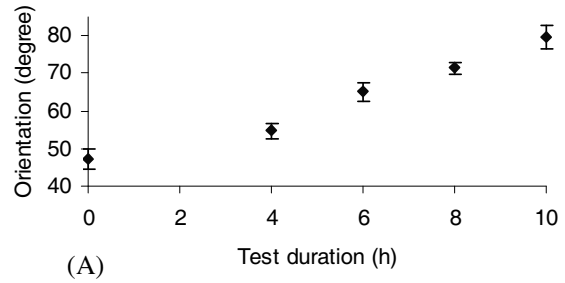


(B)

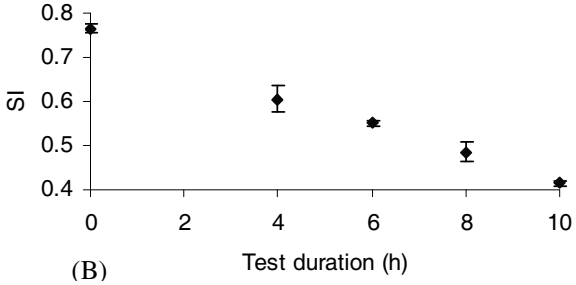
Figure 2: HUVECs arrangement on collagen coated silicon membrane (A) before and (B) after cyclic strain test. (10% strain, 1Hz frequency, 10h duration).

Topological analysis of cell images before and after the test shows significant morphological changes by application of cyclic stretch. Calculation of cell orientation parameter (θ) shows that while before the tests cells are arranged randomly on the membrane, the cyclic tensile loading leads to an average cell alignment of nearly 80° (oblique) from the axial direction of loading, along the direction of minimal deformation of elastic membrane. The reason for cellular alignment is to minimize the strain energy on cells and to reduce the stretch of microtubules and other cytoskeletal networks [32, 38, 41, 42]. The SI value decreases after test from 0.765 to 0.415.

Effects of number of cycles: Results of tensile test and the following image processing of cellular images before and after tests with different number of cycles indicate that increase of test duration (number of cycles) lead to more organized cellular network. Figure 3 shows the trend of change in SI and θ parameters by change in test duration values for load frequency of 1 Hz and strain amplitude of 10%. Results show an overall 69% increase in orientation angle and 46% reduction in shape index by increase of number of load cycles up to ten hour test duration.



(A)



(B)

Figure 3: Effect of differing number of cycles (test duration) on (A) cell orientation angle and (B) shape index (SI) for strain 10% and frequency 1Hz.

Effects of strain amplitude: Results of test with load frequency of 1 Hz, test duration of six

hours and differing strain amplitude are shown in Figure 4. Elevation of strain amplitude for constant test duration and load frequency causes reduction in SI and elevation of θ. Results of tests with different strain amplitudes indicate marked change in morphology of cells from no load condition to 47% reduction of SI and 70% elevation of θ for maximum strain amplitude.

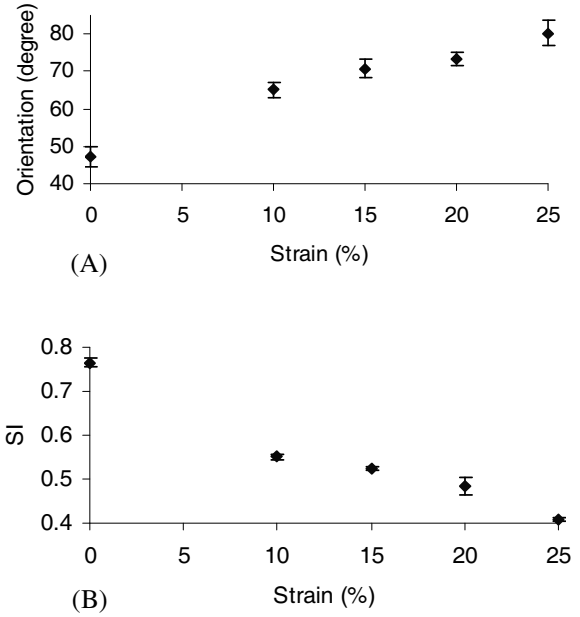


Figure 4: Effect of differing strain values on (A) cell orientation angle and (B) shape index (SI) for frequency 1Hz and test duration 6h.

Effects of load frequency: Elevation of loading frequency leads to decrease of shape index and increase of orientation angle. Results are presented in Figure 5 for 10% strain, six hour test duration and load frequencies of 1 and 1.5 Hz. Results indicate 28% decrease in SI and 29% increase in θ from no load condition to tests with 1.5 Hz frequency.

Statistical analysis: Table 1 shows average and standard deviation values of shape index (SI) and orientation angle (θ) for different strain amplitudes, frequencies and test durations.

