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Transplantation of Thioredoxin-1 Exosomes in a Murine Model of Hind-Limb Ischemia: A Novel Therapeutic Approach

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A thesis submitted to fulfill the requirements for the degree of Bachelor of Science in Allied Health Sciences with Honors

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Abstract

Peripheral artery disease is one of the most prevalent cardiovascular diseases in the United States and worldwide \((4, 5)\). It is estimated that over 8 million people in the United States are affected with peripheral artery disease \((14)\), and up to 40\% of people over 80 years old may have some degree of the disease \((5)\). Peripheral artery disease is caused by atherosclerotic occlusion of peripheral arteries, impairing perfusion in the affected areas which can lead to ischemic injury. Peripheral artery disease can increase one’s chances for having other cardiovascular co-morbidities such as coronary artery disease, among others. Current interventional strategies are endovascular treatments such as transluminal angioplasty and stenting, or in more advanced cases peripheral artery bypass grafting \((13, 14)\). Medical interventions focus on managing symptoms and treating associated biomarkers such as high blood pressure and dyslipidemia. There is a need for medical treatments that focus on neovascularization and angiogenesis to create new microvessel networks that can effectively bypass atherosclerotic blockages. One potential candidate for peripheral artery disease management is thioredoxin-1 \((\text{Trx-1})\). Trx-1 is a protein that has been shown to be an upregulator of neovascularization as well as a potent anti-oxidant \((1)\). The delivery of proteins for therapy must be achieved using an appropriate delivery vehicle. Exosomes may be an appropriate delivery vehicle, as a growing body of research has demonstrated their role in natural signaling processes, as well as their low immunogenic profile \((3, 6, 7, 8, 9, 11, 12, 19, 20)\). This study investigates the effects of transplantation of exosomes isolated from Trx-1 overexpression mice in a mouse model of hind-limb ischemia. Mice were subjected to hind-limb ischemia, and had either Trx-1 exosomes (treatment), wild type exosomes (positive control), or
phosphate buffered saline (vehicle control) injected into their semimembranosus and gastrocnemius muscles. Quantification of perfusion 28 days after induction of hind-limb ischemia revealed that Trx-1 exosome transplantation resulted in a mean perfusion ratio of 77.4%, as opposed to 38.4% in the vehicle control group. These results show that Trx-1 exosomes may be a potential target for future medical treatment of peripheral artery disease.

Introduction

Peripheral artery disease is a major cardiovascular disease that affects over eight million people in the United States (14). It is estimated that 10-25% of people over the age of 55 are affected, and approximately 40% of people over 80-years-old are affected (4, 5). In addition to coronary artery disease and stroke, peripheral artery disease (PAD) is among the leading causes of cardiovascular injury (4, 5). The risk factors for peripheral artery disease are very similar to risk factors for other cardiovascular diseases including but not limited to: cigarette smoking, diabetes mellitus, hypertension, dyslipidemia (potentially characterized by cholesterol ratio of >4), old age, and obesity (BMI>30) (4). Out of these measures, many observational studies have associated cigarette smoking with the greatest increase in risk for peripheral artery disease (4). Race may also play a role in the development of peripheral artery disease, as many observational studies have reported higher rates of PAD in the African American community (4, 5, 10). However, as is the issue with observational studies, it is unknown if these differences are due to differing racial genetic makeup, or due to socioeconomic or cultural differences at large between African American and other racial groups (4). Despite this issue, observational studies are most often the best option for epidemiologic studies as randomized controlled trials would be unethical. Peripheral artery disease is caused by the partial or complete occlusion of one or
more peripheral arteries. The specific variety of peripheral artery disease that was investigated in this study, was lower extremity peripheral artery disease (LE-PAD) (4, 10). LE-PAD is caused by partial or complete atherosclerotic occlusion of one or more arteries in the lower extremity. PAD occurs due to fatty deposit accumulation in arteries that participate in systemic circulation, resulting in a narrowing of the vessel lumen, in the process known as atherosclerosis. Narrowing of vessels results in reduced blood flow to the area which can result in ischemic injury to the area. One of the main symptoms that is associated with PAD is pain in the area near the occlusion which is known as claudication, just as patients with coronary artery occlusion may experience angina (13, 14). This pain can be caused by activity, and is often relieved upon rest. Due to pain, patients who experience intermittent claudication, may not want to or be unable to be active regularly, further exacerbating the issue. Although claudication is used as one of the primary markers of PAD, some patients with moderate PAD may not report claudication due to low activity level or other factors. Other associated symptoms could include weak or absent pulse, wounds or sores that do not heal due to low perfusion, pale or blue skin, low temperature in affected regions, or decreased nail or hair growth in affected areas (13, 14). Peripheral artery disease is progressive, and can cause critical limb ischemia resulting from severe blockages that can cause pain, sores or ulcers on the skin (13, 14). In addition to PAD and its associated symptoms, having PAD also increases one’s chances of developing stroke, transient ischemic attack, coronary artery disease, as well as having myocardial infarction (14).

