Journal of Visualized Experiments

Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58964R1
Full Title:	Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits
Keywords:	hind limb ischemia, peripheral arterial disease, peripheral vascular disease, rabbits, diabetes, hyperlipidemia, angiography
Corresponding Author:	Aaron Baker University of Texas at Austin Austin, TX UNITED STATES
Corresponding Author's Institution:	University of Texas at Austin
Corresponding Author E-Mail:	abbaker@austin.utexas.edu
Order of Authors:	Andrew D. Sligar
	Gretchen Howe
	Julia Goldman
	Patricia Felli
	Varsha Karanam
	Richard W. Smalling
	Aaron Baker
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Houston, TX USA

Aaron B. Baker, Ph. D.
Associate Professor
Fellow of the Marion E. Forsman Centennial Professorship in Engineering

Department of Biomedical Engineering
The University of Texas at Austin
107 W Dean Keeton Street, BME 5.202D, MC C0800
Phone: (512) 232-7114
Fax: (512) 471-0616
E-mail: abbaker@austin.utexas.edu

August 15th, 2018

Dear Editor:

We are honored to provided a revised manuscript, "*Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits*," for potential publication in the Journal of Visualized Experiments. We have included a detailed response to the reviewers' comments as a separate attachment.

In this work, we describe an optimized large animal model of peripheral ischemia in rabbits that have diabetes and hyperlipidemia. Despite use of this surgery as a preclinical model for many years, a detailed description of this type of surgery is lacking in the literature and there is a lack of standardization in how the surgery is performed by different groups. Moreover, the procedure is primarily performed in healthy animals rather than those with diabetes or another factor that compromises vascular regeneration. Studies using healthy animals with hind limb ischemia were used to justify clinical trials for patients with peripheral ischemia that subsequently failed to show benefit. We believe a major contributor to this failure in clinical translation stems from the use of healthy animals that have exceptional regenerative capacity in contrast to patients with PVD. The method we describe in this work addresses these issues and provides a detailed description of the ligation method to produce reproducible and comparable results between experiments. In addition, the described methods incorporate several innovations that have not been used in previous studies or have been used in separate studies and brought together in this protocol. We have also included an additional supplementary protocol for analysis of histological samples that are optimized to work on rabbit tissues as well as protocols for creating high fat diets that can provide cost savings over purchasing commercially available diets.

The Journal of Visualized Experiments is the ideal format to present the complex surgical methods described in our studies and there is a broad set of groups that would benefit from a detailed description of the methods we describe in our manuscript. The surgical method for performing hind limb ischemia in rabbits is inadequately described in the vast majority of scientific papers employing this model. We hope that this protocol will provide a more consistent preclinical model that reduces the disconnect between

results of preclinical studies and clinical trials for peripheral ischemia. Thank you for your consideration.

Sincerely,

Aaron B. Baker

Associate Professor
Fellow of the Marion E. Forsman Centennial Professorship in Engineering
Department of Biomedical Engineering
Institute for Cellular and Molecular Biology
Institute for Computational Engineering and Sciences
Institute for Biomaterials, Drug Delivery & Regenerative Medicine
University of Texas at Austin
Austin, TX 78712
Phone: (512) 232-7114
Email: abbaker@austin.utexas.edu

TITLE:

Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits

AUTHORS & AFFILIATIONS:

Andrew D. Sligar[1*], Gretchen Howe[2,3*], Julia Goldman[2,3], Patricia Felli[2,3], Varsha Karanam[1], Richard W. Smalling[2,3] and Aaron B. Baker[1,4,5,6]

[1]University of Texas at Austin, Department of Biomedical Engineering, Austin, TX
[2]Division of Cardiology, University of Texas McGovern Medical School, Houston, TX
[3]Memorial Herman Heart and Vascular Center, Texas Medical Center, Houston, TX
[4]Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX
[5]The Institute for Computational Engineering and Sciences, University of Texas at Austin, Austin, TX
[6]Institute for Biomaterials, Drug Delivery and Regenerative Medicine, University of Texas at Austin, Austin, TX
[*]These authors contributed equally

Corresponding Author:

Aaron B. Baker, Ph.D. (abbaker1@gmail.com)
Tel: 512-232-7114

Email Addresses of Co-Authors:

Andrew Sligar (asligar@utexas.edu)
Gretchen Howe (Gretchen.Howe@uth.tmc.edu)
Julia Goldman (Julia.L.Goldman@uth.tmc.edu)
Patricia Felli (Patricia.R.Felli@uth.tmc.edu)
Varsha Karanam (varshakaranam@utexas.edu)
Richard W. Smalling (richard.w.smalling@uth.tmc.edu)

KEYWORDS:

hind limb ischemia, peripheral arterial disease, peripheral vascular disease, rabbits, diabetes, hyperlipidemia, angiography

SUMMARY:

We describe a surgical procedure used to induce peripheral ischemia in rabbits with hyperlipidemia and diabetes. This surgery acts as a preclinical model for conditions experienced in peripheral artery disease in patients. Angiography is also described as a means to measure the extent of introduced ischemia and recovery of perfusion.

ABSTRACT:

Peripheral vascular disease is a widespread clinical problem that affects millions of patients worldwide. A major consequence of peripheral vascular disease is the development of ischemia. In severe cases, patients can develop critical limb ischemia in which they experience constant pain and an increased risk of limb amputation. Current therapies for peripheral ischemia include

bypass surgery or percutaneous interventions such as angioplasty with stenting or atherectomy to restore blood flow. However, these treatments often fail to the continued progression of vascular disease or restenosis or are contraindicated due to the overall poor health of the patient. A promising potential approach to treat peripheral ischemia involves the induction of therapeutic neovascularization to allow the patient to develop collateral vasculature. This newly formed network alleviates peripheral ischemia by restoring perfusion to the affected area. The most frequently employed preclinical model for peripheral ischemia utilizes the creation of hind limb ischemia in healthy rabbits through femoral artery ligation. In the past, however, there has been a strong disconnect between the success of preclinical studies and the failure of clinical trials regarding treatments for peripheral ischemia. Healthy animals typically have robust vascular regeneration in response to surgically induced ischemia, in contrast to the reduced vascularity and regeneration in patients with chronic peripheral ischemia. Here, we describe an optimized animal model for peripheral ischemia in rabbits that includes hyperlipidemia and diabetes. This model has reduced collateral formation and blood pressure recovery in comparison to a model with a higher cholesterol diet. Thus, the model may provide better correlation with human patients with compromised angiogenesis from the common co-morbidities that accompany peripheral vascular disease.

INTRODUCTION:

Peripheral arterial disease (PAD) is a common circulatory disorder in which the progression of atherosclerotic plaque formation leads to a narrowing of blood vessels in the limbs of the body. The recent increase in risk factors for atherosclerosis, including diabetes, obesity, and inactivity, has led to increasing prevalence of vascular disease[1]. Currently, it is estimated that 12%–20% of the general population over 60 years old has peripheral arterial disease[2]. A major consequence of peripheral arterial disease is the development of peripheral ischemia, most commonly found in the lower limbs. In severe cases, patients can develop critical limb ischemia, a state in which there is constant pain due to a lack of blood flow. Patients with critical limb ischemia have a 50% likelihood of having one limb amputated within one year of diagnosis. Furthermore, patients with diabetes have a higher incidence of peripheral arterial disease and poorer outcomes following interventions for revascularization[3,4]. Current therapies for peripheral ischemia include percutaneous interventions such as atherectomy and stenting or surgical bypass. However, for many patients these treatments only provide short-term benefits and many are not healthy enough for major surgical procedures. In this work, we describe a preclinical animal model for testing new treatments targeting peripheral vascular disease that incorporates the generation of peripheral ischemia in rabbits through surgical ligation in the context of the diabetic disease state.

The hind limb ischemia model in rabbits has been used as a physiological model for obstructive vascular disease and preclinical precursor to human studies for over half a century[5,6]. Rabbits are often a preferred species for studies on peripheral ischemia due to the developed musculature of the ankle and calf muscle, in contrast to common large animal models that are ungulates (animals with hooves). Several recent reviews have addressed the use of this model and others in modeling peripheral vascular disease in humans[7,8]. Similar models using hind limb ischemia in rabbits were used in preclinical studies of growth factors[9–20], gene therapy[21–44], and stem cells[45–

[51] for therapeutic neovascularization in the limbs. Unfortunately, the clinical trials that followed these successful animal studies did not show significant benefits for patients[52].

One suggested explanation of the reason for this translational failure is that the condition of peripheral ischemia in human patients is one that includes resistance to angiogenic signals[53–59]. Several studies have shown defects in angiogenic signaling pathways in diabetes and hyperglycemia. Diabetes and hyperlipidemia lead to a loss of heparan sulfate proteoglycans and an increase in enzymes that cut heparan sulfate, presenting a potential mechanism for resistance to therapeutic angiogenesis/arteriogenesis with growth factors[60,61]. Thus, a key feature of a model for peripheral ischemia should include an aspect of therapeutic resistance so that therapies may be evaluated in the context of the disease state present in human patients.

In this work, we describe a rabbit model of peripheral ischemia through surgical ligation of the femoral arteries. A lead-in period with the induction of diabetes and hyperlipidemia is incorporated into the model. We compared this model to another model that incorporates a higher fat diet without diabetes and found that the model with diabetes and lower level of hyperlipidemia was more effective in reducing blood vessel growth. Our model combines advancements that have been used by separate groups, with the goal of providing a practical and standardized method to achieve consistent results in peripheral vascular disease research.

PROTOCOL:

Studies involving animals were performed with the approval of the University of Texas at Austin and the UTHealth Science Center at Houston Institutional Animal Care and Use Committee (IACUC), the Animal Care and Use Review Office (ACURO) of The United States Army Medical Research and Materiel Command Office of Research Protections, and in accordance with NIH guidelines for animal care.

