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14. ABSTRACT

Temporary vascular shunts are essential for repairing severely damaged arterial blood vessels following extreme trauma in the combat casualty care setting. Unfortunately, recent experience in Iraq showed clotting and occlusion of existing shunts within hours. In the current research we coated shunts with a novel non-adhesive surface coating technology developed at the Wyss Institute, and tested both short and long-term patency in large animal (pig) models of thrombosis.

15. SUBJECT TERMS

Temporary vascular shunts (TVS), thrombosis, patency, Tethered Liquid Perfluorocarbon (TLP), Arteriovenous (AV), Swine model, fibrin, carotid/femoral artery

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Temporary vascular shunts are essential for repairing severely damaged arterial blood vessels following extreme trauma in the combat casualty care setting. Unfortunately, recent experience in Iraq showed clotting and occlusion of existing shunts within hours. In the current research we coated shunts with a novel non-adhesive surface coating technology developed at the Wyss Institute, and tested both short and long-term patency in large animal (pig) models of thrombosis.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Temporary vascular shunts (TVS), thrombosis, patency, Tethered Liquid Perfluorocarbon (TLP), Arteriovenous (AV), Swine model, fibrin, carotid/femoral artery

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Characterize Clotting induced by a LARGE DIAMETER, Temporary Vascular Shunt with or without a Tethered Liquid Perfluorocarbon (TLP) coating in Pigs (Wyss Institute and Boston Children's Hospital):

1.1 **Validate Methods for TLP Coating of Large Diameter TVSs:** TVSs will be obtained from Sundt (or TS-VISs from the Air Force), coated with TLP, and characterized to optimize coating conditions for prevention of blood coagulation in vitro.

1.2 **Evaluate Patency of Large Diameter TVSs in Pigs:** Juvenile Yorkshire swine will be used to mimic the clinical conditions that lead to clotting of large diameter TVSs in the presence or absence of heparin anticoagulation.

1.3 **Evaluate patency of TLP-treated TVSs in the Porcine Model:** Large diameter TVSs coated with or without the optimized TLP coating will be implanted in Juvenile Yorkshire swine and evaluated for their ability to prevent clot formation and vascular occlusion in the absence or presence of low or high doses of heparin anticoagulant for up to 72 hours

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant

results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Background:

Temporary vascular shunts are essential for repairing severely damaged arterial blood vessels following extreme trauma in the combat casualty care setting. They are required to maintain cerebral and peripheral blood flow within severed or damaged arteries (e.g., carotid, subclavian, axillary, femoral), and thereby stabilize patients with acute injury so that they can be transported to medical units for surgical reconstruction off-site. Unfortunately, recent experience with arterial shunts used in the military theater in Iraq (Rasmussen *et al.,* J. *Trauma* 2006; 61:8) and Afghanistan has revealed that clotting and occlusion of these shunts often occurs within hours after placement, particularly in distal injury locations. Moreover, this problem is accentuated because anticoagulant therapy is often counter-indicated in many patients with severe trauma. Thus, the goal of this project is to adapt an anticoagulant surface coating technology that was recently developed in our laboratory (Leslie DC *et al*. *Nature Biotech.* 2014; 32:1134) to create arterial shunts that will suppress clot formation, when under high blood flow with minimal or no soluble anticoagulation.

We believe that the major problem combat casualty care teams face today related to clotting of temporary arterial shunts can be solved by developing vascular shunts that are lined by a biocompatible anticoagulant surface coating. We recently engineered a non-adhesive surface coating that efficiently repels blood and prevents thrombosis (Leslie DC *et al*. *Nature Biotech.* 2014; 32:1134), which consists of a layer of perfluorocarbon that is tethered to the surface of a material, and then overlaid by a thin layer of liquid perfluorocarbon (**Fig. 1a**). Importantly, the perfluorocarbon we use is perfluorodecalin, which is approved for clinical use and has been demonstrated to be biocompatible with flowing blood as it was originally developed as a hemoglobin substitute.

In our past studies, we used a simple coating method to apply this tethered liquid perfluorocarbon (TLP) surface coating to more than 20 different materials that are FDA approved for clinical use, and showed that the TLP coating effectively prevented fibrin polymerization, suppressed platelet adhesion and activation, and inhibited thrombosis when in contact with either static or flowing whole human blood *in vitro* **(Fig. 1b)**. Importantly, when we TLP coated commercially available, clinical grade, arterio-venous (AV) shunts and surgically placed them in large vessels of a live pig, they maintained patency to the end of the 8-hour experiment, even in the complete absence of heparin anticoagulation **(Fig. 2)**.

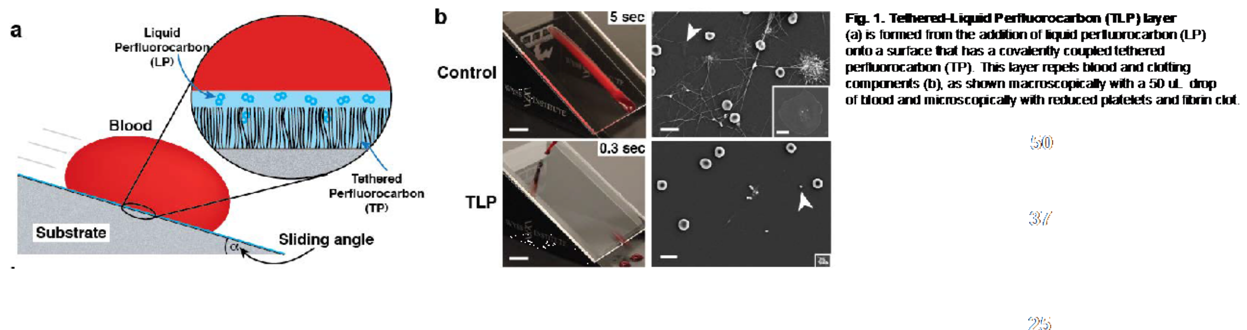


Fig. 1. Tethered-Liquid Perfluorocarbon (TLP) layer (a) is formed from the addition of liquid perfluorocarbon (LP) onto a surface that has a covalently coupled tethered perfluorocarbon (TP). This layer repels blood and clotting components (b), as shown macroscopically with a 50 uL drop of blood and microscopically with reduced platelets and fibrin clot.