Claudication is typically thought of as one of the primary indicators of PAD, but determination of pain can be a difficult task for medical professionals, and so the use of
objective techniques in PAD diagnosis and treatment is certainly critical (4). Claudication is certainly still a cardinal symptom of PAD, but subjective interpretation of pain, as well as differences in pain thresholds can make this an unreliable measure to definitively diagnose PAD (4). Primary care physicians can use a number of non-invasive techniques to assess clinical indicators of PAD. Physicians will check and compare pulses in the legs, and may listen for sound known as a bruit, or a whooshing sound through the vasculature, auscultation of which can be a warning sign for narrowed vessels (13, 14). One of the first diagnostic tests to be used would be the Ankle Brachial Index (ABI) (4, 13, 14). The ABI is a ratio of the systolic blood pressure in the arm and ankle. A significantly low ABI (<0.9) is used to diagnose PAD, and can diagnose patients who do not report experiencing claudication (4). ABI can tell physicians whether there is a discrepancy in the perfusion of the arms and legs, but will not help to identify where the narrowing of blockage is located (4, 13, 14). Another non-invasive test that may be more informative, is the Doppler ultrasound. In this diagnostic test, a physician will pass the Doppler device over the suspected areas, and the Doppler will convert soundwaves into an image that shows the position and flow of blood through the vessels, allowing visualization of any potential blockages (14). Angiography is considered the “gold standard” for diagnosing PAD, a procedure involving the administration of a contrast agent into the blood followed by X-ray imaging of blood vessels permitting visualization of potential blockages (2, 4). Although unlikely, angiography can result in damage to the arteries, or an allergic reaction to the dye used in the angiography (2). Due to the potential risks and discomfort associated with angiography, ABI is still a good preliminary measure in the diagnosis of PAD.
Treatment for PAD typically has a three-pronged approach of lifestyle changes, medical treatment, or direct intervention. Lifestyle changes include a healthier diet, smoking cessation, and gradual increase of activity levels (4, 13, 14). Physicians may prescribe statins to lower cholesterol, high blood pressure medications including ace inhibitors, beta blockers, vasodilators or calcium channel inhibitors, and medications that stop or reduce blood clots such as Plavix® (13, 14). Current interventional treatment strategies focus on endovascular or surgical methods. Endovascular treatments include percutaneous transluminal angioplasty in which a catheter is threaded to the occlusion and a balloon is inflated at the site to increase the size of the vessel lumen and flatten the plaque against the walls of the lumen (13, 14). Another endovascular treatment is placement of a stent, in which a mesh tube is placed at the site of the vessel narrowing to support the integrity of the vessel. If endovascular treatments are not appropriate for a patient, doctors may decide to use a surgical approach and perform a bypass surgery. In this procedure, surgeons make a bypass graft using a vessel from another part of the body or from a synthetic material, and allow the blood to flow around the blockage (13, 14).

Surgical procedures pose obvious risks for patients, so nonsurgical approaches are desirable. Current medical treatments manage symptoms but may not actually treat PAD. Medical treatment of PAD must be expanded from its current state, and rather than individually treating symptoms, should focus on neovascularization and angiogenesis. Formation of new microvascular networks would allow to bypass blockages without physical intervention. One potential target for peripheral artery disease therapy is thioredoxin-1 (Trx-1). Trx-1 is a 12kDa cytosolic protein, a powerful antioxidant, and is known to regulate various intracellular signaling pathways (1). Maulik et al. previously showed the therapeutic effects of Trx-1 in both
myocardial ischemia (1, 18) and hind limb ischemia (17). Maulik et al (2011) investigated the effect of Trx-1 overexpression in a mouse model of myocardial infarction, and found that overexpression of Trx-1 resulted in reduced fibrosis, oxidative stress, cardiomyocyte apoptosis as well as an increase in the formation of new blood vessels (1). Maulik et al.’s (1) discovery led to the investigation of the effects of Trx-1 overexpression in a mouse model of hind-limb ischemia. Further study by Maulik et al. (2017) investigated the effects of two overexpression models for Trx-1, transgenic overexpression and an adenoviral vector from which data supported better perfusion recovery in the overexpression groups, higher capillary and arteriolar density, as well as greater expression of common angiogenic markers (17). It has also been observed that Trx-1 expressing mesenchymal stem cells (MSCs) are capable of improving cardiac function and angiogenesis in a rat model of myocardial infarction, via paracrine signaling mechanisms (18).