1. Induction of diabetes and hyperlipidemia

1.1. Transition the New Zealand rabbits (4–6 months old) from one cup of standard alfalfa chow to 0.1% cholesterol chow over the course of four days. For days 1–5, use standard chow to cholesterol chow ratios of 1:0, 3:1, 1:1, 1:3, and 0:1, respectively. After two weeks on 0.1% cholesterol chow, induce rabbits to have diabetes using alloxan injection as described in the following steps

1.2. Sedate the rabbits using 35–75 mg/mL ketamine and 1–2 mg/mL acepromazine via subcutaneous injection and prep for an IV injection by introducing a catheter into the marginal left ear vein using a 22 g catheter.

1.3. Collect a drop of blood from the rabbits via hub of the ear vein catheter for the baseline blood glucose level (BGL) measurement. Any standard glucometer can be used. Normal glucose levels for a rabbit are typically in the range of 80 to 150 mg/dL.

1.4. Inject alloxan at 100 mg/kg in saline through the ear catheter slowly over a 10-minute period using a syringe pump.

1.5. Check the BGL every hour for the next 12 h using a standard glucometer to monitor for hypoglycemia.

1.5.1. Take blood from the rabbits by using a restrainer.

1.5.2. Anesthetize the ear with 2.5% lidocaine/2.5% prilocaine cream.

1.5.3. Take blood from the lateral ear vein using a 25 G needle and measure BGL using a standard meter.

1.6. Measure the BGL two times a day for the first 7 days. Give the rabbits an injection of insulin if the BGL reaches or exceeds 350 mg/dL.

1.7. Prepare a 3-mm stainless steel ball for implantation as a size marker during angiograms prior to the day of surgery.

1.7.1. Cut a 10-mm circular piece of silastic sheeting out of a larger sheet using a biopsy punch.

1.7.2. Mount the ball in the center of the sheet using clear silicone sealant.

1.7.3. Completely cover the ball with the sealant. Allow the sealant to cure for a minimum of 24 h.

1.7.4. Place the ball in an open 2 inch x 3 inch low density polyethylene bag and place it into a sterilization bag to be sterilized with ethylene oxide gas.

2. Preparation of rabbit for surgery

2.1. Anesthetize the rabbit using 20–40 mg/kg ketamine and 2 mg/kg midazolam via subcutaneous injection. Place the rabbit on 1.5%–3% isoflurane (typically 2%) throughout the initial sedation using a mask. Give an injection of alfaxalone to maintain anesthesia via an intramuscular injection of 3 mg/kg.

2.2. Once anesthetized, remove the mask and insert a cuffed endotracheal tube, connected to a ventilator, into the airway. Continue to administer isoflurane at 1.5%–3%.

2.3. Collect blood from the central artery from either ear for a baseline chemistry panel.

2.4. Place a 22 G ear vein catheter in the lateral ear vein for Lactated Ringer's Solution drip throughout the surgical procedure. Alternatively, normal saline (0.9% sodium chloride) can be used.

2.5. Using the lateral vein in the opposite ear, place a catheter in the vein and deliver alfaxalone at 6 mg/kg/h. Gradually increase the alfaxalone to 8 mg/kg/h while decreasing isoflurane to 0.6% during the prep period.

2.6. To limit pain and risk of infection, administer buprenorphine (0.01 mg/kg) and enrofloxacin (5 mg/kg) using a subcutaneous injection with a 25 G needle.

2.7. Trim the hair on the neck, right and left inner thighs, and back using clippers (#40 blade). Remove the hair on the back to maintain contact with the grounding pad.

2.8. Place a blood pressure cuff on each of the hind limbs and measure initial blood pressure. Place the cuff just below the knee with the probe just above the hock on the lateral surface.

2.9. Position the rabbit on the surgery table on its back and scrub and drape the surgery sites. This includes the neck for carotid artery access and inner right thigh for femoral artery access. Perform the sterilization scrub with alternating scrubs of 2% chlorhexidine and 70% ethyl alcohol. Repeat this three times, then apply a final spray with 2% chlorhexidine solution.

2.10. Place a 3-mm stainless steel ball that has been sterilized inside a low density polyethyene bag on top of the right (scrubbed) leg near the upper part of the thigh to serve as a size reference during angiogram measurements. Place a sterile drape over the leg until the time of surgery. Leave the ball inside the sterile plastic bag during the first angiogram.

3. Angiography

3.1. Expose the right common carotid artery

3.1.1. Make a 4–5 cm long incision just lateral to the trachea using a scalpel with a #15 blade.

3.1.2. Use blunt dissection to expose the carotid artery and open the incision using small Weitlaner retractors. Carefully isolate the carotid artery from the jugular vein and vagus nerve. Typically, a curved Metzenbaum scissors and a curved mosquito hemostat are used for the blunt dissection. Be sure to get full separation of the carotid artery from the nerve and jugular vein to make the ligatures only ligate the artery.

3.2. Place a ligature using a 4-0 silk suture at the proximal and distal ends of the exposed artery. Tie off the distal end of carotid with a surgeon’s knot followed by four square knots. On the proximal end, use a ligaloop to allow it to be tightened or loosened as needed. The use of a ligaloop placed at the proximal end of the exposed artery can help secure the introducer and catheter.

3.3. Administer 500 IU of heparin through the IV. Use approximately 0.5 mL of 1% lidocaine applied along the exposed carotid to dilate the vessel. One treatment is usually sufficient, but it can be repeated as needed. Place the 4-inch wire insertion tool into the artery.

3.4. Feed a 0.014 inch x 185 cm guidewire through the insertion tool to the aortic bifurcation at the iliac crest in the descending aorta. Remove the insertion tool and insert a 3F pigtail angiographic catheter over the wire.

3.5. Advance the pigtail catheter to be 2 cm proximal to the aortic bifurcation at the iliac crest in the descending aorta.

3.6. Position the tip of the catheter between the seventh lumbar and first sacral vertebrae. Test the location of the catheter by manually injecting a 2–4 mL of contrast agent.

3.7. Administer an intra-arterial injection of 100 µg nitroglycerin through the catheter to increase vasodilation.

3.8. Administer 4 mL of 1% lidocaine to the rabbit through the catheter to prevent movement during the angiogram. Attach the tubing for the injector to the catheter and remove any air bubbles in the line. Inject 8 mL of contrast media using automated angiographic injector through the catheter.

3.9. Record serial images of the hind limbs using angiography.

3.9.1. Set the power injector to inject contrast at 3 mL/sec for a total of 8 mL. Perform digital subtraction angiography at 6 frames per second.

3.9.2. Select the serial images created and alter a photo of each angiogram using approximately -40% setting to minimize appearance of bone and capture a complete picture of the vessel perfusion with contrast. An example angiogram of the vascular flow after femoral artery ligation/excision is shown in **Figure 1**.

4. Isolation of the femoral artery

4.1. Make a longitudinal incision in the skin over the right femoral artery using a scalpel (#15 blade). Ensure that the incision extends inferiorly from the inguinal ligament ending at the area just proximal to the patella (approximately 6 cm).

4.2. Use blunt dissection with curved Metzenbaum scissors or a curved mosquito hemostat to expose the femoral artery.

4.3. Use Weitlaner retractors to hold the incision open.

4.4. Add 0.5 mL of 1% lidocaine locally to reduce nerve irritation and promote vasodilation.

4.5. Continue blunt dissection of the tissues to free the entire length of the femoral artery along with all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries (**Figure 2A**).

4.6. Dissect further along the popliteal and saphenous arteries as well as the external iliac artery (**Figure 2A**). Periodically moisten the area with saline to protect from tissue damage. If the blunt dissection is performed along the femoral groove (between the muscles) there is no need to cut the muscle.

4.7. Carefully separate the artery from the vein and nerve as shown in **Figure 2B,C**. Ligate the arteries indicated by the diagram with 4.0 silk sutures by placing two ties with enough space between them to cut the artery. These ties are performed with a Surgeon's knot followed by four square knots.

4.8. Cut between the two ties on the ligated arteries using the small Metzenbaum scissors. Excise the femoral artery from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates to form the saphenous and popliteal arteries.

5. Repeat angiography

5.1. Using the attached silastic sheet, attach the 3-mm stainless steel ball into the upper part of the quadriceps muscle using 4-0 silk sutures. Pull the skin over the ball after it is in place.

5.2. Administer an intra-arterial injection of 100 µg nitroglycerin through the catheter to increase vasodilation.

5.3. If needed, administer another 4 mL of 1% lidocaine to the rabbit through the catheter to prevent movement during the angiogram.

5.4. Inject 8 mL of contrast media using an automated angiographic injector.

5.5. Perform angiography as described in step 3.11.

6. Wound closure and recovery

6.1. Remove the catheter from the right artery. Tie off the artery using the 4-0 silk suture that is already in place around the artery.

6.2. Suture all of the wounds closed. Close muscle and subcuticular layers using 4-0 polydioxanone or 3-0 polyglactin 910 on a taper needle (see **Table of Materials**) in a continuous suture pattern. Close the skin using 4-0 polydioxanone or 4-0 polyglactin 910 on a reverse cutting needle (see **Table of Materials**) in a buried continuous subcuticular suture pattern.

NOTE: If available, polydioxanone is preferred for both.

6.3. Administer intradermal injections of 0.25% bupivacaine near the incisions using a syringe with a 25 G needle. Insert the needle and inject 0.5 mL while the needle is pulled back. Give one injection per side of the wound for the incision on the neck (two injections on the neck) and two injections per side of the wound for the incision on the leg (four injections on the leg; six injections in total). The total volume injected is 3 mL (0.5 mL x 6 injections).

6.4. Administer subcutaneous injections of 0.5 mg/kg meloxicam and sustained release buprenorphine at 0.12 mg/kg.

6.5. Monitor the rabbit as it recovers from anesthesia. The rabbit will automatically start to swallow as it wakes up from anesthesia. Once the swallowing response occurs, remove the endotracheal tube. Provide close monitoring and thermal support until the rabbit is able to maintain cardiovascular function and body temperature. Return the rabbit to its enclosure once it is able to ambulate.

6.6. Employ fresh vegetables and/or syringe feeding of a critical care diet along with subcutaneous saline injections if the rabbit does not tolerate chow after the surgery. Cabbage, broccoli, cauliflower, carrots, or other in season vegetables can be used. Shred the vegetables and mix them together to aid in the rabbit returning to eating.

