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Effects of Stiffness on Endothelial Cells Behavior

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***Abstract*—**Endothelium proliferation is an important process in cardiovascular homeostasis and can be regulated by extracellular environment, as cells can actively sense mechanical environment. In this study, we evaluated endothelial cell proliferation on PDMS/alumina (Al_2O_3) composites and pure PDMS. The substrates were prepared from pure PDMS and its composites with 5% and 10% Al_2O_3 at curing temperature 50˚C for 4h and then characterized by mechanical, structural and morphological analyses. Higher stiffness was found in the composites compared to the pure PDMS substrate. Cell proliferation of the cultured bovine aortic endothelial cells on substrate materials were evaluated via Resazurin assay and 1, 1'-Dioctadecyl-1, 3, 3, 3', 3'-Tetramethylindocarbocyanine Perchlorate-Acetylated LDL (Dil-Ac-LDL) cell staining, respectively. The results revealed that stiffer substrates promote more endothelial cells proliferation to the less stiff substrates. Therefore, this study firmly hypothesizes that the stiffness elevates endothelial cells proliferation.

***Keywords*—**Bovine aortic endothelial cells, extra cellular matrix, proliferation, stiffness.

I. Introduction

BLOOD vessels as a scaffold provide special extra cellular matrix for endothelial cells. The entire vascular system has been lined by a single layer of endothelial cells which is separated from the underlying layers by connective tissue [1]-[2]. Normally, endothelial cells sit on collagen which acts as a substrate. Collagen deposition, hyper-proliferation and mineralization by calcium and phosphorous induced by risk factors like aging, atherosclerosis, diabetes mellitus and renal disorders elevate vessel stiffening [3], [4]. Alive cells follow a fact called "stiffness sensing" which means they can sense their outer environment and respond to mechanical resistivity of extracellular matrix. Endothelial cell cytoskeleton can recognize substrate mechanical properties through receptors [5]-[8]. Therefore, availability of proper stiffness profiles can elevate the feasibility of the biophysical studies on cell-substrate interaction [9]. In addition to its biocompatibility, simple fabrication, optical transparency, tunable flexibility, gas permeability, high oxidative and thermal stability and inexpensiveness have made this material attractive in cell biology [10]-[12]. From the many available means of controlling substrate-stiffness α-alumina (Al2O3) as a well-known biocompatible and stable ceramic [13] was chosen to control the substrate stiffness in this present study. Hence, this study introduces an in vitro method to show the profound role of vascular stiffness on endothelium responds in terms of adhesion, proliferation and cell morphology.

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II. Procedure

A. Preparation of Different Stiffness of PDMS Substrate

PDMS/Al2O3 composites were prepared first, by mixing PDMS gel (SYLGARD 184 Silicon Elastomer Base purchased from Dow Corning) with 5 and 10 percent of Al2O3 (supplied by Sigma) as a second phase ceramic particle using planetary ball mill (Retsch, PM200). Then a curing agent (supplied by Dow Corning along with SYLGARD 184 Silicon Elastomer) with proportion of 1:10 (v/v) (curing agent: PDMS gel) was added to PDMS/Al2O3 mixture at room temperature.

B. Stiffness Measurement

Mechanical properties of pure PDMS elastomer and its composites with different concentrations (5 and 10 wt%) of Al2O3 were characterized following the ASTM standard—ASTM D 412 test standard for vulcanized rubber and thermoplastic elastomers 16 using a universal testing machine, (Instron MicroTester, # 5848) at a constant crosshead speed of $1mm.min^{-1}$.

C. In vitro Assay

In vitro cell attachment and proliferation were determined by the Resazurin assay. This test was used to show cell metabolism. Isolated bovine aortic endothelial cells were grown in completed medium (M200) (Gibco#M200-500). Then, $1*10^4$ in passage 9 were used to seed on substrates with different stiffness which were coated on 100µl fibronectin (Sigma- Aldrich, # F1141).

Resazurin assay was performed at four time points, namely day 3, day 6, day 12, and day 18. 10% Resazurin solution were prepared at PBS. In the selected time points, medium was discarded and cells were washed with pre-warmed PBS and then incubated at 37ºC for 4h.

After incubation time, 100µl of resazurin solution were taken for absorbance measurements at 570nm and 600nm to determine the metabolism and proliferation rate of endothelial cells using the following (1):

$$\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{RED})\lambda_1 A^o\lambda_2 - (\varepsilon_{RED})\lambda_2 A^o\lambda_1}\times 100$$

where,

ε_{OX} = molar extinction coefficient of Resazurin oxidized form (BLUE)

ε_{RED} = molar extinction coefficient of Resazurin reduced form (RED)
A = absorbance of test wells
A° = absorbance of negative growth control well
λ_1 = 570nm
λ_2 = 600nm

III. Results

A. Stiffness Measurement

It was observed that Young's modulus was elevated with the addition of Al2O3 to PDMS. Stress-strain behavior of the composites was compared with the pure PDMS elastomer and the results are shown in Fig. 1.