The method to deliver a protein as large as Trx-1 must be explored in greater detail. MSCs have been used in pre-clinical models, but the safety and efficacy of these cells has not been extensively demonstrated in clinical trials (3, 12). Another candidate for drug delivery, liposomes, are small sacs are filled with water and surrounded by a lipid bilayer, thus allowing for delivery of drugs into the cytoplasm of cells. There have been concerns regarding the circulatory half-life, toxicity and immunogenicity of liposomes for drug delivery (8). Exosomes are an emerging topic in the field of drug delivery and therapeutics (3, 6, 7, 8, 9, 11, 12, 19, 20). Exosomes are cellular membrane derived vesicles which can carry proteins, DNA, mRNA, lipids and other cell products. Exosomes have proven to be potent cell-to-cell communicators, because they are naturally released from many cells in the body (3, 8, 12). The fact that
exosomes are a component of the body’s natural paracrine signaling make exosomes an intriguing option for future treatment delivery.

Therefore, exosomes isolated from Trx-1 overexpression mice may have the potential to treat murine hind limb ischemia (HLI) through effective angiogenesis, which may provide hope for a nonsurgical therapy to improve blood perfusion in patients with PAD.

Methods

Genetically engineered Thioredoxin-1 overexpression transgenic mice (Trx-1Tg/+, Gene ID: 22166) were developed in UConn Health Center’s Gene Targeting and Transgenic Facility. To determine whether Trx-1Tg/+ derived exosomes enhance neovascularization and blood perfusion in HLI, exosomes were isolated from the plasma of 8-12 week wild type (WT) control mice, as well as Trx-1Tg/+ mice. Adult 8-12-week-old C57Bl/6J mice were divided into three groups: (1) HLI + PBS (Vehicle), (2) HLI + WT Exo and (3) HLI + Trx-1Tg/+ Exo (Figure 1). Right femoral artery ligation was performed to create hind limb ischemia. Immediately after surgery, mice in the treatment group received Trx-1Tg/+ Exosomes in both the semimembranosus and gastrocnemius muscles of the right leg, whereas the left leg was used as an internal control. The left leg did not receive surgical manipulation or treatment injection. All the animals underwent Doppler imaging both pre-operatively and post-operatively for the assessment of limb perfusion. Doppler imaging was a good method to analyze perfusion as no additional manipulation was necessary to quantify the perfusion with a laser Doppler machine. Although not included in this study, immunohistochemistry was done as proof of principle, and to practice proper technique. Doppler imaging was conducted prior to, immediately following and
post-operatively at days 3, 7, 21, and 28 to ensure hind limb ischemia was successfully induced and track changes in perfusion during the recovery period.

Figure 1. Schematic representation of experimental design- HLI induction surgery was performed in WT mice and animals were injected with their groups corresponding treatment in the gastrocnemius and semimembranosus muscles of the right leg. Perfusion to the legs was visualized and quantified by laser Doppler imaging at multiple time points.

**Induction of Hind Limb Ischemia**

Mice were anesthetized in a chamber of 600 mL/min oxygen with 2.5% isoflurane, removed from the chamber and placed in a supine position on the surgical table. Anesthesia was continuously administered through a nose cone. Hair was removed from the surgical area using Nair® hair removal cream, cleaned with sterile gauze and water and subsequently sterilized with Betadine®. An incision was made near the femoral artery, distal to the knee and extending proximally to the hip. After the incision, the inferior epigastric artery was cauterized to reduce blood flow to the surgical leg. The femoral artery was ligated proximally to the
profunda and at the knee proximal to the branching of the artery. The corresponding treatment was administered to four sites in the semimembranosus and gastrocnemius muscles, depending on the group of the animal (PBS, WT exo, Trx exo). Wounds were closed with 4-0 Vicril sutures, and mice were removed from nose cone and placed under a heat lamp for post-operative monitoring.