7. Monitoring

7.1. Anesthetize the rabbits every two weeks to acquire blood pressure on both legs as described in step 2.8. Harvest blood from the central artery of the ear for use in blood chemistry assays. Alternatively, take blood from the saphenous vein or cephalic vein. Take approximately 2 mL at each time point. Use a standard blood chemistry panel for analysis. If needed, add tests for low density lipoprotein (LDL), high density lipoprotein (HDL), or hemoglobin A1c (HbA1c).

7.2. Take a very small amount of blood for BGL measurements.

8. Treatment

8.1. Prepare ten syringes with treatment, carrier, and crosslinker. Fill each syringe just prior to use with 100 µL of calcium sulfate slurry and then 100 µL of 2% sodium alginate with growth factors or other treatments such that the alginate is nearest the tip of the syringe.

8.2. Administer one prepared injection into the muscle before preparing the next. This reduces the time that the alginate interacts with the calcium sulfate in the syringe. Space the injections evenly along both sides of the femoral artery on the thigh. To achieve uniform injections, create a silicone sheet with holes to guide the injection, as described in other studies[19]. This can be easily prepared by using a biopsy punch to create holes in commercially available silicone sheeting.

9. Endpoint angiography, euthanasia, perfusion fixation and tissue harvest

9.1. At the endpoint date, perform angiography as described in step 3 but use the left carotid artery for access.

9.2. After angiography, move the animal to the necropsy table and perform perfusion fixation to preserve the hind limb tissues:

9.2.1. Increase the isoflurane to 3%–4% and perform a toe pinch to confirm the anesthesia is sufficiently deep.

9.2.2. Administer 1000 IU of heparin intravenously.

9.2.3. Create an incision along the midline of the ribcage and spanning the length of the diaphragm using a scalpel with a #20 blade.

9.2.4. With the rib cage exposed, cut the ribs just left of the midline using rib cutters. Use Weitlaner retractors to expose the heart.

9.2.5. Set up the pump with output tubing with an inner diameter of 1/8 inch and a 18G needle at the end. Preload the line with saline and have at least 600 mL of saline and formalin prepared in separate containers for the perfusion.

9.2.6. Insert the 18 G needle connected to the pump into the left ventricle via the apex of the heart. Insert another 18 G needle (unattached to anything) into the right atrium and allow blood to flow out into the downdraft of the necropsy table.

9.2.7. Use a perfusion pump to control the flow of approximately 500 mL of saline into the heart. Use a pump setting to flow 110 mL/min.

9.2.8. Once the fluid coming from the heart is clear, move the tubing from the saline reservoir to one filled with a 10% formalin solution. Twitching will occur in all four limbs if the perfusion is working properly. Pump approximately 500 mL of formalin solution into the left ventricle.

9.2.9. Turn the pump off and remove the needles from the heart.

9.3. Remove both hind limbs at the hip by cutting around the hip joint with a scalpel with #20 blade. Use a small rib cutter to remove the limbs. Use the non-ischemic limb as a control.

9.4. Store the limbs in formalin for 24 h at 4 °C and then stored in 70% ethanol at 4 °C.

9.5. For histological analysis, take multiple biopsies from the limbs. We have used eight 6-mm biopsies taken at regions across the thigh and calf in both limbs.

NOTE: While ankle blood pressure measurement and angiography are the most commonly used methods for measuring recovery of blood flow, other methods can be used to track recovery of the animals including Doppler ultrasound, laser Doppler imaging, infrared thermography[62], microsphere determined perfusion[63,64], computed tomography (CT) imaging, and magnetic resonance imaging (MRI)[65].

REPRESENTATIVE RESULTS:

Following induction of diabetes and initiation of the 0.1% cholesterol diet, the total cholesterol for the rabbits with diabetes and cholesterol diet was 123.3 ± 35.1 mg/dL (n = 6 male rabbits) averaged overall time points and rabbits. The BGL level for these rabbits was 248.3 ± 50.4 mg/dL (n = 6 male rabbits). A time course for blood chemistries and leg blood pressure ratios in a typical rabbit is shown in **Figure 3** in comparison to rabbits under a higher cholesterol diet (1% cholesterol). In non-diabetic animals, even with higher cholesterol, we found that there was increased recovery of the blood pressure in the ischemic limb and vascularity in the angiograms at the final time point (**Figure 3**). The animals on the higher cholesterol/fat diet also had increased issues with liver failure and death before the study endpoint. Thus, diabetes with a lower level of cholesterol led to more compromised perfusion at the study endpoint. Histologically, there are changes in the muscle structure consistent with edema and ischemic damage in some locations **Figure 4**. In some cases, one can observe changes/damage in the muscle fibers due to the ischemia. This can be observed as loss or disruption of the muscle fibers in the histological analysis, as has been observed in some hind limb ischemia models in mice. However, care is needed to distinguish these changes from histological artifacts of the tissue processing. Immunostaining for PECAM and αSMA can be used to identify the number of vessels and larger vessels in the tissue sections (**Figure 4**). Overall, the model using diabetes with a lower level cholesterol diet produced repeatable deficits in blood pressure and vascularization over the higher cholesterol diet model without diabetes.

FIGURE LEGENDS:

Figure 1: Angiograms for the hind limb of a diabetic and non-diabetic rabbit pre-surgery, post-surgery and after recovery for 70 days after femoral artery ligation and excision. (**A**) Angiogram of ischemic limb (left) and contralateral control limb (right). (**B**) Enlarged image of the ischemic limb at the site of ligation.

Figure 2: Induction of hind limb ischemia in rabbits through femoral artery ligation and excision. (**A**) Illustration of the vascular anatomy of the rabbit hind limb. Place ties at all the points marked to ligate the arteries. Modified and used with permission[71]. (**B**) Surgical field showing the cut down to the femoral artery prior to ligation. (**C**) Femoral arteries with ligations in place to induce hind limb ischemia.

Figure 3: Typical blood pressure and blood chemistries for the rabbits with hind limb ischemia over the course of the model. The Diabetic / MC group was induced to have diabetes and given a 0.1% cholesterol diet. The Non-Diabetic / HC group was given a 1% cholesterol diet. BGL = Blood Glucose Level. TC = Total Cholesterol. LIPA = Lipoprotein (a). BP = Blood pressure ratio between the ischemic and non-ischemic limb.

Figure 4: Histological analysis of the muscle of the hind limb in diabetic rabbits 70 days after femoral artery ligation. H&E staining as well as immunohistochemical staining for the endothelial marker, PECAM, and vascular smooth muscle cell marker, αSMA, was performed. Tissue samples were biopsied from the ischemic limb and the non-ischemic contralateral control limb.

DISCUSSION:

We have presented a preclinical model for inducing hind limb ischemia in rabbits with diabetes and hyperlipidemia. In many studies, there is ambiguity to the technique used to create hind limb ischemia in rabbits. In mice, the severity and recovery from hind limb ischemia is highly dependent on the location the ligation and technique used to induce ischemia. The significance of the technique presented in this work is that it allows for the consistent induction of ischemia that does not fully recover after 8 weeks in diabetic animals. Notably, when animals were given a higher cholesterol and fat diet, they were able to recover to near baseline levels of limb blood pressure ratio. In addition, on the higher fat diet the animals had alterations in liver enzymes suggesting liver damage. Thus, the diabetic model with a lower level of cholesterol/fat appears to be a more consistent and relevant model of chronic ischemia in the limb.

Four essential steps can be highlighted within this model including induction of diabetes, angiography, surgical ligation of the femoral arteries and application of treatment. Among these steps, the induction of diabetes was one of the most critical steps and one that may require further optimization for each laboratory. The rate of alloxan injection is a major factor that alters the toxicity and effectiveness of induction of diabetes by alloxan for rabbits. When injected too quickly, alloxan caused instability in the BGL and death in the rabbits. This can sometimes be observed as hypoglycemia that is not resolved through injections of dextrose solutions or in other cases extremely high BGL. If injected too slowly the rabbits often fail to become diabetic. It is possible that this parameter will need to be optimized for rabbits from different sources. Rabbits will typically become hyperglycemic for 1–3 h, but the BGL will then begin to drop. Therefore, usually no insulin is administered on the day of diabetes induction. However, if the BGL drops below 100 mg/dL in the first 24 h, it can be increased by injecting 10.0 mL of 5% dextrose solution subcutaneously or by changing the water supply to a 10% dextrose solution (typically overnight is sufficient). Whenever insulin is administered an extra BGL test is done to ensure the glucose levels do not drop too low. The insulin responsiveness often varies for each rabbit. Thus, individual dosing regimens are used to normalize the BGL based on how the rabbit responds to the insulin. Diabetes is typically induced after 2–3 days following the alloxan injection.

As a preclinical model of peripheral vascular disease and limb ischemia, the model presented does have some potential limitations. The induction of diabetes with alloxan is leads to rapid development of type I diabetes. This is in contrast to the chronic development of type II diabetes that is most prevalent in human patients. Moreover, ischemia is developed acutely due to surgical ligation rather than due to chronic development of vascular disease and atherosclerotic plaques. A fundamental limitation of using rabbits is their fragility as an animal model. The animals will only tolerate a limited amount of hyperlipidemia in combination with type I diabetes and optimizing the maximum amount of disease without having the animal die was a major goal

in creating this protocol. Our group has hypothesized that patients with peripheral ischemia develop therapeutic resistance to angiogenic growth factors and that this may play a major role in the failure of growth factor-based therapeutics for ischemia[66]. To this end, we have shown a loss in cell surface proteoglycans and an increase in heparanase in animal and human tissue samples[55,58,67–70]. It is unknown whether the rabbit model described here demonstrates growth factor resistance, although the observation that there is longer term ischemia with diabetes and moderate hyperlipidemia model in comparison to the high hyperlipidemia model would suggest there is some deficit in the revascularization process.

For the inclusion of treatments into the model, it is important to have a recovery period following the induction of ischemia to allow the acute healing phase to occur without intervention. If therapies are given during this time, the response would be more relevant to enhancing the response to acute ischemia rather than the chronic ischemia that characterizes peripheral vascular disease. Such a model may be relevant to acute ischemic injury in trauma or thrombosis, but would likely not provide good correlation with chronic ischemia. Given the poor correlation between positive results in preclinical models of ischemia in healthy animals and the results of clinical trials, the inclusion of diabetes or another factor that reduces vascular regeneration is essential for attempting to recapitulate limb ischemia in humans for the creation of future therapies.

