Study of Tensile Cyclic Loading on Morphology of Endothelial Cell Line in Culture Medium: A Fractal and Topological Comparison.

Samira Amini[1], Mohammad Tafazzoli-Shadpour[2], Nooshin Haghighipour[2], Mohamad Reza Hashemi Golpayegani[2], Mohammad Ali Shokrgozar[3]
[1]*Faculty of Mechanical Engineering, Ecole Polytechnique of Montreal, Montreal, Quebec, Canada*
[2]*Faculty of Biomedical Engineering, Amirkabir University of Technology (Tehran Polytechnic), Tehran, Iran*
[3]*National Cell Bank of Iran, Pasteur Institute, Tehran, Iran*

INTRODUCTION

Endothelial layer (the innermost part of blood vessels) damage might result in accumulation of blood components inside the arterial wall and consequently plaque formation. Hemodynamic forces are major indicators of the initiation and localization of endothelial injury (16). The endothelium is exposed to stresses caused by blood pressure and flow. Such stresses regulate the structure and function of blood vessels(14); however, their critical values contribute to endothelial damage (8). The pulsatile blood pressure causes pulsatile circumferential tensile stress on the arterial wall media with the maximum value on the endothelial layer(4). The degree of cell morphology in the case of excessive stress is an indicator of endothelial damage and is considered as an adaptive response to mechanical stimuli (4, 9). Effects of cyclic loading on endothelial morphology have been quantified by topological parameters (7, 8, 14). Although such parameters are indicators of morphological changes, the overall change in cellular morphology is not evaluated by them.

This study fractal geometry is used in morphology quantification of cultured endothelial cells. There is a strong relationship between complexity of the shape of objects and their fractal dimension values, however, the analytic Euclidean geometry can not quantify such complexity (12, 13). There are several ways of measuring fractal dimensions but the most common and general techniques are 'box-counting' and "Sand box" method (1, 12, 13). In addition to fractal dimension *lacunarity* of the object can be obtained by fractal analysis. Lacunarity is a measure of the heterogeneity of structure (12). Different images with the same complexity result in an identical fractal dimension value. In such cases lacunarity parameter distinguishes between images. Fractal analyses have been used in study of cell morphological changes (5, 10, 15). The scope of fractal analysis has not been extended to the study of morphological changes of cells by application of cyclic loading. Studies have shown variation of fractal dimension values in the range of 1.1-1.5 for cell morphological images (5, 6, 10, 15). Therefore in such studies changes in variables have led to small but statistically significant change of fractal related parameters.

The current study applies fractal analysis in quantification of morphological changes of cultured endothelial cells caused by mechanical loading with differing load amplitude and duration. Comparative study is performed to investigate correlation of fractal and topological analyses.

MATERIALS AND METHODS

Cell culture and test preparation

Human umbilical vein endothelial cells (HUVEC) are obtained and cultured according to the previously reported cell culture protocols (8). The cells are cultured in essential basic growth medium DMEM+Ham's F12 (Gibco) containing 20% fetal bovine serum (Seromed), 2mM L-glutamin (Gibco), 50µg/ml heparin, 50µg/ml ECGS and 1% penicillin/streptomycin (Sigma). The cell culture procedure is done in a humidified 5% CO_2 incubator at 37°C. The cultured cells are transferred on a medical grade silicon membrane coated with collagen type I for a proper cell attachment (2, 7).

Test apparatus

A specific tensile test device (Figure 1) is used for the application of axial cyclic strain on cultured cells with the ability of variation of frequency and amplitude.

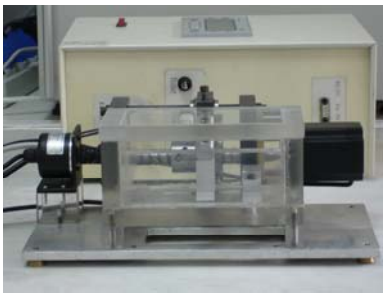

Figure 1: cyclic strain exertion apparatus

The device enables operation in an incubator limiting the size, weight and device materials. The strain frequency is in the range of 1-3 Hz and the strain value in the range of 0-25 percent.

Image analysis

To apply fractal analysis on images different image processing methods can be used. In this study

skeletonized image method is used due to higher reliability as previously reported (12).

