Supplementary Materials

Understanding the role of Vascular Stretch on Modulation of VWF and ANGPT-2 in Continuous Flow Left Ventricular Assist Device (CF-VAD) Patients

Fabrication of Stretch Devices

Theoretical analysis of percentage stretch and stress was performed using finite element analysis (FEA) through SolidWorks Simulation (Version 2024, Dassault Systemes, Waltham, MA, USA). The stretch device was composed of three main components: an outer frame, a cell-seeding chamber, and a flexible membrane. These components were individually designed, structural properties assigned and assembled using SolidWorks CAD. The mesh for FEA was generated using the Standard Solid Mesher with high mesh quality settings. An element size of 2.47514 mm and a tolerance of 0.123757 mm were employed to ensure accurate results. The mesh consisted of 15,627 nodes and 8,969 elements, with 74.2% of elements having an aspect ratio below 3 and only 1.71% above 10. The maximum aspect ratio observed was 22.186, and no distorted elements were present. The analysis was performed under 35 mmHg and 7 mmHg of vacuum for the pulsatile and continuous group, respectively. A static stress-strain analysis was performed to simulate material response at the endpoint of each cycle. Von Mises and deformation results were monitored across the structure to evaluate structural integrity, stress distribution, and total displacement of the chamber.

Molecular Techniques Details

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell lysates prepared in RIPA lysis buffer or cell culture supernatants of endothelial cells after pulsatile or continuous stretch treatment were used for the analysis of VWF and ANGPT-2 by enzyme-linked immunosorbent assay (ELISA). Human Angiopoietin-2 ELISA kit (Cat#: RAB0016, Sigma Aldrich, St. Louis, MO, USA) and Human Von Willebrand Factor kit (Cat#: RAB0556, Sigma Aldrich) were used following the instruction manuals. Each sample was measured in duplicate, and the optical density (OD) values were measured at 450 nm using Biotek Synergy HT Multi-mode Microplate Reader (Winooski, VT, USA). The VWF and ANGPT-2 values obtained from the lysate or culture supernatant were normalized to total protein measured using Barfoed reagent (Bio-Rad, Hercules, CA, USA).

Total RNA Isolation and Quantitative RT-PCR

RNeasy mini kit (Cat# 74106, Qiagen, Hilden, Germany) was used to isolate the total RNA from cultured cells. First-strand cDNA was synthesized from 500 ng of total RNA using a high-capacity cDNA reverse transcription kit (Cat# 2965267, Applied Biosystems, Foster City, CA, USA), following manufacturer's protocol. cDNA equivalent to 20 ng of total RNA was used for each qRT-PCR reactions that were performed in CFX384 real-time thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR primer sequences used in this study are listed in Table S1 in the supplemental material. Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used to amplify the target gene, following manufacturer's protocol.

Immunofluorescence Staining and Microscopy

Immunofluorescence staining was performed as described previously with slight modifications.[1, 2] Briefly, the cells were fixed in 4% paraformaldehyde in 1x phosphate buffered saline for 15 mins. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 (Thermo Fisher

Scientific, Waltham, MA, USA) for 5 mins and blocked with 2.5% donkey serum (Cat#: D9663, Sigma Aldrich) for 5 mins. Primary antibodies against VWF (15µg/ml, GA527, Dako, Santa Clara, CA, USA) and ANGPT-2 (15µg/ml, AF623, R&D, Minneapolis, MN, USA) were used. The samples were incubated in primary antibodies diluted in blocking solution of 2.5% donkey serum dissolved in PBS with 0.2% Tween 20 (Cat#: T2700, Sigma Aldrich) for 1hour at room temperature. After washing in 1x PBS with 0.2% Tween 20, matching secondary antibodies conjugated to FITC (1:200, 711-095-152, Jackson Immuno-Research, West Grove, PA, USA) or Alexa fluor 594 (1:200, A11058, Thermo Fisher Scientific) were applied and incubated for 45 mins at room temperature. Cell nuclei were counter-stained with 4′, 6-diamidino-2-phenylindole (DAPI, Invitrogen, Waltham, MA, USA) for 10 mins at room temperature. After washing with 0.2% tween 20 in 1x PBS, cell imaging was performed using a Leica stellaris 5 confocal microscope (Deerfield, IL, USA) at the UAB core facility.