ACKNOWLEDGEMENTS:

The authors gratefully acknowledge funding through the Department of Defense Congressionally Directed Research Program (DOD CDMRP; W81XWH-16-1-0582) to ABB and RS. The authors also acknowledge funding through the American Heart Association (17IRG33410888), the DOD CDMRP (W81XWH-16-1-0580) and the National Institutes of Health (1R21EB023551-01; 1R21EB024147-01A1; 1R01HL141761-01) to ABB.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Mozaffarian D. et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation.* **133** (4), e38-360 (2016).

2 Roger, V. L. et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation.* **123** (4), e18-e209 (2011).

3 Shammas, A. N. et al. Major Limb Outcomes Following Lower Extremity Endovascular Revascularization in Patients With and Without Diabetes Mellitus. *Journal of Endovascular Therapy.* **24** (3), 376-382 (2017).

4 Tunstall-Pedoe, H., Peters, S. A. E., Woodward, M., Struthers, A. D., Belch, J. J. F. Twenty-Year Predictors of Peripheral Arterial Disease Compared With Coronary Heart Disease in the Scottish Heart Health Extended Cohort (SHHEC). *Journal of the American Heart Association.* **6** (9), e005967 (2017).

5 Whiteley, H. J., Stoner, H. B., Threlfall, C. J. The effect of hind limb ischaemia on the physiological activity of rabbit skin. *British Journal of Experimental Pathology.* **34** (4), 365-375 (1953).

6 Longland, C. J. Collateral circulation in the limb. *Postgraduate Medical Journal.* **29** (335), 456-458 (1953).

7 Waters, R. E., Terjung, R. L., Peters, K. G., Annex, B. H. Preclinical models of human peripheral arterial occlusive disease: implications for investigation of therapeutic agents. *Journal of Applied Physiology.* **97** (2), 773-780 (2004).

8 Krishna, S. M., Omer, S. M., Golledge, J. Evaluation of the clinical relevance and limitations of current pre-clinical models of peripheral artery disease. *Clinical Science (London).* **130** (3), 127-150 (2016).

9 Zhou, J. et al. Therapeutic angiogenesis using basic fibroblast growth factor in combination with a collagen matrix in chronic hindlimb ischemia. *ScientificWorldJournal.* **2012**, 652794 (2012).

10 Prochazka, V. et al. Therapeutic Potential of Adipose-Derived Therapeutic Factor Concentrate for Treating Critical Limb Ischemia. *Cell Transplantation.* **25** (9), 1623-1633 (2016).

11 Cao, R. et al. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nature Medicine.* **9** (5), 604-613 (2003).

12 Doi, K. et al. Enhanced angiogenesis by gelatin hydrogels incorporating basic fibroblast growth factor in rabbit model of hind limb ischemia. *Heart and Vessels.* **22** (2), 104-108 (2007).

13 Nitta, N. et al. Vascular regeneration by pinpoint delivery of growth factors using a microcatheter reservoir system in a rabbit hind-limb ischemia model. *Experimental and Therapeutic Medicine.* **4** (2), 201-204 (2012).

14 Karatzas, A. et al. NGF promotes hemodynamic recovery in a rabbit hindlimb ischemic model through trkA- and VEGFR2-dependent pathways. *Journal of Cardiovascular Pharmacology.* **62** (3), 270-277 (2013).

15 Stachel, G. et al. SDF-1 fused to a fractalkine stalk and a GPI anchor enables functional neovascularization. *Stem Cells.* **31** (9), 1795-1805 (2013).

16 Asahara, T. et al. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation.* **92** (9 Suppl), II365-371 (1995).

17 Morishita, R. et al. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. *Hypertension.* **33** (6), 1379-1384 (1999).

18 Walder, C. E. et al. Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. *Journal of Cardiovascular Pharmacology.* **27** (1), 91-98 (1996).

19 Anderson, E. M. et al. VEGF and IGF Delivered from Alginate Hydrogels Promote Stable Perfusion Recovery in Ischemic Hind Limbs of Aged Mice and Young Rabbits. *Journal of Vascular Research.* **54** (5), 288-298 (2017).

20 Xie, J. et al. Induction of angiogenesis by controlled delivery of vascular endothelial growth factor using nanoparticles. *Cardiovascular Therapeutics.* **31** (3), e12-18 (2013).

21 Olea, F. D. et al. Vascular endothelial growth factor overexpression does not enhance adipose stromal cell-induced protection on muscle damage in critical limb ischemia. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **35** (1), 184-188 (2015).
22 Ohara, N. et al. Adenovirus-mediated ex vivo gene transfer of basic fibroblast growth factor promotes collateral development in a rabbit model of hind limb ischemia. *Gene Therapy.* **8** (11), 837-845 (2001).
23 Pyun, W. B. et al. Naked DNA expressing two isoforms of hepatocyte growth factor induces collateral artery augmentation in a rabbit model of limb ischemia. *Gene Therapy.* **17** (12), 1442-1452 (2010).
24 Kupatt, C. et al. Cotransfection of vascular endothelial growth factor-A and platelet-derived growth factor-B via recombinant adeno-associated virus resolves chronic ischemic malperfusion role of vessel maturation. *Journal of the American College of Cardiology.* **56** (5), 414-422 (2010).
25 Olea, F. D. et al. Repeated, but not single, VEGF gene transfer affords protection against ischemic muscle lesions in rabbits with hindlimb ischemia. *Gene Therapy.* **16** (6), 716-723 (2009).
26 Pinkenburg, O. et al. Recombinant adeno-associated virus-based gene transfer of cathelicidin induces therapeutic neovascularization preferentially via potent collateral growth. *Human Gene Therapy.* **20** (2), 159-167 (2009).
27 Katsu, M. et al. Ex vivo gene delivery of ephrin-B2 induces development of functional collateral vessels in a rabbit model of hind limb ischemia. *Journal of Vascular Surgery.* **49** (1), 192-198 (2009).
28 Korpisalo, P. et al. Therapeutic angiogenesis with placental growth factor improves exercise tolerance of ischaemic rabbit hindlimbs. *Cardiovascular Research.* **80** (2), 263-270 (2008).
29 Chen, F., Tan, Z., Dong, C. Y., Chen, X., Guo, S. F. Adeno-associated virus vectors simultaneously encoding VEGF and angiopoietin-1 enhances neovascularization in ischemic rabbit hind-limbs. *Acta Pharmacologica Sinica.* **28** (4), 493-502 (2007).
30 Kobayashi, K. et al. Combination of in vivo angiopoietin-1 gene transfer and autologous bone marrow cell implantation for functional therapeutic angiogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **26** (7), 1465-1472 (2006).
31 Lee, J. U. et al. A novel adenoviral gutless vector encoding sphingosine kinase promotes arteriogenesis and improves perfusion in a rabbit hindlimb ischemia model. *Coronary Artery Disease.* **16** (7), 451-456 (2005).
32 Nishikage, S. et al. In vivo electroporation enhances plasmid-based gene transfer of basic fibroblast growth factor for the treatment of ischemic limb. *Journal of Surgical Research.* **120** (1), 37-46 (2004).
33 Ishii, S. et al. Appropriate control of ex vivo gene therapy delivering basic fibroblast growth factor promotes successful and safe development of collateral vessels in rabbit model of hind limb ischemia. *Journal of Vascular Surgery.* **39** (3), 629-638 (2004).
34 Tokunaga, N. et al. Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector, gelatin. *Circulation.* **109** (4), 526-531 (2004).

35 Yamauchi, A. et al. Pre-administration of angiopoietin-1 followed by VEGF induces functional and mature vascular formation in a rabbit ischemic model. *Journal of Gene Medicine.* **5** (11), 994-1004 (2003).

36 Zhong, J. et al. Neovascularization of ischemic tissues by gene delivery of the extracellular matrix protein Del-1. *Journal of Clinical Investigation.* **112** (1), 30-41 (2003).

37 Shyu, K. G., Chang, H., Isner, J. M. Synergistic effect of angiopoietin-1 and vascular endothelial growth factor on neoangiogenesis in hypercholesterolemic rabbit model with acute hindlimb ischemia. *Life Sciences.* **73** (5), 563-579 (2003).

38 Kasahara, H. et al. Biodegradable gelatin hydrogel potentiates the angiogenic effect of fibroblast growth factor 4 plasmid in rabbit hindlimb ischemia. *The Journal of the American College of Cardiology.* **41** (6), 1056-1062 (2003).

39 Rissanen, T. T. et al. Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. *FASEB Journal.* **17** (1), 100-102 (2003).

40 Taniyama, Y. et al. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hindlimb ischemia models: preclinical study for treatment of peripheral arterial disease. *Gene Therapy.* **8** (3), 181-189 (2001).

41 Vincent, K. A. et al. Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor. *Circulation.* **102** (18), 2255-2261 (2000).

42 Gowdak, L. H. et al. Induction of angiogenesis by cationic lipid-mediated VEGF165 gene transfer in the rabbit ischemic hindlimb model. *Journal of Vascular Surgery.* **32** (2), 343-352 (2000).

43 Shyu, K. G., Manor, O., Magner, M., Yancopoulos, G. D., Isner, J. M. Direct intramuscular injection of plasmid DNA encoding angiopoietin-1 but not angiopoietin-2 augments revascularization in the rabbit ischemic hindlimb. *Circulation.* **98** (19), 2081-2087 (1998).

44 Witzenbichler, B. et al. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *The American Journal of Pathology.* **153** (2), 381-394 (1998).

45 Prochazka, V. et al. The Role of miR-126 in Critical Limb Ischemia Treatment Using Adipose-Derived Stem Cell Therapeutic Factor Concentrate and Extracellular Matrix Microparticles. *Medical Science Monitor.* **24** 511-522 (2018).