Thus, in this project we proposed to apply the TLP coating to existing clinical grade vascular shuts of various sizes, to confirm their ability to prevent thrombosis *in vitro*, and to demonstrate that they can prevent clot formation and spontaneous shunt occlusion when placed intraarterially in a pig model. Blood flow in the shunts would be analyzed using transonic flow probes, Doppler imaging and clinical assessment (e.g., distal pulses) at the time of placement and after surgical closure of the animals. Blood samples would be collected at the same times to assess coagulation status (and clot formation within the shunts will be visualized and quantified at the end of the 72-hour experiment. We proposed to compare 3 different commercial sources of vascular shunts in the presence of low (ACT 200-350), full (maintain ACT >450), or no heparin anticoagulation therapy during the course of surgery. Large diameter shunts 8-10 mm inner diameter) were to be analyzed first, and then once we demonstrated feasibility, we planned to extend these studies to include smaller pigs to determine the smallest diameter shunt that can be maintained patent using the TLP coating.

Temporary vascular shunts occlude readily, with only 12% of distal shunts remaining patent after 2 hours in military experience in Iraq (Rasmussen *et al.,* J. *Trauma* 2006; 61:8). In that study, the lack of patency of the distal vessels did not lead to loss of limbs at early time points; however, unpublished observations of military surgeons to whom we have spoken is that thrombosis and loss of patency of vascular shunts requires extensive thrombectomy, complicates vascular surgery, delays recovery and likely leads to a higher incidence of limb loss. Thus, the development of a vascular shunt technology that can be surgical placed in injured arteries and maintain limb perfusion for up to 3 days with minimal or no heparin anticoagulation would save limbs and patient lives. The same approach could be used to place shunts in veins to decrease venous hypertension, tissue ischemia and bleeding from the injured extremity.

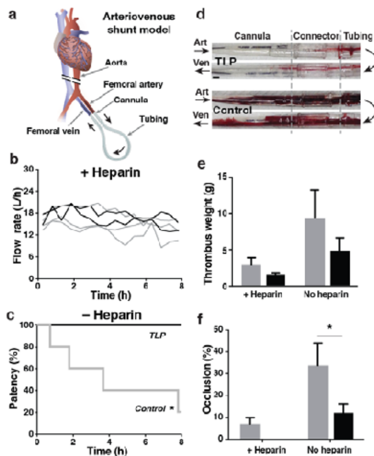


Fig. 2. Thrombogenicity of TLP-coated circuits in a porcine arteriovenous shunt model. **(a)** Schematic of the porcine arteriovenous shunt model showing placement of the cannulae. Arrows indicate direction of blood flow. **(b)** Real-time measurements of flow rate (10 s average) at the midpoint of the circuit showing that in heparinized animals (+ heparin), flow rate is maintained for 8 h in both control (gray lines, n = 3) and TLP (black lines, n = 2). **c)** Kaplan-Meier curve of patency from real-time flow-rate measurements in non-heparinized animals [illegible] **(d)** Photographs of polyurethane cannulae, polycarbonate connectors and PVC tubing of TLP (top) and control (bottom) circuits after 8 h of blood flow. Arrows indicate direction of blood flow through arterial (Art) or venous (V en) cannula. [illegible] **(e)** Thrombus weight in the TLP [illegible]

1.1 Validate Methods for TLP Coating of Large Diameter TVS:

1.1.1 Deliverables/Milestones: Development of an optimal method for TLP coating of TVS that prevents blood coagulation in vitro.

In order to compare TLP coated shunts with standard medical grade TVS shunts, we first needed to develop methods to generate reproducible clotting and then to quantify the coagulation on shunts. For in-vitro studies we used a laboratory - scale peristaltic pump flowing anticoagulated blood through peristaltic tubing and TVS shunts at 70 mL/min while for in vivo studies, we tested TVS shunts from large animal models of blood coagulation in shunts as described in section 1.2.

To measure coagulation, we initially used the protein stain- Coomassie Blue to measure fibrinogen and accumulated fibrin polymers in thrombi in the TVS shunts generated in vitro and in vivo. This was only partially successful. Following the generation of thrombi, the TVS shunts were washed gently with saline and then stained with Coomassie blue using a peristaltic pump flowing for 1 hour at 11 ml/hr (**Fig. 3A**).

Coomassie Blue staining of TVS shunts after in vivo experiments showed dark red/purple staining of the thrombus in control (CNTRL) shunt (**Fig 3B**), while the TLP shunt was brilliant blue (**Fig. 3C**). The purple color in the CNTR-Stained (**Fig. 3B bottom**) might be indicative of glycoprotein in thrombi while the Brilliant Blue on the TLP-stained (**Fig. 3C bottom**) could be protein formation such as fibrinogen, or simply staining of the hydrophobic surface. We quantified Coomassie-stained areas using ImageJ/FIJI.

To utilize the observation of the hydrophobic staining of TLP to our advantage, we developed a protocol (**Fig. 4A**) using a hydrophobic dye - Rhodamine 110 which stains the hydrophobic TLP layer green. Samples explanted from pig experiments show that CTRL shunts do not have a hydrophobic layer (**Fig. 4B**), whereas TLP shunts maintain hydrophobicity and show strong green fluorescence (**Fig. 4C**).

Blinded analysis from 6 pig cases revealed that Coomassie Blue staining does not distinguish CTRL shunts from TLP shunts (**Fig. 5 Top**), whereas Rhodamine 110 staining readily distinguishes these two treatment conditions (**Fig. 5 Bottom**). This shows that Rhodamine 110 staining is a useful method for estimating hydrophobicity of TLP coating in pre and post implatation of shunts and was used in subsequent experiments.

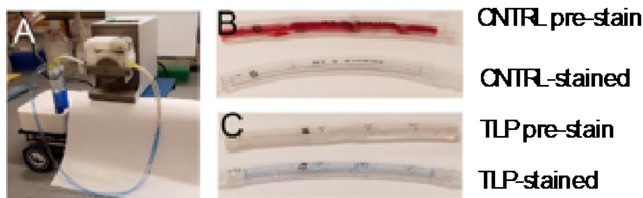


Fig. 3. Coomassie Blue staining.

A: Setup for Coomassie Blue staining under flow.
B: (Top) A CTRL shunt with thrombus before staining. (Bottom) The CNTRL shunt thrombus turns purple (which is difficult to see in the photograph).
C: (Top) A patent TLP shunt before staining. (Bottom) The patent TLP shunt stains brilliant blue.

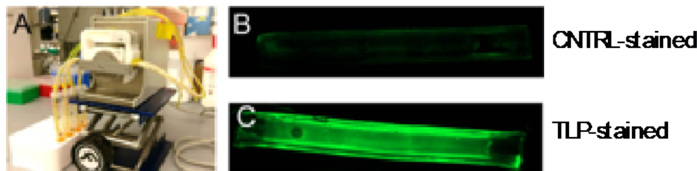


Fig. 4. Rhodamine 110 staining of patent shunts.

A: Set up for Rhodamine 110 staining.
B: A "stained" CTRL shunt.
C: A stained TLP shunt.
The different in fluorescence intensity of the two shunts clearly shows the different hydrophobicity of the coatings.