Western Blot Analysis

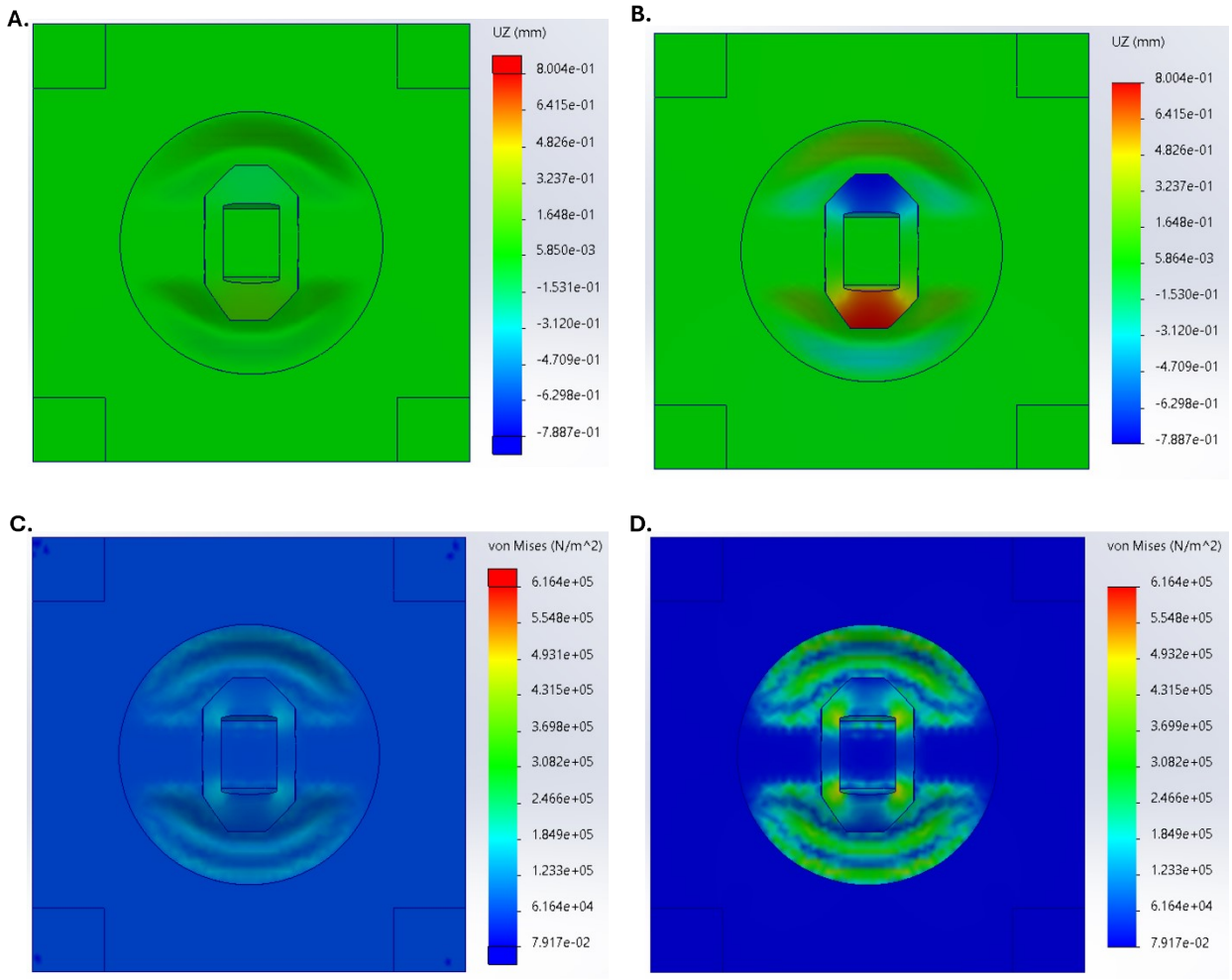
Cells were lysed with freshly prepared cell lysis buffer by mixing Pierce™ RIPA Buffer (Cat#: 89900, Thermo scientific, Rockford, IL, USA) and 20µl/ml protease and phosphatase single use inhibitor cocktail (Cat#: 78442, Thermo Scientific). Proteins were concentrated from culture medium by using 20% Trichloroacetic acid dissolved in water, following the protocol described by Faoro, 2011.[3] The protein concentration was measured using Barfoed reagent (Cat#: 500-0205, Bio-Rad, Hercules, CA, USA) following instructional manual. Western blot analysis was performed as described previously with slight modification.[1, 4] Briefly, the cell lysates or media concentrate were separated by 8-10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Cat#: IPVH00010, Millipore, Tulla green, CC, Ireland). The membranes were blocked with 5% skim milk (Cat#: 1706404, Bio-Rad) in 1x tris buffer saline containing