**Figure 2.** Pictures from surgical procedure. From left- initial anesthesia chamber, mouse in surgical position inserted into nose cone, site of incision, and wound closure.

*Doppler Imaging*

Mice were anesthetized in chamber with 600 mL/min oxygen with 2.5% isoflurane. Upon successful anesthetization, mice were removed from the chamber and placed in a prone position on a heating pad and inserted into nose cone for continuous administration of anesthesia. Heating pads are necessary for the Doppler, as cold temperature can lead to microvessel constriction which will negatively impact the amount of perfusion measured by the Doppler. Once inserted into the nose cone, the hind legs were extended straight backward, parallel with the tail. The Doppler machine was positioned so that the laser was targeted at the base of the tail along the middle axis of the body. Using PIMSOFT® software, the distance between the animal and Doppler machine was set to 16-18 cm. On the PIMSOFT® software, the target area was drawn to include the entire length of both hind legs. The procedure was
repeated with the mouse in a supine position, positioning the Doppler laser between the base of the tail and the genitalia. This was necessary as Doppler imaging only measures perfusion at a depth of 2-3mm from the surface of the skin. Mice were returned to the cage following imaging.

*Tissue Processing*

Mice were euthanized using an overdose of ketamine (100 mg/mL) and xylazine (100 mg/mL) in saline for delivery. The overdose was defined as 3 times the dose used for anesthetic purposes, resulting in injection of 0.3 mL per 20 grams of body weight. Upon successful euthanization, the hind limbs were dissected and the gastrocnemius and semimembranosus muscles were sampled. The tissues were fixed overnight in a 10% PBS buffered formalin (HT501128 SIGMA) in 4 °C. Formalin fixation was achieved by infiltrating the tissue and crosslinking proteins, resulting in greater rigidity and preservation of morphology and protein markers. Formalin fixation was chosen because formalin preserves protein integrity and reactivity, allowing for antigen specific staining. Upon fixation, the samples were put in between two flat sponges and inserted into a plastic cassette. Multiple tissue cassettes were put into a stainless-steel carrier and attached to the arm of an automatic tissue processor. In the automatic tissue processor, the cassettes were dehydrated in containers of increasing graded concentrations of 70%-100% ethanol for one hour each. Paraffin wax is not miscible in water therefore ethanol was used to displace the water in the tissue with gradual increase of concentration of ethanol to preserve the integrity of the tissue. Ethanol and paraffin are not miscible, so tissue cassettes were cleared in Histoclear® 3 x 1 hour. Subsequent to clearing, sponges surrounding the tissues were removed. Paraplast® was melted at 60 °C and the tissue cassettes were placed in a container of molten
wax. Tissues were paraffinized for 3x30 minutes to displace Histoclear®. Paraffinization imparts physical properties similar to wax, allowing for sectioning. The tissues were embedded in a wax block and allowed to solidify on a cold plate. Tissue was sliced at 5 microns on the microtome, warmed in a 40-50 °C water bath, scooped onto a microscope slide, and dried overnight at 37 °C.

**Immunohistochemistry**

Immunohistochemistry was not done on samples collected from animals in this study. However, staining was done as proof of concept, and to provide direction for future experiments. One useful stain for this experiment was 3,3′-Diaminobenzidine (DAB) staining, commonly used in the staining of nucleic acids and proteins. The theory behind this staining protocol is that DAB will be oxidized in the presence of hydrogen peroxide and peroxidase to produce a brown alcohol insoluble precipitate. It is important for the precipitate to be alcohol insoluble as the slides will be submerged in ethanol during subsequent dehydration.