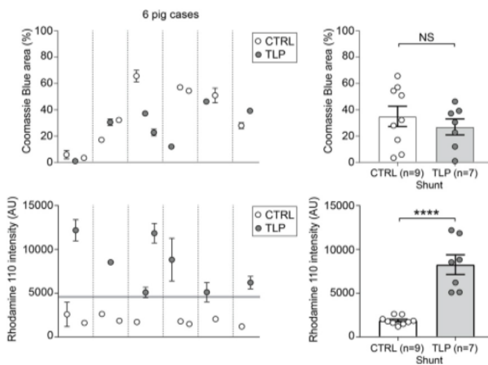


Fig. 5. Blinded analysis of explanted shunts.

2 or 3 shunts were implanted in each of 6 pigs (9 CTRL and 7 TLP shunts).
15/16 shunts remained patent in these short term (6 hr) studies.
The results show Mean ± SEM.

Top: Coomassie Blue stained
(Left) area of staining.
(Right) unpaired t-test between CNTRL and TLP showing no significant difference

Bottom: Rhodamine 110 staining
(Left) staining intensity.
The thick gray line is the mean fluorescence intensity of all shunts, and all TLP shunts are above this mean. .
(Right) Significantly different between two shunt treatments by unpaired t-test.

We developed two *in vitro* thrombotic models for studying the underlying mechanisms of thrombus formation. We hypothesize that high shear rate is one of the underlying mechanisms of thrombus formation (Casa and Ku, 2017, Annu. Rev. Biomed. Eng.). Using a Chandler Loop (**Fig. 6A**), a shunt is connected to silicon tubing, which is filled with Na citrate treated porcine whole blood. CaCl2 is mixed with the porcine blood to a final concentration of 2.5, 5, or 10 mM $CaCl_2$ and the entire tubing circuit is rotated at ~60 rpm overnight at room temperature. This rotation creates shear stress and results in thrombus formation in 14/16 shunts. In the other 2 shunts, thrombus was formed but this slipped out from the shunt at the end of the experiment. Based on the thrombus condition, we term the former as anchored thrombus and the latter as non-anchored thrombus (**see Fig. 12** for the pictures of in vivo explanted shunts). The other *in vitro* model is based on our newly developed *in vivo* porcine thrombotic model (see

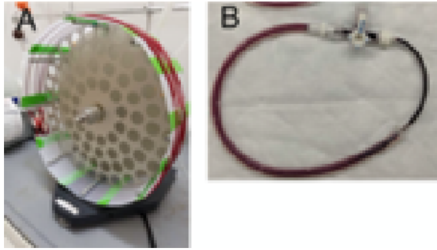


Fig. 6. Two in vitro thrombotic models.

A: Chandler Loop model. Porcine blood mixed with CaCl2 is rotated overnight. A majority of thrombi are ***anchored thrombus.***

B: Thrombin model. Porcine blood mixed with thrombin is incubated. Depending on incubation time, either anchored or non-anchored thrombus is formed .

below), in which we reduce blood flow (Zhang et al., 2017, Ultrasound Med. Biol.) to increase a chance of thrombus formation by accumulated coagulant and inflammatory factors. To mimic this, Na citrate treated porcine whole blood is mixed with 1 or 2 units/ml of thrombin (a coagulant factor) in a shunt connected to silicon tubing (**Fig. 6B**). The shunt is incubated at 37°C for 5 to 72 hours. The shunts with 5 to 7 hours incubation formed non-anchored thrombi (3/3), whereas incubation for 24 to 72 hours formed anchored thrombi (4/4).

Based on these *in vitro* thrombotic models, we studied the inner surface of shunts (2 anchored and 2 non-anchored thrombi) using scanning electron microscopy (SEM). **Fig. 7** shows representative examples of each case. In vitro anchored thrombus had thin layers of a sheet that trap activated platelets and red blood cells (RBCs) (**Fig. 7 left**). On the other hand, non-anchored thrombus had aggregated RBCs trapped by fibers (**Fig. 7 right**). Based on these observations, we hypothesize that anchored thrombus is formed by activated platelets and their ligand, von Willebrand Factor (vWF), a coagulant factor, whereas non-anchored thrombus is mainly formed by RBCs and fibrinogen/fibrin polymers.
To analyze the composition of the thrombi from the in vitro and in vivo experiments. We developed immunohistochemistry (IHC) analysis of platelets, red blood cells (RBCs), Von Willebrand factor (VWF), and fibrinogen in thrombi from in vivo experiments based on our previous studies on in vitro thrombi. Our analysis revealed similar composition of thrombi formed in vitro and in vivo (**Fig. 8**).

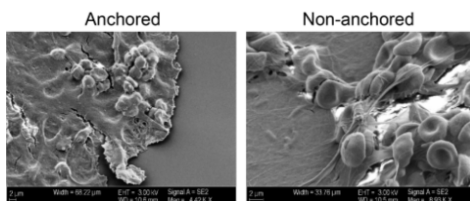
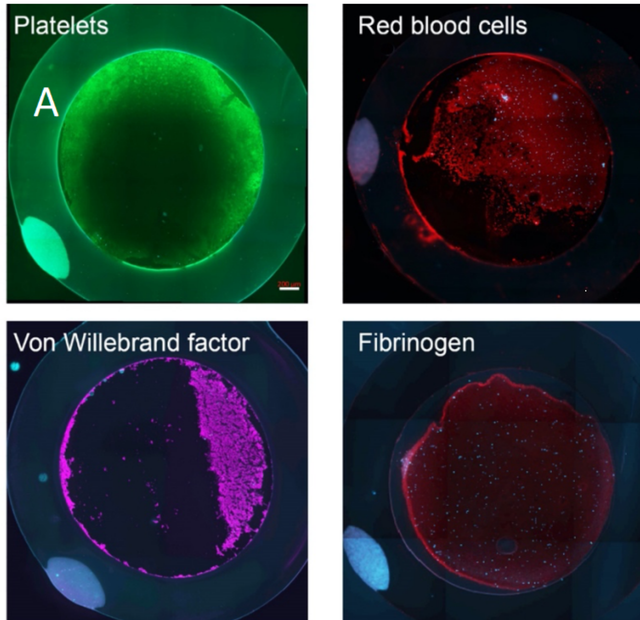


Fig. 7. Scanning electron microscopic (SEM) images.
(Left) A representative example of in vitro anchored thrombus by thrombin model. Activated platelets and some RBCs are seen on the surface. The thin layers of a sheet might be created by activated platelets.