46 Wang, J. et al. A cellular delivery system fabricated with autologous BMSCs and collagen scaffold enhances angiogenesis and perfusion in ischemic hind limb. *Journal of Biomedical Materials Research Part A.* **100** (6), 1438-1447 (2012).

47 Hao, C. et al. Therapeutic angiogenesis by autologous adipose-derived regenerative cells: comparison with bone marrow mononuclear cells. *American Journal of Physiology - Heart and Circulatory Physiology.* **307** (6), H869-879 (2014).

48 Nemoto, M. et al. Adequate Selection of a Therapeutic Site Enables Efficient Development of Collateral Vessels in Angiogenic Treatment With Bone Marrow Mononuclear Cells. *Journal of the American Heart Association.* **4** (9), e002287 (2015).

49 Mikami, S. et al. Autologous bone-marrow mesenchymal stem cell implantation and endothelial function in a rabbit ischemic limb model. *PLoS One.* **8** (7), e67739 (2013).

50 Wang, S. et al. Transplantation of vascular endothelial growth factor 165transfected endothelial progenitor cells for the treatment of limb ischemia. *Molecular Medicine Reports.* **12** (4), 4967-4974 (2015).
51 Yin, T. et al. Genetically modified human placentaderived mesenchymal stem cells with FGF2 and PDGFBB enhance neovascularization in a model of hindlimb ischemia. *Molecular Medicine Reports.* **12** (4), 5093-5099 (2015).
52 Annex, B. H. Therapeutic angiogenesis for critical limb ischaemia. *Nature Reviews Cardiology.* **10** (7), 387-396 (2013).
53 Das, S. et al. Syndesome Therapeutics for Enhancing Diabetic Wound Healing. *Advanced Healthcare Materials.* **5** (17), 2248-2260 (2016).
54 Jang, E., Albadawi, H., Watkins, M. T., Edelman, E. R., Baker, A. B. Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (5), 1679-1684 (2012).
55 Monteforte, A. J. et al. Glypican-1 nanoliposomes for potentiating growth factor activity in therapeutic angiogenesis. *Biomaterials.* **94** 45-56 (2016).
56 Das, S. et al. Syndecan-4 Enhances Therapeutic Angiogenesis after Hind limb Ischemia in Mice with Type 2 Diabetes. *Advanced Healthcare Materials.* **5** (9), 1008-1013 (2016).
57 Das, S., Majid, M., Baker, A. B. Syndecan-4 enhances PDGF-BB activity in diabetic wound healing. *Acta Biomaterialia.* **42** 56-65 (2016).
58 Das, S., Singh, G., Baker, A. B. Overcoming disease-induced growth factor resistance in therapeutic angiogenesis using recombinant co-receptors delivered by a liposomal system. *Biomaterials.* **35** (1), 196-205 (2014).
59 Kikuchi, R. et al. An antiangiogenic isoform of VEGF-A contributes to impaired vascularization in peripheral artery disease. *Nature Medicine.* **20** (12), 1464-1471 (2014).
60 Shafat, I., Ilan, N., Zoabi, S., Vlodavsky, I., Nakhoul, F. Heparanase levels are elevated in the urine and plasma of type 2 diabetes patients and associate with blood glucose levels. *PLoS One.* **6** (2), e17312 (2011).
61 Wang, Y. et al. Endothelial cell heparanase taken up by cardiomyocytes regulates lipoprotein lipase transfer to the coronary lumen after diabetes. *Diabetes.* **63** (8), 2643-2655 (2014).
62 Fan, C. L. et al. Therapeutic angiogenesis by intramuscular injection of fibrin particles into ischaemic hindlimbs. *Clinical and Experimental Pharmacology and Physiology.* **33** (7), 617-622 (2006).
63 Liddell, R. P. et al. Endovascular model of rabbit hindlimb ischemia: a platform to evaluate therapeutic angiogenesis. *Journal of Vascular Interventional Radiology.* **16** (7), 991-998 (2005).
64 Gowdak, L. H. et al. Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. *Circulation.* **102** (5), 565-571 (2000).
65 Zhang, H., Wang, X., Guan, M., Li, C., Luo, L. Skeletal muscle evaluation by MRI in a rabbit model of acute ischaemia. *The British Journal of Radiology.* **86** (1026), 20120042 (2013).
66 Jang, E., Albadawi, H., Watkins, M. T., Edelman, E. R., Baker, A. B. Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation,

migration, and neovascularization of ischemic muscle. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (5), 1679-1684 (2012).

67 Das, S. et al. Syndesome Therapeutics for Enhancing Diabetic Wound Healing. *Advanced Healthcare Materials.* **5** (17), 2248-2260 (2016).

68 Das, S., Majid, M., Baker, A. B. Syndecan-4 enhances PDGF-BB activity in diabetic wound healing. *Acta Biomateriala.* **42** 56-65 (2016).

69 Baker, A. B. et al. Regulation of heparanase expression in coronary artery disease in diabetic, hyperlipidemic swine. *Atherosclerosis.* **213** (2), 436-442 (2010).

70 Das, S. et al. Syndecan-4 Enhances Therapeutic Angiogenesis after Hind Limb Ischemia in Mice with Type 2 Diabetes. *Advanced Healthcare Materials.* **5** (9), 1008-1013 (2016).

71 Popesko, P., Rajtová, V., Horák, J. i. *A Colour Atlas of the Anatomy of Small Laboratory Animals*. Wolfe Publishing, London. (1992).