On the first day of staining slides were deparaffinized in Histoclear® for 2x2 minutes. The slides were rehydrated by being transferred into containers of decreasing graded concentrations of 100%, 90%, 80%, 70%, 60%, 50% ethanol for two minutes each, and then rinsed in running water for 10 minutes. 1X Antigen retrieval solution (Biogenex® HK086-9K) was pre-warmed in steam-bath for 20 minutes. The slides incubated in the antigen retrieval solution for 20 minutes in the steam bath to break crosslinks formed by formalin fixation, effectively exposing antigens. The slides were removed from the antigen retrieval solution and cooled in running water for 20 minutes. The slides were washed in 1X PBS 3x5 minutes, and the sections were subsequently circled with a hydrophobic ink pen. 100 uL of 0.3% hydrogen peroxide was
added to each of the slides and allowed to sit for 30 minutes, and subsequently washed in 1X PBS 3x5 minutes. The blocking serum (Thermo-37520) was then added for 20 minutes at room temperature. Rabbit polyclonal caveolin-1 (SC-894) primary antibody was applied at a 1:100 dilution in 1X PBS and allowed to incubate overnight at 4 °C. Caveolin-1 is an endothelial cell marker and is a good marker for capillaries (Figure 3). On day 2 of staining, slides were washed in 1X PBS 3x5mins, incubated with goat anti-rabbit antibody with horseradish peroxidase (PI-1000) for 30 minutes at room temperature and washed in 1X PBS 3x5minutes after incubation. DAB solution (SK-4100) was prepared as follows: 5 mL water, 2 drops of PBS buffer stock, 4 drops of DAB, and 2 drops of H₂O₂. The tissue sections were covered with the DAB solution using a Pasteur pipette and once the solution began to turn brown, the slides were placed in a running water bath for 10 minutes. Slides were gradually dehydrated in 50%, 60%, 70%, 80%, 90%, 100% ethanol, and then were mounted with coverslips (0.17 mm x22 mm) using Permount® (SP15-500) medium.

**Figure 3.** Left- DAB staining slide for myocardium. Right- DAB staining slide for gastrocnemius. Darkly stained regions represent endothelial cells, thus allowing for quantification of capillary density.

*Isolation of Exosomes*
Blood was harvested from WT and Trx-1 overexpression mice and was centrifuged at
3000 g to remove cells and isolate plasma. The supernatant was transferred to a sterile tube,
and appropriate amount of System Biosciences ExoQuick Exosome Precipitation Solution® (Cat
# EQPL10A-1) was added (67uL ExoQuick/250uL Biofluid). The tube was mixed by inversion and
flicking the bottom of the tube. The tubes were incubated on ice for 30 minutes and
subsequently centrifuged at 1500g at 4 °C. The supernatant was poured off, and residual
ExoQuick solution was removed by additional centrifugation at 1500 g for 5 minutes. The pellet
was suspended using 250-500 uL Resuspension Buffer®. One unit of ExoQuick® beads was
pretreated with 400 uL of Resuspension Buffer® and vortexed for 3 minutes, and centrifuged at
3000 g for 3 minutes. The liquid was removed and the wash was repeated two more times. The
exosome suspension was added to the washed beads and was mixed at room temperature
using a shaker for 15 minutes. The sample was centrifuged at 6000 g for 5 minutes and the
resulting supernatant contained purified exosomes.

Characterization of Exosomes

Exosomes were visualized and photographed using a transmission electron microscope
(Figure 4). The size and diameter of the exosomes were determined using Nanoparticle Tracking
Analysis instrument (NS500; Malvern Instruments, Westborough, MA). The exosome samples
were diluted 100-fold with phosphate buffered saline (1X PBS) and 500 uL was injected into the
NS500 instrument. Video recordings of the Brownian motion of the particles were analyzed by
the NTA software to determine vesicle size (Figure 4).
Figure 4. Left- Screen capture of NTA video recording. Right- Frequency distribution of vesicle sizes. High frequency of vesicles smaller than 150nm suggest the presence of exosomes.
Results

Perfusion ratios were analyzed and compared at post-operative day 0, as well as post-operative day 28 to quantify the amount of perfusion recovery. This method was used so that the perfusion values were standardized to each animal based on their internal control non-ischemic leg. Following calculation of perfusion for animals in each group (n=4), data showed that treatment with PBS resulted in a mean perfusion ratio of 0.384, wild type exosomes resulted in mean perfusion ratio of 0.5222, and Trx-1 exosomes resulted in a mean perfusion rate 0.774 (Figure 6). Newman Keuls multiple comparison showed a significant difference in the means of Trx-1 exosome treated group with those treated with PBS (P<0.05), however there was no significant difference between the wild type exosome treated group and Trx-1 exosome treated group