(Right) A representative example of in vitro non-anchored thrombus by Chandler Loop model. All cells are presumably RBCs that are trapped by fibers



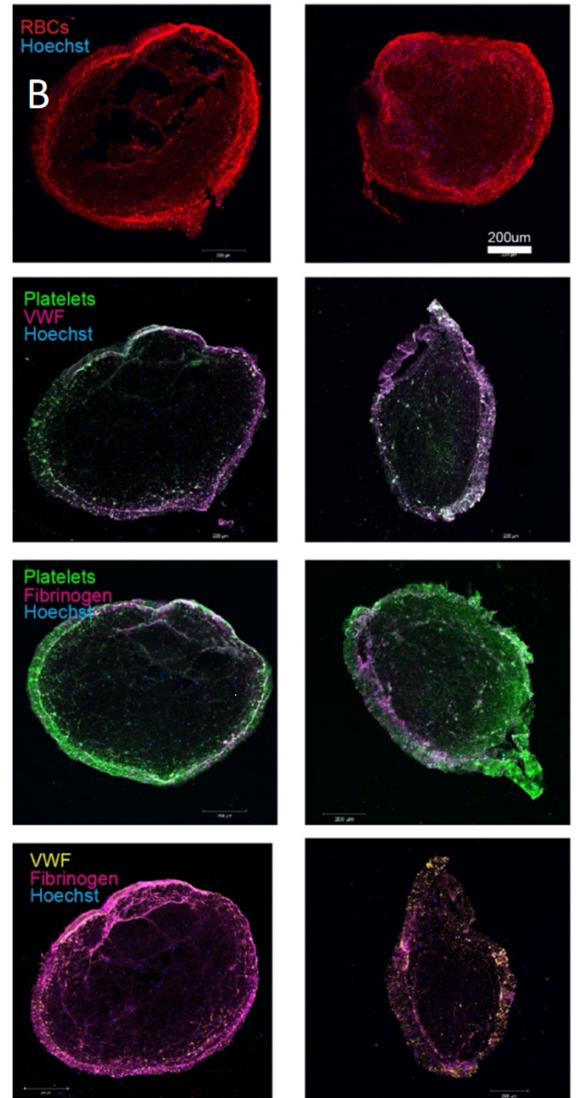


Fig. 8A. IHC of platelets, RBCs, VWF, and fibrinogen in vitro thrombi. Platelets were stained using 1:20 FITC conjugated mouse anti-porcine CD61 Ab. RBCs were stained using 1:500 rabbit anti-human RBC Ab and 1:1000 Alexa 594 goat anti-rabbit IgG. VWF was stained using 1:50 sheep anti-human VWF Ab and 1:500 donkey anti-sheep IgG. Fibrinogen was stained using 1:50 rabbit anti-human fibrinogen β Ab and 1:200 Alexa 647 goat anti-rabbit IgG. Nuclei were counter stained with Hoechst Blue. Scale: 200 µm.

Fig. 8B. IHC of RBCs, platelets, VWF, and fibrinogen in vivo comparing anchored and non-anchored thrombi
1,2. RBCs and nuclei (Hoechst blue); 3,4. triple staining of platelets (green), VWF (red), and nuclei (blue); 5,6.platelets (green), fibrinogen (red), and nuclei (blue); 7,8. VWF (yellow), fibrinogen (red), and nuclei (blue). RBCs were stained using 1:500 rabbit anti-human RBC antibody (Ab) and 1:500 Alexa 594 goat anti-rabbit IgG. Platelets were stained using 1:50 mouse anti-porcine CD61 Ab and 1:400 Alexa 488 goat anti-mouse IgG. VWF was stained using 1:50 sheep anti-human VWF Ab and 1:400 Alexa 647 donkey anti-sheep IgG. Fibrinogen was stained using 1:50 rabbit anti-human fibrinogen β Ab and 1:200 Alexa 647 goat anti-rabbit IgG. Nuclei were counter stained with Hoechst Blue.
White areas in triple staining indicate co-localization of platelets and proteins or two proteins. Scale: 200 µm.

We analyzed the shunts from in-vivo studies, to determine whether there were discernable differences between the adhered and non-adhered clots produced in the shunts. During coagulation, platelets interact with VWF and fibrinogen, and so we analyzed the clots for these factors. We found little difference between anchored and non-anchored thrombi for platelet and VWF colocalization: In anchored thrombi platelets colocalized with VWF 58.3 ± 6.3% while in non-anchored thrombi 58.8 ± 5.4% non-anchored (mean ± SD; two animals; 2-4 sections in each case). Similarly, there was little difference in platelets colocalization with fibrinogen (anchored vs non-anchored; 44.7 ± 2.8% vs 43.4 ± 6.6%). So, unlike the in-vitro experiment, we were unable to differentiate the anchored from unanchored thrombi produced in vivo using IHC staining patterns.

In testing alternate materials, proprietary shunts and lubricant were obtained from FreeFlow Medical Devices Inc and tested *in vitro* for thrombus formation using the Chandler loop setup and Coomassie staining as described above.

The results from this experiment are below (**Fig. 9.**) Images were taken before and after staining with SimplyBlue Coomassie (G250). In the pre-stain images, the control shunts contained anchored thrombus formation after overnight exposure but treated shunts with lubricant had little to no thrombus formation. Both stained blue with SimplyBlue Coomassie which stains the hydrophobic surfaces. No purple-stained thrombi were detected on the FreeFlow shunts containing both the tethered Perfluorocarbon (TP) and Liquid perfluorocarbon (LP), while these are readily seen in control shunts with only TP.

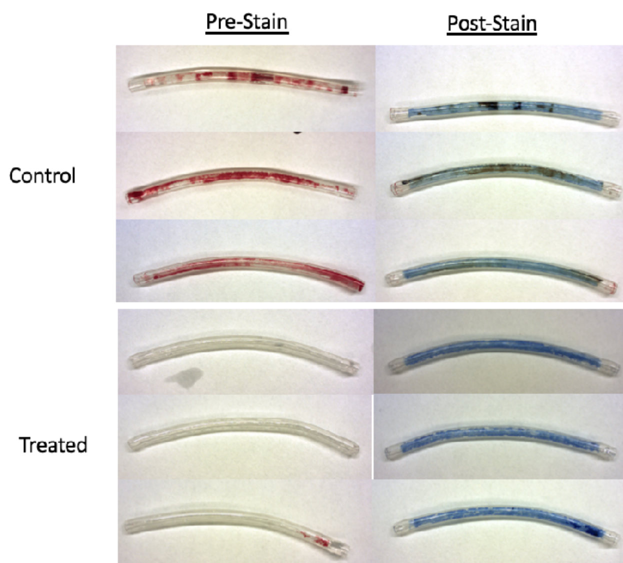


Fig. 9. Pre-stain and post stain images of control and treated FreeFlow Medical shunts. Anchored thrombi were found on the control shunts but not on the treated shunts. All shunts stained blue with the SimplyBlue Coomassie (which stains the hydrophobic surface of the shunts).

