Video Article

Preclinical Model of Hind Limb Ischemia in Diabetic Rabbits

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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[9,10,11,12,13,14,15,16,17,18,19,20], gene therapy[21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44], and stem cells[45,46,47,48,49,50,51] 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[53,54,55,56,57,58,59]. 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. 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
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.
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.
4. Inject alloxan at 100 mg/kg in saline through the ear catheter slowly over a 10-minute period using a syringe pump.
5. Check the BGL every hour for the next 12 h using a standard glucometer to monitor for hypoglycemia.
   1. Take blood from the rabbits by using a restrainer.
   2. Anesthetize the ear with 2.5% lidocaine/2.5% prilocaine cream.
   3. Take blood from the lateral ear vein using a 25 G needle and measure BGL using a standard meter.

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.
7. Prepare a 3-mm stainless steel ball for implantation as a size marker during angiograms prior to the day of surgery.
   1. Cut a 10-mm circular piece of silastic sheeting out of a larger sheet using a biopsy punch.
   2. Mount the ball in the center of the sheet using clear silicone sealant.
   3. Completely cover the ball with the sealant. Allow the sealant to cure for a minimum of 24 h.
   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

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

1. Expose the right common carotid artery
   1. Make a 4–5 cm long incision just lateral to the trachea using a scalpel with a #15 blade.

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.

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. 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.
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.
5. Advance the pigtail catheter to be 2 cm proximal to the aortic bifurcation at the iliac crest in the descending aorta.
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.
7. Administer an intra-arterial injection of 100 µg nitroglycerin through the catheter to increase vasodilation.
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.
9. Record serial images of the hind limbs using angiography.
    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.
    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

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).
2. Use blunt dissection with curved Metzenbaum scissors or a curved mosquito hemostat to expose the femoral artery.
3. Use Weitlaner retractors to hold the incision open.
4. Add 0.5 mL of 1% lidocaine locally to reduce nerve irritation and promote vasodilation.
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**).
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.
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.
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

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.
2. Administer an intra-arterial injection of 100 µg nitroglycerin through the catheter to increase vasodilation.
3. If needed, administer another 4 mL of 1% lidocaine to the rabbit through the catheter to prevent movement during the angiogram.
4. Inject 8 mL of contrast media using an automated angiographic injector.
5. Perform angiography as described in step 3.11.

6. Wound closure and recovery

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

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).
2. Take a very small amount of blood for BGL measurements.

8. Treatment

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.
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