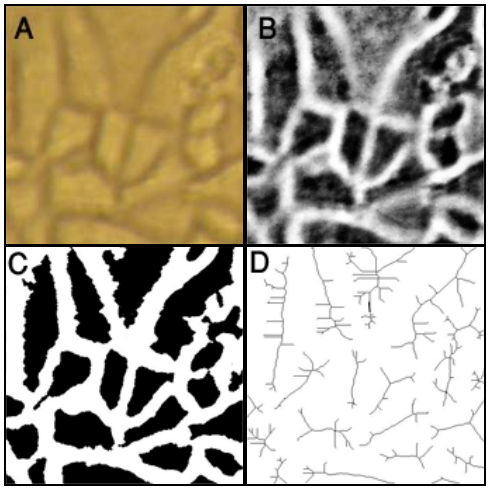

Figure 2: A: Original Image, B: Grayscale of the Original Image, C: Binary Thresholding Image, D: The Skeleton of cell network

Images of the cultured endothelial cells are captured using an invert microscope (Zeiss ID03) and a digital camera (Sony DSC-W7) before and after tests to study effects of cyclic loading on cell morphology. The images are processed using a computer-assisted image analysis code designed in MATLAB 7.2 (R2006a) image processing toolbox.

The results of image processing on a typical sample of human umbilical vein endothelial cells are shown in Figures 2. After application of gradient detection algorithm to the grayscale of original image (Figure 2-B), binary thresholding is performed on the gradient image by selecting its mean gray value as the threshold value (Figure 2-C). This allows a proper identification of the cell profiles. To remove small remaining artifacts from the resulting binary image, a geometric filter is applied to select profiles with area greater than specific number of pixels. By using binary thinning procedures the skeleton (Figure 2-D) of the filtered image is obtained.

Fractal analysis

Fractal dimension

To measure the fractal dimension of cellular structures two major methods are used. The first method is the box-counting method which measures the distances between points on the border of the binary image. It is based on the concept of covering the border (6, 12, 14). Mass-related method is another approach for measuring fractal dimension which counts border pixels located in a concentric discs or boxes of various sizes which are randomly centered on the image border pixels within the radius of gyration of the image (12). The resultant fractal dimensional parameter is called 'sand-box' or the cumulative mass dimension (1, 12).

In current study fractal dimension of the binary skeleton is estimated using both methods based on the designed code in MATLAB and results of two methods are compared.

Lacunarity

If different images lead to an identical fractal dimension value due to their similar complexity, fractal dimension can not distinguish between such images (12). For a proper analysis, measurement of lacunarity distinguishes such objects. The procedure used in current study to measure the lacunarity is to calculate the normalized standard deviation of the number of pixels in each measuring element (12). The code was designed in MATLAB.

Topological analysis

In addition to fractal analysis, topological analyses are performed for evaluation of change in shape index (SI) as an indicator of cell elongation, and orientation angle (θ) of cellular network by cyclic loading. The binary image of the cell images is processed to calculate the topological parameters. The mean shape index of the image containing number of cells is defined as $SI = 4\pi A_{total} / P_{total}^2$. In which A_{total} and P_{total} represent the total area and total perimeter of the cells in a selected cellular network region respectively (3, 17). Cell orientation parameter (θ) is defined as the angle (in degrees) between the direction of stretching and the major axis of the ellipse which covers the cell geometry. Similar to calculation of fractal parameters a specific code is designed in MATLAB to estimate the topological parameters.

Experiment Protocol

Two sets of experiments are performed based on independent variables including strain amplitude and test duration (number of load cycles).The cultured cells are stretched by applying cyclic strain through the tensile apparatus on the silicone membrane. The images of cultured cells before and after the test are captured and processed to obtain fractal and topological parameters. For each test, the images of four major zones of the cells are distinguished and for each zone four randomly chosen fields of the image are analyzed (5). The resultant fractal and topological parameters are calculated. Statistical t-test is performed to investigate significance of change in mean values of resultant parameters by alteration of independent variables. The correlation between resultant topological and fractal parameters are obtained for validation of fractal analysis.

RESULTS AND DISCUSSION

The first set of experiments is performed using 10% strain and frequency of 1 Hz with differing number of cycles. Samples are under cyclic loading for 4, 6, 8, and 10 hours. Figure 3 shows images of cellular samples before and after test with 10 hours duration.

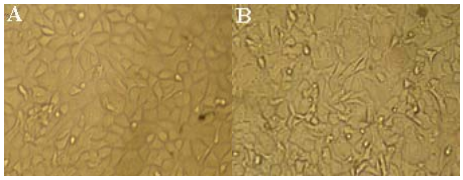

Figure 3: cell morphology before (A), after (B) the test (10% strain, 1 Hz frequency and 10 hours duration)

The second set of experiments is performed with a different strain amplitude equivalent to 20% and the same frequency for 4 and 6 hours. Cultured endothelial cells elongated and realigned over time in response to both 10% and 20% cyclic strain with 1 Hz frequency.

Fractal dimension analysis

The fractal dimension values (D) of the cellular images for both sets of experiments are calculated. Figure 4 indicates the average fractal dimension of HUVEC in response to 10% cyclic strain with 1 Hz frequency. The results of t-test show that there are significant differences between mean values of results for all test groups. The calculated P value for comparison of before test and after test groups are smaller than 0.05 Results confirm the significant effect of cyclic loading on cellular morphology. The comparative results are presented in Figure 4.