Multiple measurement analyses of variance (ANOVA) indicate significant differences between average values of topological parameters of tests before and after loading with different values of variables (test duration, frequency, and amplitude) with P values smaller than 0.05.

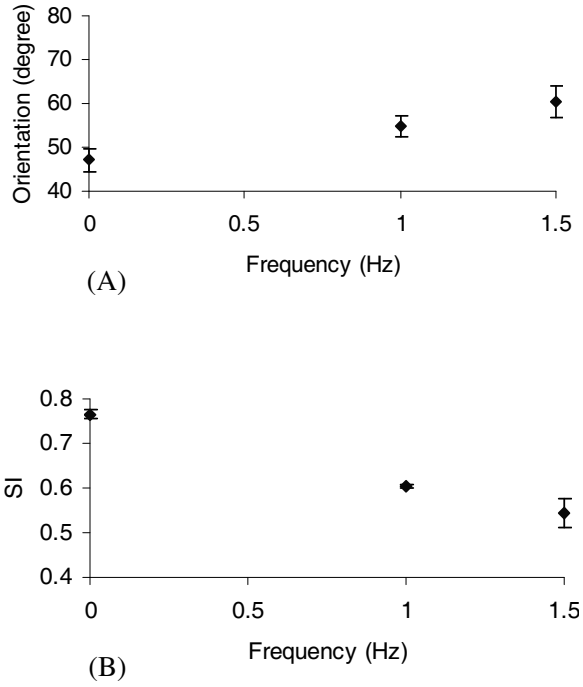


Figure 5: Effect of differing loading frequency on (A) cell orientation angle and (B) shape index (SI) for strain 10% and test duration 6h.

The correlation coefficients between SI and θ are calculated for different sets of experiments. The correlation coefficients are equal to -97.4% for test duration experiments, -.99.5% for strain amplitude tests, and -99.9% for load frequency experiments. Results indicate strong correlation between increase in orientation angle and reduction of shape index by change in experimental variables. The high correlation indicates that both parameters are indicators of morphological changes by loading.

Effects of strain waveform: In addition to parameters such as load amplitude and frequency, the rate of extension and relaxation of membrane during the load cycle period which determines the strain profile is considered. For results presented in Table 1 such ratio is equal to one indicating a harmonic strain wave. Results show different SI

Table 1: Cellular shape index (SI) and orientation angle (θ) values for different strain amplitudes, test durations and frequencies.

Morphological Parameters								
Strain (6h, 1HZ)	SI	θ (degree)	Test durations (10%, 1HZ)	SI	θ (degree)	Frequency (10%, 6h)	SI	θ (degree)
0%	0.765 (Std=0.01)	47.1 (Std=2.6)	0h	0.765 (Std=0.01)	47.1 (Std=2.6)	0 Hz	0.765 (Std=0.01)	47.1 (Std=2.6)
10%	0.55 (Std=0.005)	65 (Std=2.4)	4h	0.605 (Std=0.003)	54.8 (Std=2)	1 Hz	0.55 (Std=0.005)	65 (Std=2.4)
15%	0.524 (Std=0.005)	70.7 (Std=4.1)	6h	0.55 (Std=0.005)	65 (Std=2.4)	1.5 Hz	0.525 (Std=0.03)	66.7 (Std=3.5)
20%	0.485 (Std=0.002)	73.2 (Std=3.3)	8h	0.486 (Std=0.002)	71.3 (Std=1.7)			
25%	0.408 (Std=0.005)	80.15 (Std=2.1)	10h	0.415 (Std=0.006)	79.6 (Std=3.3)			

and θ values with differing extension to release ratio. For the load frequency of 1HZ, strain amplitude of 10% and test duration of 6 hours, the resultant parameters for the extension to relaxation time ratios of one and 3/7 are significantly different by t-test analysis with P value smaller than 0.02. The ratio of stretch to release in the altered load wave is comparable to the ratio of systolic to diastolic pressure portions for a typical cardiac cycle. This ratio has also been used for study of CAMP production in endothelial cells [20]. Results show 9.7% decrease in SI and 10.8% increase in θ values for the altered load wave compared to harmonic wave. This indicates that not only the strain wave amplitude determines the cell morphology, but also the strain wave form is a determinant. Different pressure waveforms in different physiological and pathological situations influence the morphology of endothelial cells and their functions.

Effects of PH value: Endothelial cell are remodeled by application of cyclic tension through increase in orientation angle and decrease in shape index. Such remodeling is compatible with function of endothelial cells. In addition to remodeling the pathology of endothelial cells can also be studied by change in topological parameters. Such event can be modeled by application of cyclic load in a harsh environment.