For Quality Control (QC) testing of shunts, the translation of the water droplet (colored blue) through a shunt was measured on a leveled goniometer by changing the surface pitch through tilt angles of ± 15°. In control shunts, a 5 uL drop of water immediately “pins” to the surface and does NOT move with the translation of the goniometer. Pinning has been observed in all control shunts. In TLP-treated shunts, the blue water droplet slides along the surface for tilts in the range of 3°- 10°. All TLP shunts passed this tilt angle testing and were released for testing *in vivo.*

Evaluate Patency of Large Diameter TVSs in Pigs:

1.2.1 ***Deliverables/Milestones:*** Development of a porcine model of large diameter TVS clotting in the absence or presence of low or high doses of heparin anticoagulant.

Flow Probe testing: We first tested the accuracy of the Transonic flow probes in vitro by volumetric analysis and confirmed the accuracy which had been factory calibrated to the fluid temperature and shunt dimensions. In initial in vivo studies, we compared the Transonic probe with the Doppler Ultrasound unit from the animal facility and detected flow in carotid and femoral arteries in pigs using both technologies. The flow rate measured by the Transonic probe ranged from 50 to 200 ml/min, which is in the range expected for pig carotid arteries. Therefore, we concluded that the Transonic probe was sufficiently sensitive for use in this in vivo TVS shunt study.

The Transonic Probe had been factory calibrated for 12Fr shunts. In order to use this technology with shunts of different diameters, we needed to calibrate these in-house using 8 Fr, 10 Fr, and 12 Fr shunts Following re-calibration, the transonic flow sensors had ±5% error from the measured flow rate (volume/min) in the required flow range of <15-250 ml/min. Therefore, we were able to use this technology to accurately measure blood flow rate, estimate TVS patency, and monitor thrombus formation in vivo for the 3 sizes of shunts. We successfully applied this Transonic probe technology to shunts implanted in carotid and femoral arteries in 6 pigs. We compared flow measurements in vivo between CTRL and TLP Covidien Argyle shunts using the Transonic probe. In this pilot study, one CTRL shunt clotted immediately which was detected in real time using the Transonic probe and confirmed post-mortem on the explanted shunt (Fig. 5).

Porcine Thrombotic Model:

A major challenge in this project was the production of thrombi in vivo paired with detailed laboratory analysis of these clots. Initially, we placed 6-inch COVIDIEN Argyle shunts in the carotid artery with or without the TLP coating. To accurately quantify coagulation within the shunts during the in-life experiment (and to collect both anchored and non-anchored thrombi), we heparinized animals immediately before necropsy, rapidly exposed the full length of the shunt, and tied off the vessels at either end to avoid disturbing any thrombus that had formed in the shunt during the experiment. However, because the 6-inch shunts were so long (and reached close to the heart), we were unable to rapidly expose the vasculature at both ends of the shunt at necropsy for this tie-off step. Therefore, we tested different shunts and determined that 3 inches as the optimum length. While this aided surgery, and dissection at the end of the in-life study, the thrombus formation was reduced in these shorter shunts - probably due to the smaller surface area in contact with the blood, necessitating development of mechanical methods for inducing thrombi.

In one method of thrombus generation (Wysokinski et al., 1999, Arterioscler. Thromb. Vasc. Biol.), we damaged endothelial cells to induce release of pro-coagulant and inflammatory factors. We tested 2 methods of damaging endothelial cells: 2 arteries were scraped using a plastic microbiology loop for 50 sec and 1 artery was injured using a hemostat vessel (3 replicates of a 10-sec clamping separated by 5 sec intervals); animals were monitored under anesthesia for 6-8 hours. Thrombus formation was monitored in real time by measuring blood flow rate in vivo using Transonic probes and further confirmed by visual inspection of explanted shunts at the conclusion of the 8 hour experiments. We placed 3-inch 8 Fr COVIDIEN Argyle shunts into the left and right carotid arteries of 2 animals (3 CTRL shunts) without heparin administration. These methods were unsuccessful as all 3 shunts were patent.

Next, while using Wyss funding to develop surgical methods, we optimized the technique for damaging endothelial cells proximal to the shunt and added an umbilical band distal to the shunt. We tested brushes with nylon bristles (0.05 inch in diameter) and a stainless steel handle (Key Surgical, channel cleaning brush) (**Fig. 10**). We used a 6-inch 8 Fr shunt as a guide to advance the brush into the vessel to a point proximal to the 3-inch shunt placement (**Fig. 10A**). Once at the desired location, we extended the brush through the guide shunt, brushed back and forth 3 times and rotated the brush 3 times (**Fig. 10B**). We

also placed a band using cotton umbilical tape [Ethicon Inc.) distal to the shunt placement to reduce blood flow rate to enhance thrombus formation (Zhang et al., 2017, Ultrasound Med. Biol.), using a 16G catheter as a guide to tie the tape to the desired diameter. We placed CTRL shunts in the left and right carotid arteries and right femoral arteries of 2 pigs (6 shunts). The degree of damage to the endothelial cell layer was assessed by postmortem immunohistochemistry using anti-CD31 antibody, fluorescence microscopy, and image processing (**Fig. 11**). Immunohistochemistry of the intact endothelial cell layer is shown in Fig.11A, while the damaged layer is shown in Fig.11B.

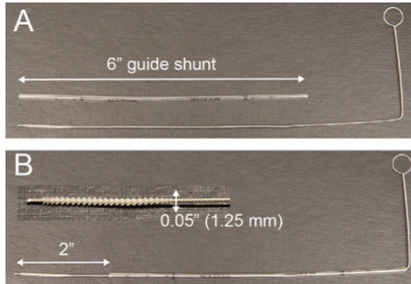


Fig. 10. Experimental damaging the endothelial cell layer in the artery. A: a 6-inch long guide shunt and brush. **B:** Brush is extended outside the guide shunt and can be moved back and forth and rotated to damage the endothelial cell layer. Inset: Enlarged view of brush with a diameter of 0.05 inch (1.25 mm).

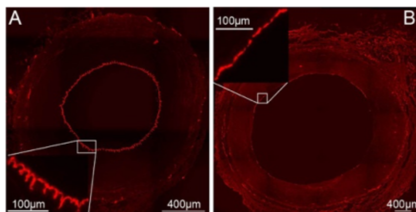


Fig. 11. Immunohistochemistry of the endothelial cell layer in the carotid arteries. Due to auto-fluorescence of porcine tissues, Alexa 594 is used for the conjugation of secondary antibody. **A:** Intact endothelial cell layer in the distal carotid artery. 0% damage. **B:** Damaged endothelial cell layer in the posterior carotid artery. 88% damage. Insets: Enlarged view of the endothelial cells.