Figure 1

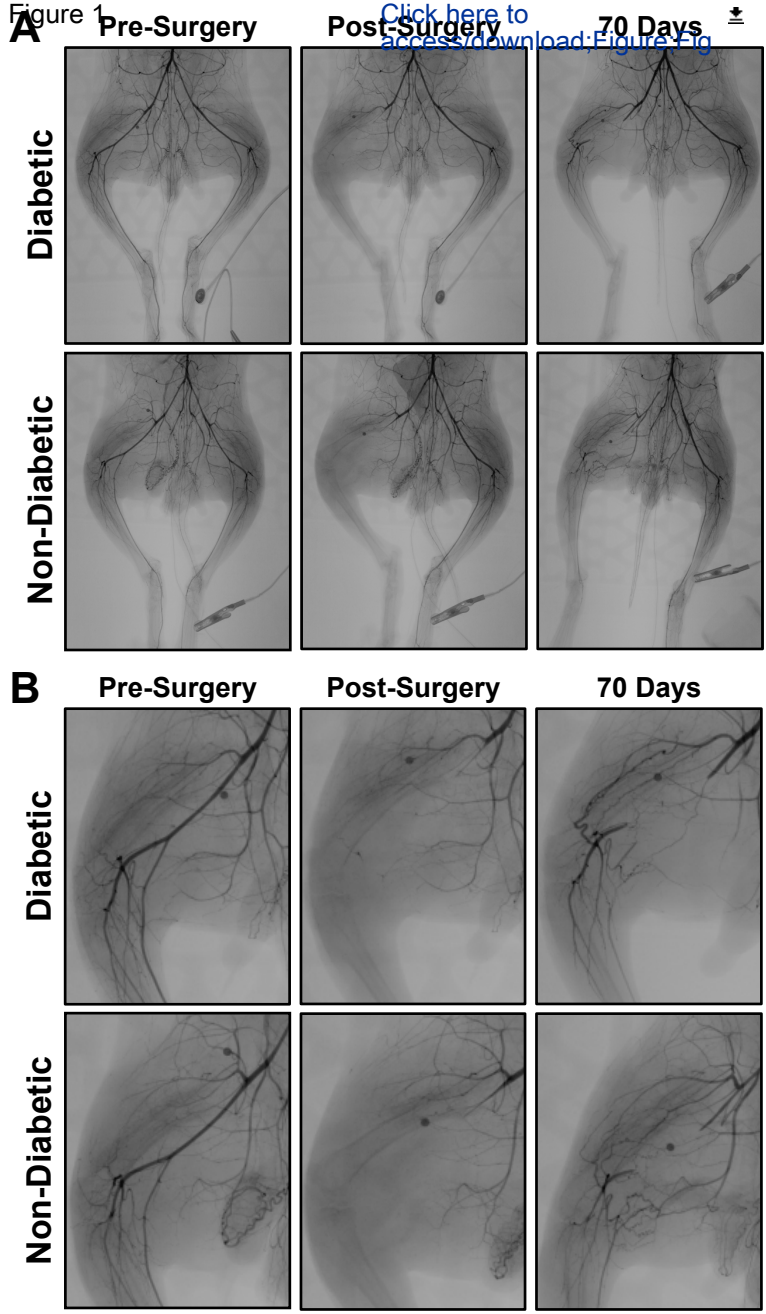


Figure 2

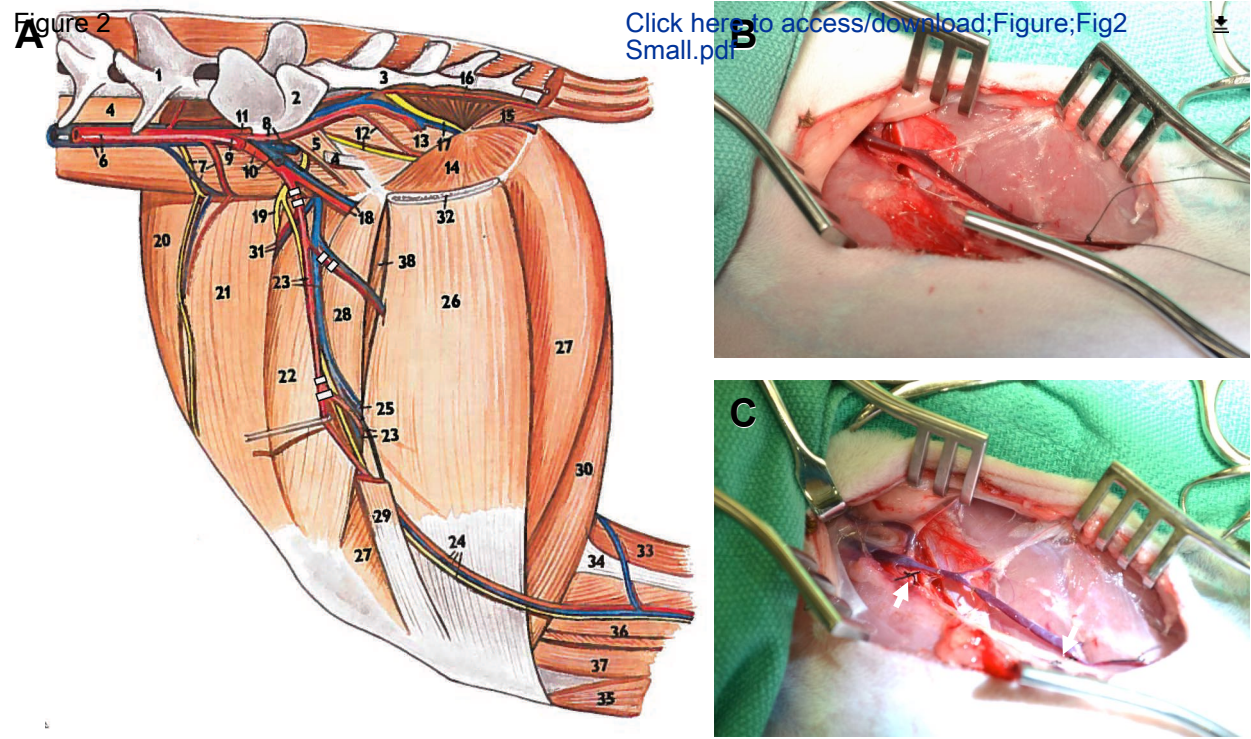
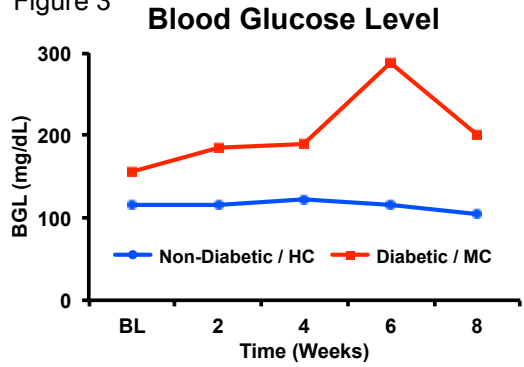
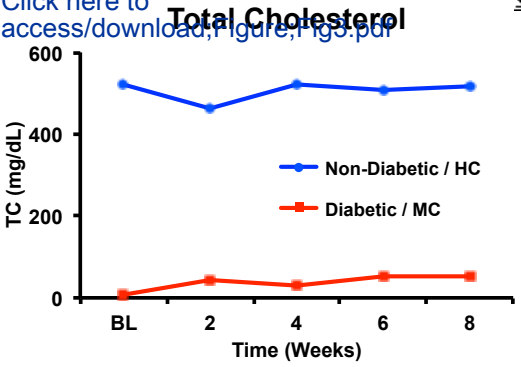
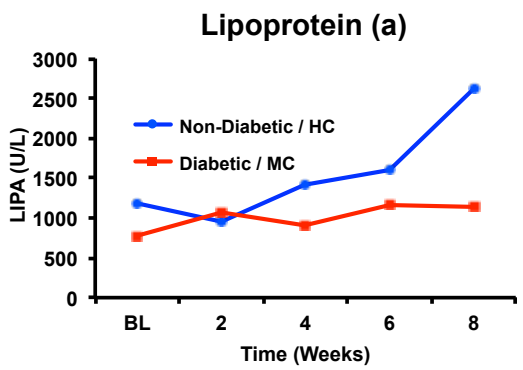


Figure 3







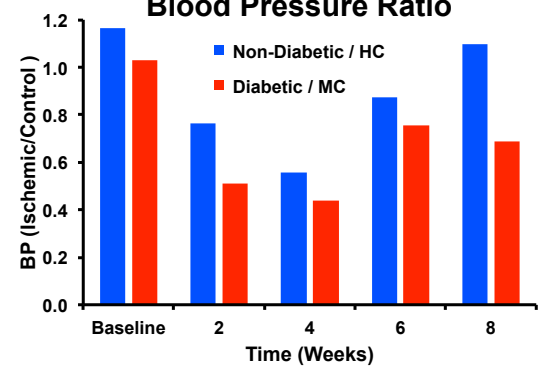
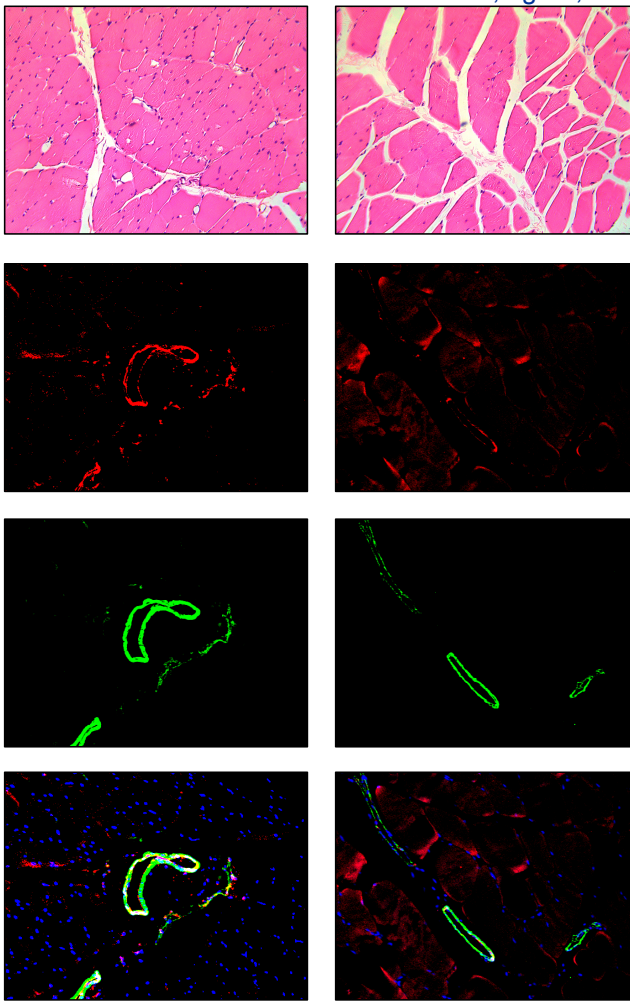


Figure 4



Name	Company	Catalog Number
0.9% Sodium Chloride	Henry Schein Medical	1537468 / 1531434
1 mL Syringe	VWR	BD309628
10 mL Syringe	VWR	BD309695
10% Formalin	Fisher-Scientific	23-245684
18G Needle	VWR	89219-294
20G Needle	VWR	89219-340
25G Needle	VWR	89219-290
27G Needle	VWR	89219-288
5 mL Syringe	VWR	BD309646
5% Dextrose	Patterson Veterinary	07-800-9689
Acepromazine	Patterson Veterinary	VEDC207
Alfaxalone	Patterson Veterinary	07-891-6051
Alginate	Sigma-Aldrich	PHR1471-1G
Alloxan Monohydrate	Sigma-Aldrich	A7413
Angiography Equipment	Toshiba	Infinix-i
Angiography Injector	Medrad	
Anti-Mouse Ab Alexa 594	Thermo Fisher Scientific	A-11032
Anti-Rabbit Ab Alexa 488	Thermo Fisher Scientific	A-11008
a-SMA Antibody	Abcam	ab5694
Baytril	Bayer Animal Health	724089904201
Blood Chemistry Panel	IDEXX	2616
Blood Pressure Cuff	WelchAllyn	
Blood Pressure Monitor	Vmed Technology	
Bupivacaine	Henry Schein Medical	6023287
Buprenorphine	Patterson Veterinary	42023017905
Buprenorphine SR	ZooPharm	
Calcium Sulfate	CB Minerals	
Chlorhexidine Scrub	Patterson Veterinary	07-888-4598

Chloroform	Fisher-Scientific	C298-4
Cholesterol	Sigma-Aldrich	C8503
DAPI	Thermo Fisher Scientific	62248
Ear Vein Catheter	Patterson Veterinary	SR-OX165
Endotracheal tube	Patterson Veterinary	
Glucometer	Amazon	B001A67WH2
Glucometer Test Strips	McKesson Medical-Surgical	788222
Guidewire	Boston Scientific	39122-01
Hair Clippers	Amazon	B000CQZI3Q
Heating Pad	Cincinnati Subzero	273
Heating Pad Pump	Gaymar	
Hemostat	Fine Science Tools	13009-12
Heparin	Patterson Veterinary	
Insertion Tool	Merit Medical Systems	MAP550
Insulin	HPB Pharmacy	
Insulin Syringes	McKesson Medical-Surgical	942674
Introducer	Cook Medical	G28954
Isoflurane	Henry Schein Medical	1100734
Ketamine	Patterson Veterinary	856440301
Lactated Ringers	McKesson Medical-Surgical	186662
Lidocaine	McKesson Medical-Surgical	239936
Lidocaine/Prilocaine cream	McKesson Medical-Surgical	761240
Ligaloop	V. Mueller	CH117 / CH116
Mazola Corn Oil	Amazon	B0049IIVCI
Medrad Syringe	McKesson Medical-Surgical	346920
Meloxicam	Patterson Veterinary	
Metal ball sutures	Ethicon-Johnson & Johnson	K891H
Metzenbaum Scissors	Fine Science Tools	14019-13
Midazolam	Henry Schein Medical	1215470

Nitroglycerin	McKesson Medical-Surgical	927528
PECAM Antibody	Novus Biologicals	NB600-562
Perfusion Pump	Masterflex	
Pigtail Catheter	Merit Medical Systems	1310-21-0053
Polydioxanone (PDS II) suture	McKesson Medical-Surgical	129271
Polydioxanone (PDS II) suture	McKesson Medical-Surgical	129031
Polyglactin 910 (Vicryl) suture	Butler	7233-41
Polyglactin 910 (Vicryl) suture	McKesson	104373
Rabbit Chow (Alfalfa)	LabDiet	5321
Rabbit Restrainer	VWR	10718-000
Rib Cutters	V. Mueller	
Scalpel	Fine Science Tools	10003-12
Scalpel Blade	Fine Science Tools	10015-00
Silk Sutures	Ethicon-Johnson & Johnson	A183H
Stainless Steel Ball	McMaster-Carr	1598K23
Surgical Drapes	Gepco	8204S
Syringe Pump	DRE Veterinary	
Visipaque contrast media	McKesson Medical-Surgical	509055
Weitlaner Retractor	Fine Science Tools	17012-13