An alteration in PH value is examined as an environmental disturbance, resulting in malfunction of cells. Experiments are performed for samples under cyclic loading for 10 hours with 10% strain, frequency of 1 Hz and differing PH values of medium. Resultant topological parameters for normal PH (7.4), elevated PH (8.2) with applied load and no load conditions are compared with pre-test parameters. The PH value of 8.2 leads to disturbance of the function of cultured cells which may alter the remodeling of cells. Microscopic views of cells show that the number of dead cells is not increased significantly after elevation of PH value. This is also shown by remodeling of cells after application of loading. However, the degree of remodeling is different due to the fact that the cells in disturbed environment are not functioning properly.

Table 2 shows the resultant mean values of topological parameters for normal and disturbed conditions. The increase of PH without application of cyclic load leads to 16% increase of SI and 10% decrease in θ, describing disturbed cellular organization. Application of cyclic tensile load on cells in both conditions causes cellular alignment, however such alignment is markedly more significant in normal condition compared to disturbed condition. While loading causes 46% reduction in SI in normal condition, such value is significantly lower in elevated PH condition with 7% reduction in SI compared to cells incubated in elevated PH without applied load.

Results confirm lack of remodeling of endothelial cells in elevated PH environment defined by reduction of θ and elevation of SI. The high value of PH in medium results in loss of cell alignment compared to pre-load situation.

4 Discussion

A new apparatus is designed and manufactured for imposing cyclic tensile loads on cultured cells and soft tissues suitable for the study of cellular responses to physiological and pathophysiological loading conditions. The results obtained from the use of this new device are applicable in elucidation of the mechanisms of cellular regulation and adaptation to mechanical forces *in vivo* such as blood pressure.

Application of new design leads to evaluation of HUVEC alignment and elongation by cyclic stretching. While the cells are proliferated in random directions, applying cyclic load causes alignment of cells in a direction which strain energy is minimized. Shape index reduction is due to the elongation of cells by cyclic loading together with reduction of cell area and increase in cell perimeter to minimize the strain energy [30, 43]. Elevation of cell perimeter results in an elongated cell interface with adjacent cells, leading to altered physical and chemical environments of elongated cells. By increase of cell to cell interface area, functional parameters such as cell adhesion, membrane tension, and cytoskeletal struc-

Table 2: Cellular shape index (SI) and orientation angle (θ) values for normal PH and elevated PH with strain 10%, frequency 1 Hz and test duration 10h.

Morphological Parameters				
Topological Parameters	Before Test	After Test Normal PH	Static with Elevated PH	After Test Elevated PH
SI	0.77	0.42	0.89	0.83
θ (degree)	47.1	79.6	42.3	41.3

ture are affected and altered. Through such mechanisms cellular functions are adjusted for differing physiological conditions resulting in cellular adaptation and remodeling. For critical values of SI and/or θ endothelial damage and dysfunction are expected.

Elevation of number of strain cycles, strain amplitude and loading frequency results in statistically significant reorientation and elongation of cells. Alteration of variables leads to different cell alignment. Elevation of load frequency, amplitude, and duration results in different degrees of cellular alignment, described by reduced shape index and increased orientation angle with non-linear variation. By increase of variables shape index is reduced with a non-linear trend toward an asymptote, i.e. further alteration of test variables does not lead to further alignment and remodeling of the cells. This can be assumed as the threshold of cell damage, beyond which the cell is not capable of remodeling by intensified loading conditions.

The cell injury might also initiate by disturbed environment, leading to dysfunctioning of cell remodeling. Application of cyclic load in an environment with elevated PH –as an example of systemic disturbance- results in dysfunctioning of cells with overall reduction of orientation angle and increase of shape index, disorder of cell network and cell injury. Cells under such conditions are not capable of proper remodeling by applied tensile load.

Excessive loading either in the case of high load pulse amplitude or long duration of loading may contribute to endothelial dysfunction and injury. In arterial system these situations are correspondent to hypertension and aging. Geometrical complexities such as arterial bifurcations cause further stress concentration. Endothelial cells exposed to hypertension or aging minimize the pathological consequences by structural adaptation and remodeling, but in severe situations specially in critical locations such as arterial junctions the endothelial damage is unavoidable resulting in plaque formation and atherosclerosis; and clinical consequences such as plaque rupture and eventually stroke.

It is concluded that tensile loading of endothelial cells influences cell morphology and alignment a mechanism for structural regulation, functional adaptation and remodeling. The scope of this study will be expanded to study of effects of cyclic loading on cytoskeletal fibers of cells such as actin filaments and cell proliferation. In addition to mechanical parameters, effects of parameters such as cell confluence, cell age and membrane material on cellular morphology are other scopes of study.

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