1. At the endpoint date, perform angiography as described in step 3 but use the left carotid artery for access.
2. After angiography, move the animal to the necropsy table and perform perfusion fixation to preserve the hind limb tissues:
    1. Increase the isoflurane to 3%–4% and perform a toe pinch to confirm the anesthesia is sufficiently deep.
    2. Administer 1000 IU of heparin intravenously.
    3. Create an incision along the midline of the ribcage and spanning the length of the diaphragm using a scalpel with a #20 blade.
    4. With the rib cage exposed, cut the ribs just left of the midline using rib cutters. Use Weitlaner retractors to expose the heart.
    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.
    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.
    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.
    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. Turn the pump off and remove the needles from the heart.
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.
4. Store the limbs in formalin for 24 h at 4 °C and then stored in 70% ethanol at 4 °C.
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.
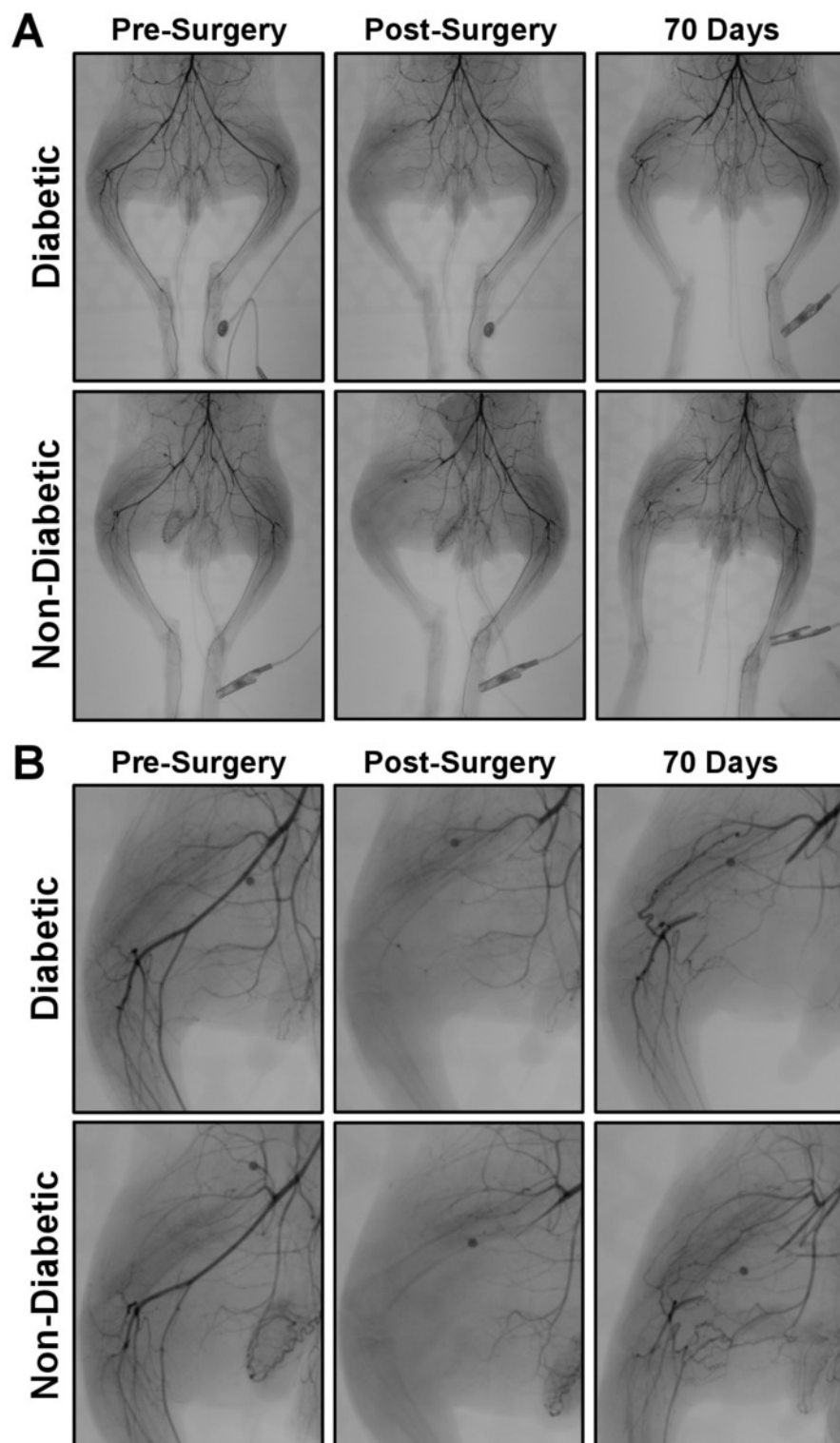
Figure 1: Angiograms for the hind limb of a diabetic and non-diabetic rabbit pre-surgery, post-surgery and after recovery for 70 days after femoral artery ligation and excision. (**A**) Angiogram of ischemic limb (left) and contralateral control limb (right). (**B**) Enlarged image of the ischemic limb at the site of ligation. Please click here to view a larger version of this figure.
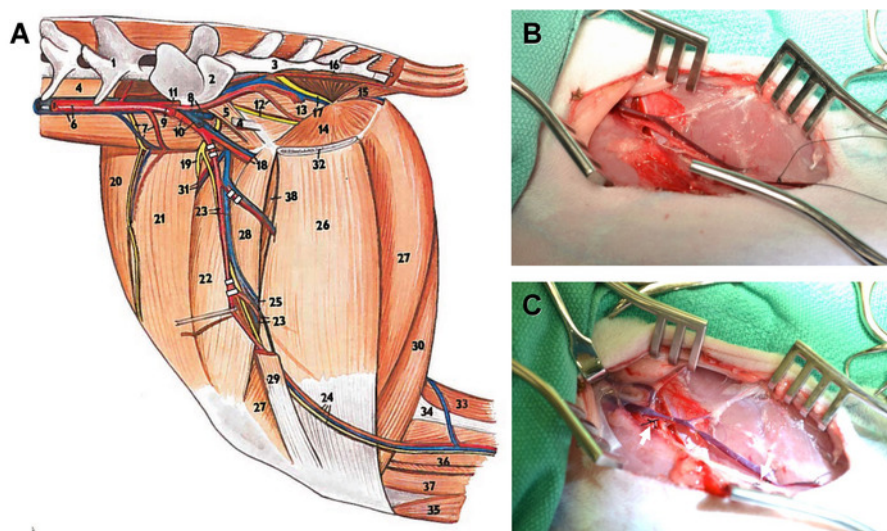
Figure 2: Induction of hind limb ischemia in rabbits through femoral artery ligation and excision. (**A**) Illustration of the vascular anatomy of the rabbit hind limb. Place ties at all the points marked to ligate the arteries. Modified and used with permission[71]. (**B**) Surgical field showing the cut down to the femoral artery prior to ligation. (**C**) Femoral arteries with ligations in place to induce hind limb ischemia. Please click here to view a larger version of this figure.
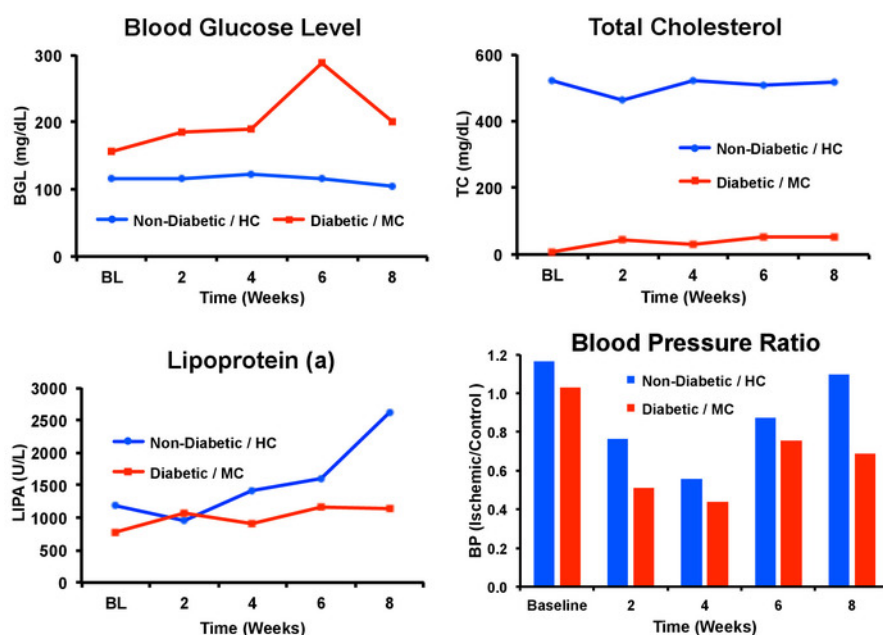