Figure 5. Representative laser Doppler images of phosphate buffered saline (PBS, control), WT-Exo and Trx1-Exo treated mice group taken at pre-operative and post-operative days 0 and
28 are provided to demonstrate the scale of perfusion level in color from high (red) to minimum/low (blue/black) and levels in between as green and yellow

**Figure 6.** Perfusion ratios of mice in PBS (vehicle control), WT-Exo (positive control), and Trx1-Exo, (n=4, all groups). (PBS vs Trx1-exo, p<0.05) (PBS vs WT-exo, p>0.05) (WT-exo vs Trx1-exo, p>0.05)

**Discussion**

In the present study, it was demonstrated that treatment with Trx-1 exosomes could attenuate hind limb ischemic injury in a murine model of hind-limb ischemia. We found that treatment with Trx-1 exosomes restored limb perfusion to a mean 77.4% perfusion recovery, when compared to the 38.4% perfusion recovery in the vehicle control group (Figure 6). The results show that exosomes loaded with treatment can promote perfusion recovery. This study supports a growing body of evidence that support the role of exosomes in treatment delivery, and improvement of ischemic injury outcomes (3, 6, 7, 8, 9, 11, 12, 19, 20).
Hu et al. (2015) showed positive results using induced mesenchymal stem cell (iMSC) exosomes based on previous evidence that bone marrow derived mesenchymal stem cells (BMSCs) are a good source of cell-based therapy (9). However, the disadvantage to using BMSCs is that only a small number of cells can be taken from a donor because the cells are adult somatic cells which can lose their ability to self proliferate with age or after several cell cultures. Emerging evidence has suggested that therapeutic effects of stem cells are mediated by the exosomes secreted by those cells. Hu et al. (9) discovered that transplantation of iMSC-exo into mice with hind-limb ischemia resulted in higher perfusion and micro vessel density. The microvessel density was analyzed by staining with CD-31 and evaluating how many vessels appeared in a square millimeter of tissue. Increased microvessel density specifically supports the idea that exosomes are able to restore perfusion via therapeutic angiogenesis. In vitro experiments using human umbilical vascular endothelial cells (HUVEC) cultured with iMSC-exo expressed higher levels of angiogenesis related genes particularly vascular endothelial growth factor (VEGF) A and B, and angiogenin (9). Hu et al. (2015) found that the IMSC-exo cultured HUVEC cells secreted more of VEGF A and B, angiogenin, and other angiogenesis related proteins into the medium, further cementing the role of exosomes in paracrine signaling (9).

The therapeutic effects of exosomes are not limited to hind limb ischemia injury. There have been many studies that have shown the benefits of exosome transplantation in recovery of myocardium. Vincencio et al. (2015) investigated the effects of exosome transplantation in an in vivo model of myocardial infarction in rats. Vicencio et al. (2015) found that administration of exosomes via a tail vein injection 15 minutes prior to surgery, resulted in a
significant reduction of the infarct size (19). Animals in the vehicle control group had an average infarct size of 48% of the risk area, while animals in the treatment group had an average infarct size of 25% of the risk area (19). Vincencio et al. (2015) demonstrated the therapeutic effects of naturally occurring exosomes. Studies such as these (6, 7, 11, 19, 20) strongly suggest a role for exosomes as endogenous signaling molecules, and due to their potency, exosomes are excellent candidates for the delivery of exogenous treatments.

A shortcoming of our study is that the exosomes were injected immediately following induction of hind-limb ischemia; as mentioned earlier, one of the biggest challenges in treating peripheral artery disease is the lack of symptoms in the early stages of the disease. To account for the limitation, additional experiments could test the efficacy of transplanting exosomes hours, or even days after the initial induction of hind limb ischemia. Further studies should include protein and mRNA characterization of exosome surface markers, as well as the contents inside. The effect of Trx-1 exosomes on micro vessel density should also be analyzed using DAB staining, or any similar staining method for common endothelial cell including but not limited to CD31 and CD34. Further investigation is required to see where the injected exosomes circulated, and to see if there was any preferential localization in the body.

We have demonstrated that injection of exosomes isolated from Trx-1 overexpression mice significantly recovered perfusion in comparison to a vehicle control group. Our results have significant translational value because novel non-invasive intervention for peripheral artery disease could help to improve outcomes in clinical presentations of peripheral artery disease. Non-invasive interventions will be particularly beneficial to those patients who have
extensive atherosclerosis beyond the level of stenting, but who are not healthy enough to undergo open bypass surgery.

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