After explanting the shunts, we observed 3 different types of thrombi: anchored, non-anchored, and patent (**Fig. 12**). In the CTRL shunts in the carotid artery, we observed 1 anchored thrombus (**Fig. 12A**), 1 non-anchored (**Fig. 12B**), and 2 patent (**Fig. 12C**). In the CTRL shunts in the femoral artery, we observed 1 anchored thrombus and 1 patent (Pig 447 and 448 in Table 1). Building on these results, in later studies we increased the number of brushing and rotations to 10 each, and changed the banding technique from umbilical tape to a silk tie, and the catheter from 16G to 20G for the distal carotid artery, to increase coagulant and release of inflammatory factors from damaged arteries and enhance thrombus formation.

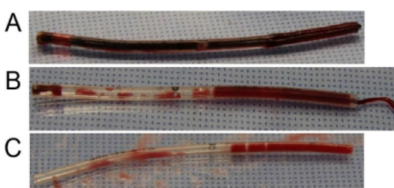


Fig. 12. 3 types of thrombus in explanted CTRL shunts in vivo. A: Anchored thrombus. **B:** Non-anchored thrombus. Thrombus is formed but slipped out from a shunt during postmortem preparation. **C**: Patent. Liquid blood is readily absorbed into a paper towel.

In the next set of studies, we implanted CTRL shunts in the left and right carotid arteries and the left or right femoral arteries of 2 pigs (Pig #453 and #454, total of 6 shunts). The carotid artery CTRL shunts resulted in 1 anchored thrombus, 1 non-anchored thrombus, and 2 were patent. In the femoral artery: 1 anchored thrombus and 1 non-anchored one (**Table 1**).

Then we placed CTRL shunts in the left and right carotid arteries and right femoral arteries of 4 pigs (Pig #433, #434, #455, & #456 a total of 11 shunts). This study resulted in formation of 6 anchored and 2 non-anchored thrombi in CTRL shunts within the carotid artery. CTRL shunts in the femoral artery shunts were found to contain 2 anchored and 1 non-anchored thrombi (**Table 1**). All in vivo results are summarized in **Table 1** and **Fig. 13**. Our success in consistently forming thrombi in control shunts improved with refinement of the methods of endothelial damage - increasing brushing from 3 to 10 times, and catheter from 16 to 20 Fr. (**Table 1). Fig. 13A** illustrates the distribution of 3 types of thrombus in the CTRL shunts.

We tested whether Coomassie Blue staining could distinguish these 3 types of thrombus (**Fig. 13B).** While the Coomassie Blue was able to distinguish the anchored thrombi from those that were non-anchored or patent; it did not distinguish between non-anchored and patent thrombi.

Pig ID	Artery	Brushing	Rotating	Catheter	Banding	Thrombus
447	LCA	3 times	3 times	16G	Umbilical tape	Anchored
	RCA	3 times	3 times	16G	Umbilical tape	Patent
	RFA	3 times	3 times	NA	NA	Patent
448	LCA	3 times	3 times	16G	Umbilical tape	Patent
	RCA	3 times	3 times	16G	Umbilical tape	Non-anchored
	RFA	3 times	3 times	NA	NA	Anchored
453	LCA	10 times	10 times	20G	Umbilical tape	Non-anchored
	RCA	10 times	10 times	20G	Umbilical tape	Patent
	RFA	10 times	10 times	20G	Silk tie	Anchored
454	LCA	10 times	10 times	20G	Umbilical tape	Patent
	RCA	10 times	10 times	20G	Umbilical tape	Anchored
	RFA	10 times	10 times	20G	Silk tie	Non-anchored
433	LCA	10 times	10 times	20G	Silk tie	Anchored
	RCA	10 times	10 times	20G	Silk tie	Non-anchored
	RFA	10 times	10 times	20G	Silk tie	Non-anchored
434	LCA	10 times	10 times	20G	Silk tie	Anchored
	RCA	10 times	10 times	20G	Silk tie	Anchored
455	LCA	10 times	10 times	20G	Silk tie	Anchored
	RCA	10 times	10 times	20G	Silk tie	Anchored
	RFA	10 times	10 times	20G	Silk tie	Anchored
456	LCA	10 times	10 times	20G	Silk tie	Anchored
	RCA	10 times	10 times	20G	Silk tie	Non-anchored
	LFA	10 times	10 times	20G	Silk tie	Anchored

Table 1. Testing thrombosis in CNTRL shunts in new porcine model
3 types of thrombus are categorized into different highlighted colors as in Fig. 11. All CTRL shunts are 8 Fr. LCA; left carotid artery, LFA; left femoral artery, RCA; right carotid artery, RFA; right femoral artery, NA; not available

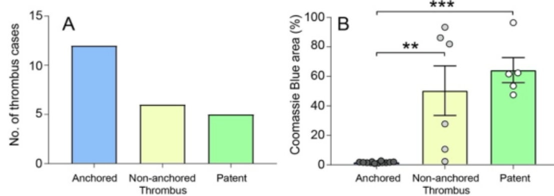


Fig. 13. A: Distribution of 3 types of thrombus in CTRL shunts. Each bar color corresponds to Table 1.
B: Coomassie Blue stained areas in 3 types of thrombus. Coomassie Blue stained areas in anchored thrombus cases are significantly lower than two other thrombus cases. Unpaired t-tests adjusted by Bonferroni correction for multiple comparisons. **: $p<0.01$, ***: $p<0.001$

Pig ID	Artery	Shunt	Brushing	Rotating	Catheter	Banding	Thrombus
457	LCA	TLP	10 times	10 times	20G	Silk tie	Patent
	RCA	CTRL	10 times	10 times	20G	Silk tie	Anchored
	LFA	TLP	10 times	10 times	20G	Silk tie	Patent
458	LCA	CTRL	10 times	10 times	20G	Silk tie	Anchored
	RCA	TLP	10 times	10 times	20G	Silk tie	Non-anchored
	RFA	CTRL	10 times	10 times	20G	Silk tie	Anchored
466	LCA	TLP	10 times	10 times	20G	Silk tie	Non-anchored
	RCA	CTRL	10 times	10 times	20G	Silk tie	Anchored
470	LCA	CTRL	10 times	10 times	20G	Silk tie	Non-anchored
	RCA	TLP	10 times	10 times	20G	Silk tie	Patent
452	LCA	CTRL	10 times	10 times	20G	Silk tie	Non-anchored
	RCA	TLP	10 times	10 times	20G	Silk tie	Patent
460	LCA	TLP	10 times	10 times	20G	Silk tie	Anchored
	RCA	CTRL	10 times	10 times	20G	Silk tie	Non-anchored

Table 2. Summary of CTRL vs. TLP shunts in a new porcine thrombotic model. 3 types of thrombus are categorized into different highlighted colors as in Fig. 13. All shunts are 8 Fr. LCA; left carotid artery, LFA; left femoral artery, RCA; right carotid artery, RFA; right femoral artery.