0.1% Tween 20 (TBS-T) for 1 hour with gentle rocking. The membranes were incubated overnight with primary antibodies at 4°C with gentle rocking. Secondary antibodies conjugated with horseradish peroxidase were applied to the membranes, which were then incubated for 2 hours at room temperature with gentle rocking. After washing with 1x TBS-T, the membranes were incubated with SuperSignal™ West Pico Plus Chemiluminescent substrate (Cat#: 34580, Thermo Scientific, Rockford, IL, USA) and the signal was recorded immediately using an Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK). Antibodies against VWF (1:1,000 dilution, GA527, Dako, Santa Clara, CA, USA), ANGPT-2 (1:1000 dilution, AF623, R&D, Minneapolis, MN, USA), β-Actin (1:1000 dilution, Cat#: 4970, Cell Signaling Technology, Danvers, MA, USA), Anti rabbit HRP-linked (1:3000 or 1:5000 dilution Cat#: 7074, Cell Signaling Technology), Anti-Rat HRP-linked (1:5000 dilution, Cat#: 7077, Cell Signaling Technology), Anti-Mouse HRP-linked (1: 5000 dilution, Cat#: 7076, Cell Signaling Technology) were used.

Figures with Legends

Supplementary Figure 1: Validation of uniaxial stretch PDMS device with SolidWorks. Finite Element Analysis (FEA) results for theoretical validation of the Uniaxial PDMS Stretch Device. **(A, B)** depict the stretch of the seeding chamber along the Z- plane (relative plane parallel to the device). Peak stretch expected on devices in the continuous group **(A)** is ~4%, while the expected peak stretch on the cyclic group **(B)** is 20%. Direction of stretch parallel to the cell monolayer is color coded. **(C, D)** depict a Von Misses Stress test. Analysis of Von Misses results for continuous **(C)** and cyclic **(D)** groups show that most of the stretch-induced stress is dissipated by the seeding chamber, which avoids the induction of non-physiological stress into the monolayer of cells.

Supplementary Figure 1: Validation of uniaxial stretch PDMS device with SolidWorks.



Supplementary Table 1. DNA Sequences of Quantitative RT-PCR Primers

Gene		Primer Sequence
VWF	**Sense**	5′-GCAGTGGAGAACAGTGGTG-3′
	Antisense	5′-GTGGCAGCGGGCAAAC-3′
ANGPT-2	**Sense**	5'-AACTTTCGGAAGAGCATGGAC-3'
	Antisense	5'-CGAGTCATCGTATTCGAGCGG-3'

References

1. Sah, J.P., et al., *Ectonucleotide pyrophosphatase 2 (ENPP2) plays a crucial role in myogenic differentiation through the regulation by WNT/β-Catenin signaling.* Int J Biochem Cell Biol, 2020. **118**: p. 105661.
2. Sah, J.P., et al., *MBP-FGF2-Immobilized Matrix Maintains Self-Renewal and Myogenic Differentiation Potential of Skeletal Muscle Stem Cells.* Int J Stem Cells, 2019. **12**(2): p. 360-366.
3. Faoro, V. and G. Stanta, *Trichloroacetic Acid (TCA) Precipitation of Proteins*, in *Guidelines for Molecular Analysis in Archive Tissues*, G. Stanta, Editor. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 257-258.
4. Haglund, T.A., et al., *Evaluation of flow-modulation approaches in ventricular assist devices using an in-vitro endothelial cell culture model.* The Journal of Heart and Lung Transplantation, 2019. **38**(4): p. 456-465.