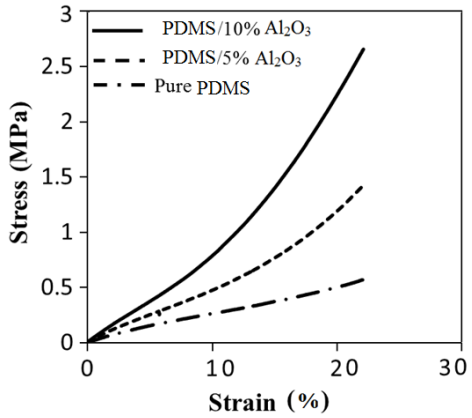

Fig. 1 Stress vs. strain behavior of PDMS /Al2O3 composites compare to the pure PDMS elastomer

As a result of direct relation between young modulus with stiffness, the trend of elevation in stiffness was exactly same with young modulus (Table I).

TABLE I
Young's Modulus and Stiffness Variation in PDMS Membrane and PDMS/Alumina Composites

Sample code	PDMS (wt %)	Al_2O_3 (Wt %)	Young's modulus (MPa)	Stiffness (N/m)
Pure PDMS	100	0	0.326±0.008	2.4
PDMS/5% Al_2O_3	95	5	0.459±0.02	3.5
PDMS /10% Al_2O_3	90	10	1.083±0.01	8.1

B. Cell Adhesion Assay

Cells showed a reasonable proliferation on substrates. Endothelial cells behavior for days 3, 6, 12, and 18 has been shown in Fig. 2. As stiffness were enhanced through adding alumina to PDMS, cell adhesion and proliferation were increased, since the highest absorbance in resazurin test belongs to PDMS/10% Alumina and pure PDMS has the lowest absorbance.

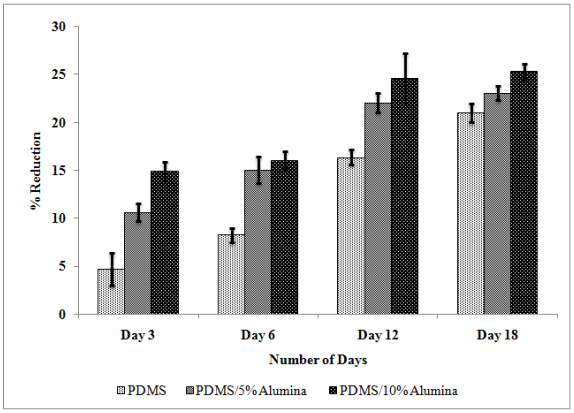

Fig. 2 % Reduction of Resazurin as a function of adhesion and proliferation of endothelial cell cultured on different composites during various time points

IV. Discussion

Endothelial cells are lined on collagen as an extracellular matrix in blood vessel [4]-[6]. Collagen deposition, excessive collagen proliferation, calcium and phosphorous mineralization of collagen arrays which happen in some disorders are stiffness inducers in blood vessel. On the other side, cells can sense substrate properties and respond to it through their receptors. Therefore, this study aimed to evaluate regulation of endothelial cells in respond to stiffness of substrate as extracellular environment.

Stiffness has impact on endothelial cells proliferation as its stiffness elevates. This issue was studied through preparation of different stiffness from PDMS through adding 5% and 10% Alumina [13], [14]. Therefore, PDMS /10% Al2O3 composite shows stiffer substrate of 1.1 MPa compared to pure PDMS which shows lowest stiffness of 300 KPa. It was observed because of the strong interfacial bonding between the alumina particles and PDMS matrix. Manipulation in Al2O3 concentration can alter the stiffness of substrate; hence, it might be a potential scaffold for the studies on the effects of stiffness on cell responds in terms of attachment, proliferation and morphology.

The properties of scaffold in terms of topography and mechanical properties can affect cell responds [15]. Moreover, researchers have confirmed that substrates rigidity depend on substrate stiffness can affect cells behavior [16]. These findings later on approved by studies done on fibroblasts. Fibroblast cells represented high tempt for growing on stiff substrates. Endothelial cells revealed stress fiber and well spread shape on stiff substrate [9].

Consequently, cells respond to extra cellular matrix by their cytoskeleton, which sense the substrate forces, and modify their biological and mechanical functions [4]. In this study, time-dependent characteristic of substrate stiffness on cell function were evaluated at various time intervals. Our results indicate that the cell proliferation in stiff substrate is higher than those of soft substrate. The cell proliferation (i.e., function of absorbance percentage) increases over the time for

PDMS /10% Al2O3 composite (since it is stiffer substrate) and it remained higher till the end of the study, day-18, compared to the other time points.

V. Conclusion

Increase in PDMS stiffness is possible through adding Alumina as an inert ceramic. This kind of stiffness affects cell proliferation in a period of 18 days.

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