We placed both CTRL and TLP 3-inch shunts (COVIDIEN Argyle Carotid Artery, Radiopaque Straight) in the left and right carotid arteries and the left or right femoral artery of 6 animals (7 CTRL and 7 TLP shunts) (**Table 2**). The results from CTRL shunts in the carotid artery were 4 anchored and 3 non-anchored thrombi. The results from TLP shunts in the carotid artery were 1 anchored thrombus, 2 non-anchored thrombi, and 4 patent (**Table 2 and Fig. 13A**). While Coomassie Blue staining does not distinguish the difference between patent and occluded shunts, Rhodamine 110 staining readily distinguishes the difference (**Fig. 13B and C**). The same results are further broken down into the 3 types of thrombi. We did not perform any statistical test due to the small sample size in each type. While Coomassie Blue staining may distinguish anchored thrombus from non-anchored thrombus and patent in both CTRL and TLP shunts (**Fig. 13D**), Rhodamine 110 staining readily distinguishes between CTRL and TLP treatments (**Fig. 13E**).

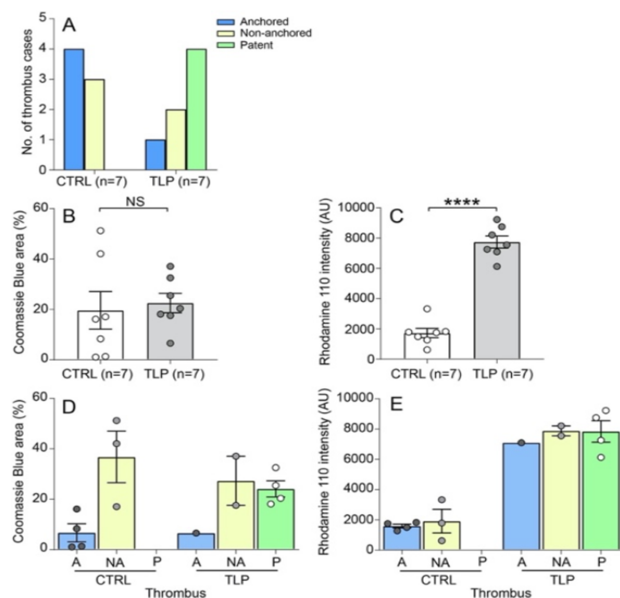


Fig. 13. A: Distribution of 3 types of thrombus in CTRL and TLP shunts. **B:** No significant difference (NS) in Coomassie Blue stained area by paired t-test. **C:** Significantly different in Rhodamine 110 intensity by paired t-test. ****: p<0.0001. **D-E:** Coomassie Blue stained area and Rhodamine 110 intensity in 3 types of thrombus between CTRL and TLP shunts. No statistical test is performed. All results show Mean ± SEM. A; anchored thrombus, NA; non-anchored thrombus, P; patent.

The results of the short-term studies under anesthesia (8-12) hours using the refined scraping and banding technique & COVIDIEN Argyle shunts are summarized in **Table 1&2** and **Fig. 14**. These data confirm that TLP coatings produce a statistically significant (chi-square, $p < 0.05$) reduction in thrombus formation and flow occlusion in TVS shunts for at least 8-12 hours *in vivo*.

We investigated the underlying mechanisms of 3 types of in vivo thrombus. Below are examples of SEM images of each in vivo thrombus types (**Fig. 14**). All cells that appear in these SEM images are probably RBCs. A major difference in these 3 types seems to be the number of fibers except for anchored thrombus in which there are also thin layers of a sheet (presumably made from activated platelets) as in vitro anchored thrombus.

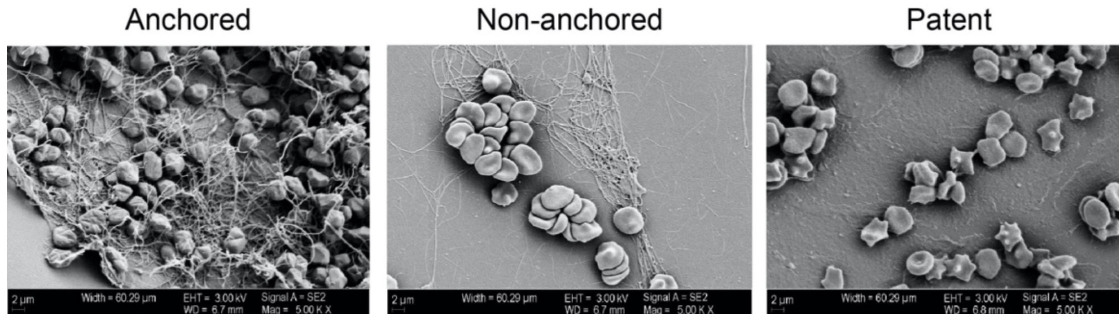


Fig. 14. SEM images of 3 types of in vivo thrombus. (Left) An example of anchored thrombus in a CTRL shunt of pig 466 right carotid artery. A vast majority of cells are RBCs. As in vitro anchored thrombus, thin layers of a sheet are seen under aggregated RBCs. Fibers trap RBCs. (Center) An example of non-anchored thrombus in a CTRL shunt of pig 452 left carotid artery. Clusters of aggregated RBCs and fibers are observed. (Right) An example of patent in a TLP shunt of pig 452 right carotid artery. Small clusters of RBCs are visible

We also tested a new TLP-coated shunt material from FreeFlow Medical Devices Inc. with the brushing injury model we had established in short term (8 hr) study (n=6 animals). Shunts were placed in the left and right carotid arteries. While the tethered layer alone did not prevent the formation of thrombi, as expected, the tethered layer with the addition of the liquid perfluorocarbon layer prevented clot formation in four of the six shunts (**Fig. 15**).

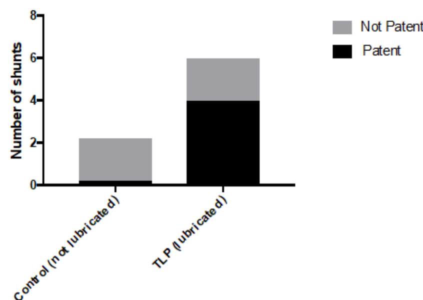


Fig.15. Explanted shunt results from *in vivo* 8-hour studies using new TLP-coated shunt material from FreeFlow Devices Inc. TLP coating suppresses thrombus formation for at least 8 hours in this model.