Figure 3: Typical blood pressure and blood chemistries for the rabbits with hind limb ischemia over the course of the model. The Diabetic / MC group was induced to have diabetes and given a 0.1% cholesterol diet. The Non-Diabetic / HC group was given a 1% cholesterol diet. BGL = Blood Glucose Level. TC = Total Cholesterol. LIPA = Lipoprotein (a). BP = Blood pressure ratio between the ischemic and non-ischemic limb. Please click here to view a larger version of this figure.
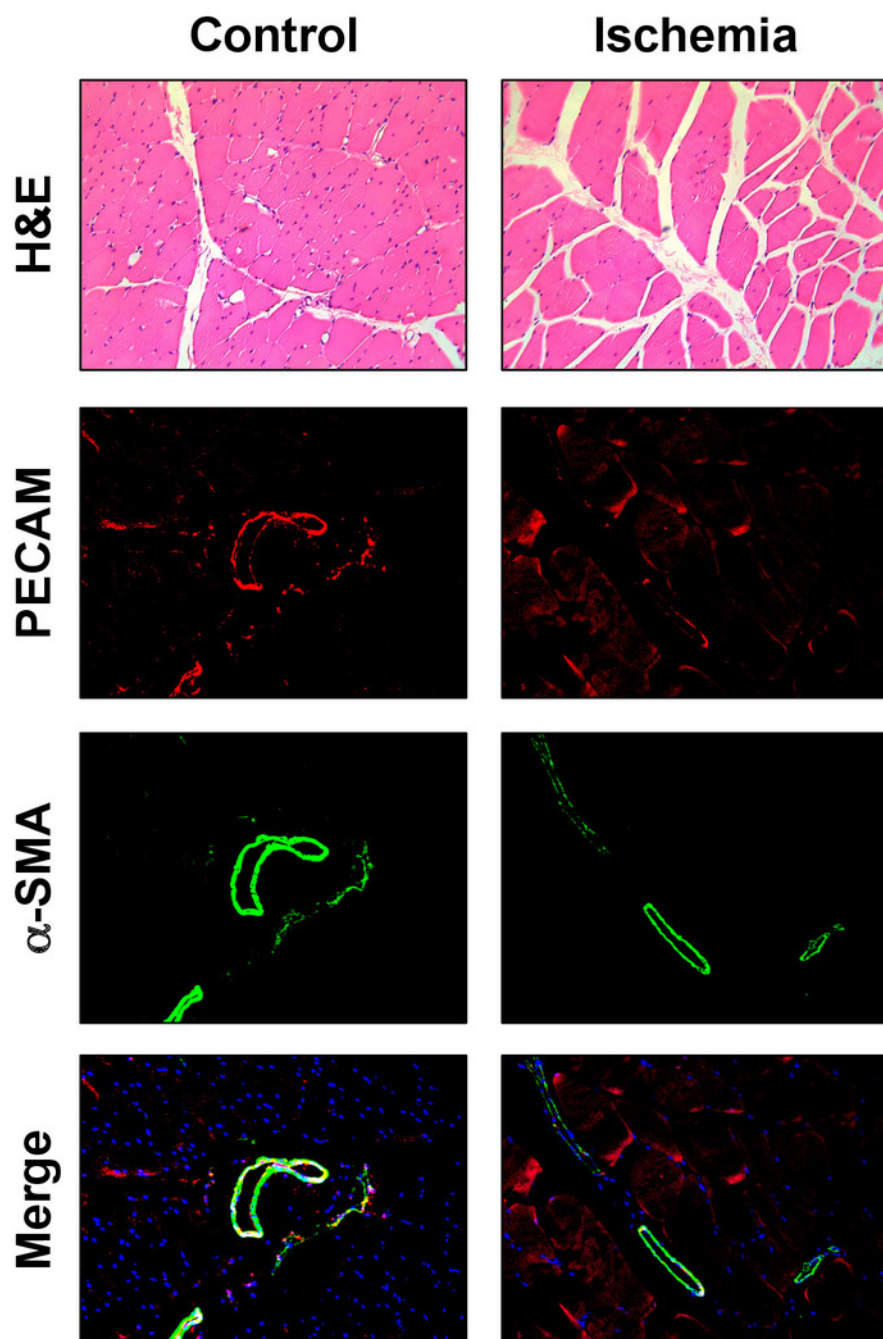
Figure 4: Histological analysis of the muscle of the hind limb in diabetic rabbits 70 days after femoral artery ligation. H&E staining as well as immunohistochemical staining for the endothelial marker, PECAM, and vascular smooth muscle cell marker, αSMA, was performed. Tissue samples were biopsied from the ischemic limb and the non-ischemic contralateral control limb. Please click here to view a larger version of this figure.

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[55,58,67,68,69,70]. 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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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).