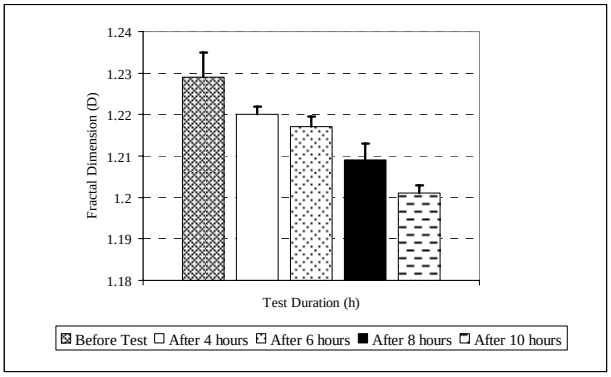

Figure 4: Comparative fractal dimensions in response to 10% cyclic strain with 1Hz frequency.

The mean fractal dimension of the image samples before performing the test is 1.229 (std=0.006) with low variations among different samples. After the test, reduction in fractal dimension (D = 1.201, std=0.002) is observed. The reduction in fractal dimension indicates less complexity in the image and more order of cellular network which is correlated with the orientation of cells after application of cyclic loading. The alignment of cells after cyclic loading with fractal analysis describes the adaptation of endothelial cells to mechanical stimuli.

Results of fractal dimension value for strain amplitude of 20% with four and six hour durations show same trend with P values smaller than 0.01. The mean fractal dimension evaluated before performing the test is 1.24. After applying 4 and 6 hour cyclic strain on the HUVEC, the fractal dimension decreases to 1.218 and 1.21 respectively (data not shown).

The result of correlation analysis of sand box method versus box-counting method for the results indicate a relationship between them with a correlation coefficient equal to 0.95 which is compatible with published data for fractal analysis of cell morphology(12). The high value of correlation coefficient between two fractal methods confirms fractal analysis as a proper tool for comparison of cell morphology before and after test.

Lacunarity Analysis

Figure 5 indicates the average and standard deviation of lacunarity value of HUVEC images in response to 10% cyclic strain with 1 Hz frequency. T-tests results show significant difference with P values smaller than 0.02.

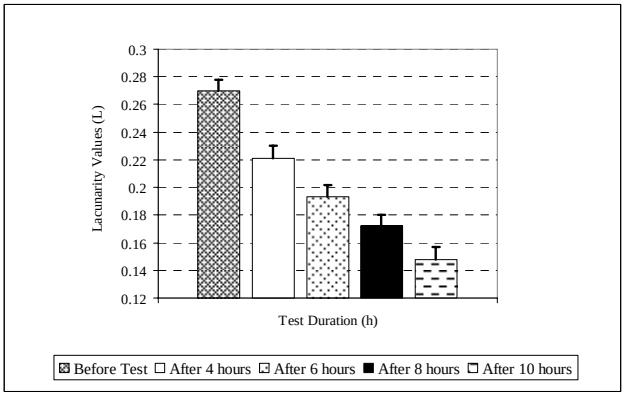

Figure 5: Comparative lacunarity values in response to 10% cyclic strain with 1Hz frequency.

The mean lacunarity value of the image samples before the test is 0.27 with low variations among different samples (Std = 0.008). After the test, although the reduction in fractal dimensions mean value were small, lacunarity value decrease further more (L = 0.148) indicating reduction in the non-uniformity (heterogeneity) of HUVEC structure during the test and more organized structure in image which is correlated with the orientation of cells after application of cyclic loading. Hence, lacunarity can be considered as a measure of the non-uniformity, potentially useful to detect situations in which spatial

distribution of the endothelial cells pattern is altered by cyclic strain.The same trend is observed for change in lacunarity values for strain amplitude of 20% (data not shown). Statistical analysis show significant difference with P value smaller than 0.01.

Topological Analysis

Topological analysis of cell shape index (SI) shows a statistically significant change by increase of test duration with 10% cyclic strain and 1 HZ frequency. Results indicates the reduction of cell shape index with the test duration which is compatible with published data(3, 11, 17) (data not shown).

Cell orientation angle shows an increasing trend (data not shown) which is compatible with published data for endothelial cell morphology under strain(11).

Significant statistical correlations between resultant parameters of topological and fractal analyses in this study verify use of fractal analysis in study of endothelial cell morphological change under mechanical loading. The result of correlation analysis on endothelial cells under 10% strain with 1 Hz frequency show that fractal dimension is significantly correlated with both the cell shape index (SI) (R=0.977) and cell orientation angle (θ) (R=-0.983). The relationship between fractal dimension and cell shape index and orientation angle for 20% strain with 1Hz frequency is also evaluated. The correlation coefficients are equal to 0.989 and -0.903 respectively confirming that fractal analysis can be used as a compact descriptor of the shape of the endothelial cells under cyclic strain in vitro.