The porcine model of thrombus production by damaging the arterial endothelium *in situ* and reducing blood flow through the shunt induces accumulation of procoagulant and inflammatory factors, resulting in full or partial occlusion. We have showed that TLP coating reduces thrombus formation in these 8-12 hour studies, in anesthetized animals (**Fig.16**).

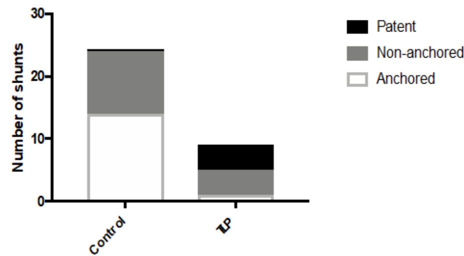


Fig. 16. Explanted shunt results from *in vivo* 8-12 hour studies. All vessels received endothelial cell scraping and banding prior to shunt placement. TLP coating suppresses thrombus formation for at least 8-12 hours in this model.

In our short term 8-hour studies under anesthesia (with banding and scraping to replicate traumatic vessel injury), TLP shunts performed significantly better than previously reported shunts Rasmussen *et al.* (J. *Trauma* 2006; 61:8). In our study, 89% (8/9) of the TLP shunts remained either fully patent (44%) or partially patent (contained only non-anchored thrombi) after 8 hours while 12 of 24 controls (50%) were occluded and a further 12 (50%) had non-anchored thrombi. The TLP patency is well are well above the reported 12% of shunts that remained patent after 2 hours based on military experience from Iraq (Rasmussen *et al.,* J. *Trauma* 2006; 61:8). Thus, results from our short-term study with traumatic vessel injury suggest that the TLP shunts might be useful in a military setting.

1.3.1 ***Deliverables/Milestones:*** *Demonstrate that a large diameter TVS coated with TLP remains patent for at least 24 hours with minimal or no heparin in a porcine model.*

In 1.2.1 we had developed a short-term model to test the TVS shunt where the entire 8-hour experiment (including the banding, endothelial damage and shunt placement in the carotid arteries) was conducted on anesthetized pigs. Due to the cardio-suppressive effects of the anesthesia, we could not keep animals under anesthesia for the full 24 hours, therefore, our original intent in 1.3.1 was to simply wake these animals after the banding, scraping and shunt placement & continue the 24 hour experiment. Unfortunately, these animals (n=3) experienced prolonged recovery from anesthesia in which the animals did not regain the ability to ambulate normally and had elevated temperatures (>105F) and animals had to be euthanized to avoid pain and suffering. It is likely that the increased pressures, blood flow and stress of waking up from anesthesia and moving about had caused thrombi we had induced by the banding and scraping to dislodge and travel to the brain, resulting in these neurological complications.

Based on these results, we did NOT use the endothelial cell layer damage to the carotid arteries for the long-term shunt survival model and additional studies were conducted WITHOUT endothelial cell layer damage. However, without this reproducible formation of thrombi, we were forced to rely on endogenous clot formation due to the shunt material. Initially, we tested the 3 inch shunts (placed without scraping and banding) in 3-day studies, and while we did not see the neurologic effects, we obtained mixed results as controls were not clotting consistently. Therefore, we extended these studies to 14 days, with Covidien Argyle Shunts, to determine whether the difference between treated and controls would be more apparent.

In the 14-day survival, we used two animals. We placed one TLP-treated Covidien shunt in the left carotid artery in one animal. While in the other animal, we placed a TLP coated Covidien shunt in the right carotid and an untreated Covidien shunt in the left carotid. Both animals survived surgery, and remained on study for the full 14 days. However, the animal with the untreated shunt had difficulty during recovery and appeared agitated during recovery, necessitating treatment with diazepam, midazolam and banamine treatment. While both animals showed evidence of clotting in the shunts on explant, it is interesting to note that the control shunt was associated possible neurological and respiratory complications, whereas the TLP shunt was not.

One of the lessons we learned in the course of this study is that placing shunts in healthy, young pigs will not reliably produce thrombi even in the control shunts. Mimicking the trauma that may accompany the need to place an arterial shunt in the field is hard to accomplish in a lab setting. This is why we attempted

numerous methods to reliably induce thrombosis in vivo and test different shunts. In future studies, we would instead propose to investigate different types of anesthesia that would allow prolongation of the short term anesthetized model for up to 24 hours. Alternatively, the test shunts would be placed in vessels distant from the brain (to avoid the immediate neurological problems of carotid arteries) using catheter lab technologies to place expanding shunts, and catheter balloons to induce endothelial damage such as published by the LoGerflo group.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Trained Shanda Lightbown to perform the large animal shunt placements in carotid and femoral arteries.

Amanda Jiang was trained to manage anesthesia during large animal shunt cases.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

These results were shared at MHSRS meetings

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

"Nothing to Report"

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We developed a large animal model of clotting (thrombosis) in Yorkshire swine using techniques imitating battlefield trauma including vaso-occlusion and damage to the endothelial cell (inner layer) of blood vessels. Using this model, we tested the efficacy of our non-thrombotic slippery surface in both short term (hours) and long term (days) of study. In our study nearly half of the slippery catheters (44%) were fully patent (open) at the end of the 8 hour study, while half (50%) the controls were clotted off. This is far better than the 12% of shunts that remained patent in the 2 hour Iraq study (Rasmussen et al., 2006). We were not able to prove the long-term effect of this coating, due to issues with the aggressive thrombosis model in awake, ambulatory pigs.

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

"Nothing to Report"

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Our TLP technology has been licensed by a new startup (FreeFlow Devices Inc.) which plans to commercialize it for medical applications.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

"Nothing to Report"

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

"Nothing to Report"

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

"Nothing to Report"

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

"Nothing to Report"

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

"Nothing to Report"

Significant changes in use of biohazards and/or select agents

"Nothing to Report"

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  "Nothing to Report"

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  "Nothing to Report"

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

  "Nothing to Report"

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  "Nothing to Report"

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  "Nothing to Report"

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Patents on TLP
  Licensing to Freeflow Medical

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

"Nothing to Report"

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

What individuals have worked on the project?

Name: Donald Ingber, MD, PhD
Project Role: PI
Nearest person month worked: 0.3
Contribution to Project: No Change

Name: Mike Super, PhD
Project Role: Co-PI
Nearest person month worked: 0.6
Contribution to Project: No Change

Name: Shanda Lightbown
Project Role: Technician
Nearest person month worked: 3
Contribution to Project: No Change

Name: Thomas Doyle
Project Role: Technician
Nearest person month worked: 3
Contribution to Project: No Change

Name: Sami Rifai
Project Role: Technician
Nearest person month worked: 3
Contribution to Project: No Change

What other organizations were involved as partners?

Organization Name: Boston Children's Hospital
Location of Organization: Boston, MA
Partner's contribution to the project

- Subcontractor