Comments
250 mL bag / 1000 mL irrigation btl
Secondary Antibody for IHC
Secondary Antibody for IHC
Primary Antibody for IHC
Enrofloxacin
Rabbit Panel
Flexiport Disposable BP Cuff-infant size 7
Vmed Vet-Dop2
Food and Pharmaceutical Grade USP and FCC

Surflo IV catheters
Sheridan Brand, Depends on Rabbit Size
Accu-Chek Aviva
Accu-Chek Aviva Plus
Oster #40 blade
Gaymar T/Pump
Curved Mosquito Hemostat
metal wire insertion tool
Novalin R & Novalin N
3F Check Flo Performer Introducer
White Mini / Yellow Mini
150 mL
4-0 silk C-1 30"

Primary Antibody for IHC
3F pigtail
4-0 taper RB-1 (needle comes on suture)
4-0 reverse cutting FS-2
3-0 taper RB-1
4-0 reverse cutting FS-2
#15 blade
4-0 silk ties 18"
3-mm diameter
Versaflow VF-300

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits

Author(s): Andrew D. Sligar, Gretchen Howe, Julia Goldman, Patricia Felli, Varsha Karanam, Richard W. Smalling and Aaron B. Baker

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [√] Standard Access [ ] Open Access

Item 2 (check one box):

[√] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Aaron Baker
Department:	Biomedical Engineering
Institution:	University of Texas at Austin
Article Title:	Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits
Signature:	 Date: 8/17/18

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Response to Reviewers' Comments

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have reread the manuscript thoroughly and corrected any grammatical errors found.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: We have obtained copyright permission for reusing an illustration in one of our figure and have modified the legend to include a reference to the source.

3. Please provide an email address for each author.

Andrew Sligar: asligar@utexas.edu
Gretchen Howe: Gretchen.Howe@uth.tmc.edu
Julia Goldman: Julia.L.Goldman@uth.tmc.edu
Patricia Felli: Patricia.R.Felli@uth.tmc.edu
Varsha Karanam: varshakaranam@utexas.edu
Richard W. Smalling: richard.w.smalling@uth.tmc.edu
Aaron B. Baker: abbaker1@gmail.com

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Ziploc, Medrad, etc.

Response: We have removed commercial language from the body of the paper.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response: An ethics statement has been added at the end of the introduction.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Response: All steps have been switched to the imperative tense.

7. 1.4, 1.5, 1.7, 3.11, 7.1, etc.: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Response: The steps have been shortened or split up to make directions that are more clear and discrete.

8. As Figure 4 shows immunostaining results, please consider moving details in Appendix A in the protocol (after step 9.13). Please note that some of the shorter Protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Response: One of the reviewers suggested that we remove this protocol from the paper. However, we feel it is an important protocol to have with the paper because it took considerable effort to find antibodies/methods that work for staining blood vessels in rabbits. We also feel that it would be distracting to have all of the steps within the main protocol. Thus, having the protocol in the appendix seems a good compromise for these issues.

9. Please also consider moving details in Appendix B in the protocol; for instance, step 1.1 that mentions the cholesterol chow may be appropriate.

Response: We also feel this protocol would be best to be included as an appendix. The reason is that it is not essential and high cholesterol food can be purchased commercially. However, for many labs trying to reduce costs this protocol would be very useful and we did not know of another paper that describes this method.

10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have highlighted essential the steps that would be included in the video.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We have now highlighted complete steps that are imperative commands.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: We have highlighted all sub-steps of highlight steps.

13. Figure 1: Please explain the difference between the upper and bottom panels.

Response: We have now included an explanation of the upper and lower panels for this figure in the figure legend.

14. Figure 4: Please describe what the different panels are.

Response: we have now described the different panels in the figure legend.

15. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: We have revised the discussion extensively to roughly follow this outline.

16. References: Please do not abbreviate journal titles.

Response: We have removed the abbreviations from the references.

Reviewers' comments
Reviewer #1
Manuscript Summary:
In this interesting manuscript, Sligar and Howe et al show that hind limb ischemia model in rabbits with diabetes and hyperlipidemia may be a useful preclinical model. Although the research method was described, there are some information that need to be added in order to aid in the understanding of the reader:

Major Concerns:
1. If possible, could you add angiograms of non-diabetic model in Figure 1?

Response: We have added an angiogram of a non-diabetic rabbit to the figure.

2. Is there a significant difference to the blood pressure at 8 weeks in Figure 3?

Response: We did not perform statistics on these samples as the data was from a single animal for each group as an illustration of the typical response of each model.

Minor Concerns:
3. What does "Control" mean in Figure 4? Non-diabetic rabbit, diabetic rabbit no ischemia?

Response: We have added the details of the control to the legend of figure 4.

Reviewer #2

Manuscript Summary

In this nice paper the authors have perfected a new angiogenesis model. This model has reduced collateral formation and blood pressure recovery in comparison to a model with a higher cholesterol diet. Thus, the model may provide better correlation with human patients with compromised angiogenesis from the common co-morbidities that accompany peripheral vascular disease.

Major Concerns:

- a laser doppler additional evaluation could be useful to make the model more available.

Response: We have added a sentence to refer the reader to references to other imaging methods that can be used to assess recover from ischemia including Doppler ultrasound, laser Doppler imaging, infrared thermography, microsphere determined perfusion, computed tomography (CT) imaging, and magnetic resonance imaging (MRI). These modifications can be found on page 13 of the revised manuscript.

Minor Concerns:

- none.

Reviewer #3:

Manuscript Summary:

This manuscript describes in an excellent way the induction of hind limb ischemia in a clinical highly relevant animal model with comorbidities frequently seen in patients and therefore is a welcome contribution the field, namely a rabbit model with diabetes. The procedure is clearly described and raises just a few remarks.

Minor Concerns:

The restoration of the blood flow after ligation of the femoral artery mainly depends on collateral formation, and to a much lesser extent on angiogenesis in the distal ischemic tissue. Therefore, the authors should speak about therapeutic neovascularization instead of therapeutic angiogenesis since this term covers both arteriogenesis and angiogenesis.

Response: We have modified the text to refer to therapeutic neovascularization rather than therapeutic angiogenesis.

Section 1.4 line 2 : change ' major factor the alters ' to ' major factor that alters' .

Response: We have corrected this sentence in the revised manuscript.

Comment
Section 4.6: Ligate all of the above arteries with 4.0 silk sutures: it is unclear what is meant with " all of the above arteries" since in the section above two different sets of arteries are described that need to be isolated:
'all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries (Fig. 2A). Dissect further along the popliteal and saphenous arteries as well as the external iliac artery (Fig. 2A)'

Response: **We have modified the statement in 4.6 to refer to the diagram that shows the specific locations for ligation.**

Comment
9.1 how is the end point day determined or decided upon?

Response: The time point after an extensive review of the literature for studies that could observe recovery of the animals after induction of ischemia. The appropriateness of the time point was also supported by the presence of significant ischemia in the diabetic group at the end of the study.

Comment
The appendices on the immunohistochemistry protocol and on chow preparation are redundant and can be left out.

Response: In our own experience, we have had difficulty staining for blood vessel in formalin fixed paraffin sections on samples from rabbit muscle tissue. We screened through many primary antibodies to create the current protocol and felt it would save other groups time and money if we include our optimized protocol.

For the chow preparation, we also felt this could provide considerable cost savings and did not know of a published protocol that described this method of creating high fat rabbit chow.

Appendix A. Immunostaining on Paraffin Sections

1. Warm slides for 10 minutes at 60 °C on slide warmer.
2. Deparaffinize the sections.
    a. Soak slides for 2 minutes in histology grade xylene.
    b. Move slides to fresh xylene for 2 minutes.
    c. Move slides to fresh xylene for 2 minutes.
    d. Move slides to fresh xylene for 2 minutes.
3. Rehydrate the sections.
    a. Soak slides in 100% ethanol for 2 minutes.
    b. Move slides to fresh 100% ethanol for 2 minutes.
    c. Move slides to 95% ethanol for 2 minutes.
    d. Move slides to 70% ethanol for 2 minutes.
    e. Move slides to ddH2O for 2 minutes.
    f. Move slides to PBS for 5 minutes.
4. Conduct antigen retrieval
    a. Move slides to target retrieval solution.
    b. Place the histology cup with slides and target retrieval solution in a water bath at 85°C for 2 hours 40 minutes.
5. Remove the histology cup from the water bath and cool for 20 minutes.
6. Wash the slides twice for 5 min in PBS.
7. Block the sections with 20% fetal bovine serum in PBS for 45 minutes at room temperature.
    a. For many slides, this can be done in a histology staining cup.
    b. For fewer slides, use a hydrophobic pen and apply the solution onto the flat section.
8. Wash the slides twice for 5 min in PBS.
9. Use a hydrophobic pen to draw circles around the tissue sections on the slides if they have not been drawn already.
10. Apply the primary antibodies at the appropriate concentration in PBS-1% BSA to the tissue sections. Use 1:25 for the PECAM-1 antibody and 1:100 for the □-SMA antibody.
11. Incubate the slides overnight at 4°C in a closed container with damp towels to ensure drying does not occur.
12. Wash the sections with PBS twice quickly, then three times allowing 5 minutes per wash.
13. Label the sections with secondary antibodies at 1:1000 dilution in PBS-1% BSA with a 1:1000 dilution of DAPI (from a stock solution of 1 mg/ml). Incubate for 75 minutes at room temperature protected from light.
15. Wash the sections with PBS twice quickly, then three times allowing 5 minutes per wash.
16. Put a drop of hard set mounting media without DAPI on each of the sections.
17. Carefully apply a glass coverslip, ensuring bubbles do not form over the section
18. Allow the hard set mounting media to dry overnight.