CONCLUSION

The use of fractal geometry in biology and medicine is well established. Fractal related parameters are considered as quantitative morphological descriptors. Due to the strong relationship between structure and function in biology fractal analysis can be used to study function and malfunction of cells based on their morphological descriptions. Endothelial cells are prone to cyclic stretch due to pulsatile blood pressure and their injury is the main source of cardiovascular pathology. Endothelial function and pathology can be monitored by their morphology. Fractal analysis is a quantitative method of study of morphological change and consequently functions of endothelial cells. This study quantifies change in cultured endothelial cell morphology under stretch by cyclic loading. Results indicate statistically significant reduction in fractal dimension of well functioning cells by application of cyclic loading describing alignment of cells for optimized performance. The strong correlations of resultant parameters obtained from fractal analysis and topological analysis verify the use of fractal analysis in study of cellular response to mechanical stimuli. It is shown that fractal analysis can be used as a suitable method in tissue engineering for evaluation of cell morphology.

REFERENCES

1. Caserta, F., H. E. Stanley, W. D. Eldred, G. Daccord, R. E. Hausman, and J. Nittmann. 1990. Physical mechanisms underlying neurite outgrowth: A quantitative analysis of neuronal shape. Phys Rev Lett 64:95-98.
2. Cevallos, M., G. M. Riha, X. Wang, H. Yang, S. Yan, M. Li, H. Chai, Q. Yao, and C. Chen. 2006. Cyclic strain induces expression of specific smooth muscle cell markers in human endothelial cells. Differentiation 74:552-61.
3. Dieterich, P., M. Odenthal-Schnittler, C. Mrowietz, M. Kramer, L. Sasse, H. Oberleithner, and H. J. Schnittler. 2000. Quantitative morphodynamics of endothelial cells within confluent cultures in response to fluid shear stress. Biophys J 79:1285-97.
4. Fukushima, S., H. Fujioka, and K. Tanishita. 2003. Shear stress distribution on the surface of endothelial cells during flow-induced morphological remodeling. JSME International Journal, Series C: Mechanical Systems, Machine Elements and Manufacturing 46:1275-1283.
5. Guidolin, D., A. Vacca, G. G. Nussdorfer, and D. Ribatti. 2004. A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay in vitro. Microvasc Res 67:117-24.
6. Jelinek, H. F., and E. Fernandez. 1998. Neurons and fractals: how reliable and useful are calculations of fractal dimensions? J Neurosci Methods 81:9-18.
7. Joung, I. S., M. N. Iwamoto, Y. T. Shiu, and C. T. Quam. 2006. Cyclic strain modulates tubulogenesis of endothelial cells in a 3D tissue culture model. Microvasc Res 71:1-11.
8. Lelkes, P. I. 1999. Mechanical Forces and the Endothelium, vol. six. Harwood academic publishers, New York.
9. Malek, A. M., and S. Izumo. 1996. Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. J Cell Sci 109 (Pt 4):713-26.
10. Orlowski, D., Z. Soltys, and K. Janeczko. 2003. Morphological development of microglia in the postnatal rat brain. A quantitative study. Int J Dev Neurosci 21:445-50.
11. Owatverot, T. B., S. J. Oswald, Y. Chen, J. J. Wille, and F. C. Yin. 2005. Effect of combined cyclic stretch and fluid shear stress on endothelial cell morphological responses. J Biomech Eng 127:374-82.
12. Smith, T. G., Jr., G. D. Lange, and W. B. Marks. 1996. Fractal methods and results in cellular morphology--dimensions, lacunarity and multifractals. J Neurosci Methods 69:123-36.
13. Smith, T. G., Jr., W. B. Marks, G. D. Lange, W. H. Sheriff, Jr., and E. A. Neale. 1989. A fractal analysis of cell images. J Neurosci Methods 27:173-80.
14. Stamenovic, D., and N. Wang. 2000. Invited review: engineering approaches to cytoskeletal mechanics. J Appl Physiol 89:2085-90.
15. Takeda, T., A. Ishikawa, K. Ohtomo, Y. Kobayashi, and T. Matsuoka. 1992. Fractal dimension of dendritic tree of cerebellar Purkinje cell during onto- and phylogenetic development. Neurosci Res 13:19-31.
16. Wang, J. H., P. Goldschmidt-Clermont, J. Wille, and F. C. Yin. 2001. Specificity of endothelial cell reorientation in response to cyclic mechanical stretching. J Biomech 34:1563-72.
17. Yamaguchi, T., Y. Yamamoto, and H. Liu. 2000. Computational mechanical model studies on the spontaneous emergent morphogenesis of the cultured endothelial cells. J Biomech 33:115-26.