Appendix B. Preparation of Cholesterol Diet

1. Fill a bucket with 9 kg of rabbit chow.
2. Prepare a cholesterol/corn oil/chloroform solution in a chemical fume hood. The recipe below creates a high fat diet with 0.1% cholesterol. For other high fat diets use can be created by changing the amounts of cholesterol and/or corn oil.

a. Weigh out 9 g of cholesterol and put it in a large beaker
b. Add 200 mL of chloroform to the beaker and stir
c. Add 45 mL of corn oil to the beaker
d. Fill the beaker up to the 1000 mL marker with chloroform and stir

Note: be sure to use appropriate personal protective equipment when handling chloroform. The brand of corn oil we used (see Table 1) worked well for this purpose. Other corn oils may work as well but the results will depend on the fat content of the corn oil.

3. Spread the food out on a stainless steel tray inside a chemical fume hood.
4. Pour the cholesterol/corn oil/chloroform solution over the food in the hood.
5. Wearing heavy-duty chemical resistant gloves and using a stainless steel spoon, mix the food and the solution until no puddles are present and the food appears an even color.
6. Lower the sash of the hood to leave a two-inch gap and allow the solution to evaporate in the hood for 2 days.

The University of Texas at Austin
Andrew Sligar
107 W. Dean Keeton St
TX, United States

OFFICIAL RECEIPT

Our reference:	4745410971792520
Account Name :	Andrew Sigar
Invoice Number	RP018045

3-Nov-2018

Dear Sir/Madam,

We herewith acknowledge the receipt of your credit card payment ending in 7024 on 02-Nov-2018 in the amount of USD 118.50 as full payment towards invoice RP018045.

We trust this information meets with your approval.

Yours sincerely,

Ian Brent I. Miran

e-mail: ianbrent.miran@yahoo.com

UTMAIL

Andrew D Sligar <asligar@utexas.edu>

Credit Card Payment - Elsevier_ RP018045

3 messages

Mata, Jesus S. (REPH-MNL) <jesus.mata@reedelsevier.com> Thu, Nov 1, 2018 at 1:35 PM
To: "asligar@utexas.edu" <asligar@utexas.edu>
Cc: "Maranan, Mary Jane A. (REPH-MNL)" <jane.maranan@reedelsevier.com>

Dear Andrew,

Thanks for the payment.

This is to confirm that we have successfully charged USD 118.50 via MasterCard ending in 7024. The payment has been posted against invoice RP018045.

Payment Accepted

Your payment was accepted

Payment Details

```
Payment Reference : RP018045
Reference at PSP : 4745410971792522
```

Kindly advise us any issues/concerns about the statement so we can address them accordingly.

Should you have further questions, please feel free to get in touch.

Kind Regards,

Jesús S. Mata
Credit Control Administrator, Credit Risk & Receivables

ph: +1 937 247 2339
fax: +1 877 223 1436
jesus.mata@reedelsevier.com

ELSEVIER

As a valued customer we appreciate your feedback on the service we deliver to you. If you would like to share your comments please send an email to the Global Head O2C, O2CUSCollections@ReedElsevier.com.

Drew Sligar <asligar@utexas.edu> Thu, Nov 1, 2018 at 1:47 PM
To: "Livingston, Jennifer G" <Jenna.L@austin.utexas.edu>

---------- Forwarded message ---------
From: **Mata, Jesus S. (REPH-MNL)** <jesus.mata@reedelsevier.com>
Date: Thu, Nov 1, 2018 at 1:35 PM
Subject: Credit Card Payment - Elsevier_ RP018045
To: asligar@utexas.edu <asligar@utexas.edu>
Cc: Maranan, Mary Jane A. (REPH-MNL) <jane.maranan@reedelsevier.com>

Dear Andrew,

Thanks for the payment.

This is to confirm that we have successfully charged USD 118.50 via MasterCard ending in 7024. The payment has been posted against invoice RP018045.

Kindly advise us any issues/concerns about the statement so we can address them accordingly.

Should you have further questions, please feel free to get in touch.

Kind Regards,

As a valued customer we appreciate your feedback on the service we deliver to you. If you would like to share your comments please send an email to the Global Head O2C, O2CUSCollections@ReedElsevier.com.

--
Drew Sligar

4 attachments

image001.png
12K

image002.png
38K

image002.png
38K

image001.png
12K

Livingston, Jennifer G <Jenna.L@austin.utexas.edu> Thu, Nov 1, 2018 at 1:48 PM
To: "asligar@utexas.edu" <asligar@utmail.utexas.edu>

Woohoo!

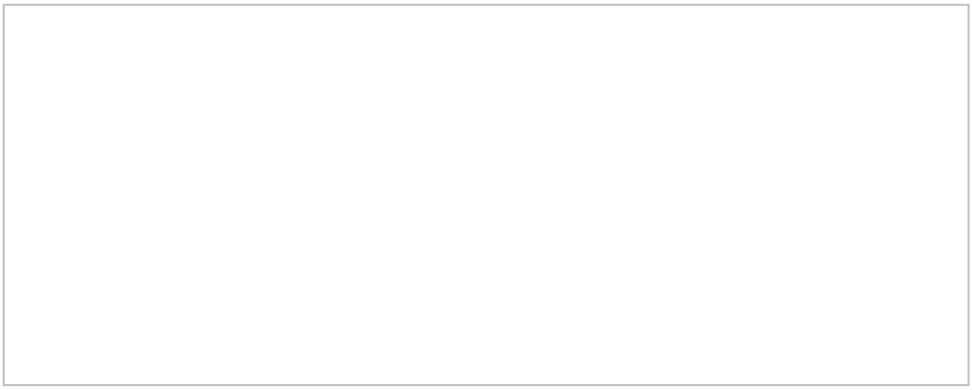
From: Drew Sligar <asligar@utexas.edu>
Sent: Thursday, November 1, 2018 1:47 PM
To: Livingston, Jennifer G <Jenna.L@austin.utexas.edu>
Subject: Fwd: Credit Card Payment - Elsevier_ RP018045

---------- Forwarded message ---------
From: **Mata, Jesus S. (REPH-MNL)** <jesus.mata@reedelsevier.com>
Date: Thu, Nov 1, 2018 at 1:35 PM
Subject: Credit Card Payment - Elsevier_ RP018045
To: asligar@utexas.edu <asligar@utexas.edu>
Cc: Maranan, Mary Jane A. (REPH-MNL) <jane.maranan@reedelsevier.com>

Dear Andrew,

Thanks for the payment.

This is to confirm that we have successfully charged USD 118.50 via MasterCard ending in 7024. The payment has been posted against invoice RP018045.



Kindly advise us any issues/concerns about the statement so we can address them accordingly.

Should you have further questions, please feel free to get in touch.

Kind Regards,

As a valued customer we appreciate your feedback on the service we deliver to you. If you would like to share your comments please send an email to the Global Head O2C, O2CUSCollections@ReedElsevier.com.

--

Drew Sligar

INVOICE NO. RP018045

Our Ref: UKRPINV/SR Fee & Permission/jbsubr/B9780702026997
GR Ref: AG

29 October 2018 **Due date: 30 Dec 2018**

The University of Texas at Austin
attn: Andrew Sligar
107 W. Dean Keeton St
Austin, 78712
TX, United States
asligar@utexas.edu
4176693356

COLOUR ATLAS OF ANATOMY OF SMALL LABORATORY ANIMALS (VOLUME 1) 2003, ISBN: 9780702026997, Rajtova et al ed, 1 figure only.

Proposed use: To be used in a journal/magazine "JoVE, Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits".

Payment due: USD 118.50

REMITTANCE SLIP – PLEASE RETURN WITH PAYMENT TO:

PAYMENT OPTIONS

1. By *Bank transfer*
Remit to: ING Bank NV, Bijlmerplein 888, 1102 MG Amsterdam, The Netherlands, Swift Code: INGBNL2A. *You must quote the Elsevier Invoice Number as a reference.*
Bank account:
0020158157 (USD) $ IBAN: NL17INGB0020158157
0007151730 (EUR) € IBAN: NL81INGB0007151730
0020158165 (JPY) ¥ IBAN: NL92INGB0020158165

2. By *Cheque* made payable to Elsevier Ltd and sent with a copy of the invoice to addresses:

For Non-US Customers
Elsevier Ltd
PO Box 1270
1000 BG Amsterdam
The Netherlands

For US Customers
Elsevier Ltd
PO Box 7247-6140
Philadelphia, PA 19170-6140
USA

3. By *Credit Card*. Fill in your credit card details below and send to:

For Non-US Customers
Elsevier Ltd
PO Box 1270
1000 BG Amsterdam
The Netherlands
Fax: (+31) 20 485 2620

For US Customers
Elsevier Ltd
Credit and Collections
3251 Riverport Lane
Maryland Heights, MO 63043 USA
Fax: +1-877-223-1436

|__| Visa Card |__| Access/Euro/Mastercard |__| American Express

Card no |__|__|__|__| - |__|__|__|__| - |__|__|__|__| - |__|__|__|__|

Expiry Date Month |__|__| Year |__|__|

Signature _____

Name _____

TERMS AND CONDITIONS

INVOICE NO. RP018045
29 October 2018

The University of Texas at Austin
attn: Andrew Sligar
107 W. Dean Keeton St
Austin, 78712
TX, United States
asligar@utexas.edu
4176693356

COLOUR ATLAS OF ANATOMY OF SMALL LABORATORY ANIMALS (VOLUME 1) 2003, ISBN: 9780702026997, Rajtova et al ed, 1 figure only.

Proposed use: To be used in a journal/magazine "JoVE, Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits".

Permission to republish the material is granted subject to the following conditions:

- Payment of the copyright fee of USD 118.50
- If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained, then that material may not be included in your publication/copies.
- Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows: "This article was published in Publication title, Vol number, Author(s), Title of article, Pages Nos, Copyright Elsevier (or appropriate Society name) (Year)".
- Reproduction of this material is confined to the purpose for which permission is hereby given and for use on a non-commercial basis of handicapped persons and the blind.
- This permission is for one time use and is granted for non-exclusive **English** world rights only. For other languages, please reapply for permission separately. Permission EXCLUDES use in an electronic form other than as specified above.