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TITLE:

Large Animal Model for Evaluating the Efficacy of the Gene Therapy in Ischemic Heart

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SUMMARY:

Myocardial gene therapy for ischemic heart disease holds great promise for future therapeutics. Here, we introduce a large animal model for evaluating the efficacy of gene therapy in the ischemic heart.

ABSTRACT:

Coronary artery disease is one of the significant causes of mortality and morbidity worldwide. Despite the progression of current therapeutics, a considerable proportion of coronary artery disease patients remain symptomatic. Gene therapy-mediated therapeutic angiogenesis offers a novel therapeutic method for improving myocardial perfusion and relieving symptoms. Gene therapy with different angiogenic factors has been studied in few clinical trials. Due to the novelty of the method, the progress of myocardial gene therapy is a continuous path from bench to bedside. Therefore, large animal models are needed for evaluating the safety and efficacy. The more the large animal model identifies the original disease and the endpoints used in clinics, the more predictable outcomes are from clinical trials. Here, we introduce a large animal model for evaluating the efficacy of the gene therapy in the ischemic porcine heart. We use clinically relevant imaging methods such as ultrasound imaging and $^{15}H_2O$-PET. For targeting the gene transfers into the desired area, electroanatomical mapping is used. The aim of this method is: (1) to mimic chronic coronary artery disease, (2) to induce therapeutic angiogenesis at hypoxic areas of the heart, and (3) to evaluate the safety and efficacy of the gene therapy by using relevant endpoints.

INTRODUCTION:

Coronary artery disease is accountable for the vast proportion of mortality and disease burden worldwide[1]. Current treatment strategies are percutaneous interventions, pharmacological treatment, and bypass surgery[2]. However, despite the progression of these current therapeutics, many patients suffer from so-called refractory angina, underlining the unmet need for novel treatment approaches[3]. Gene therapy-mediated therapeutic angiogenesis could target this

patient group.

Myocardial gene therapy is most often delivered by using different viral vectors, most commonly replication-deficient adenovirus[4]. As therapeutic genes, various angiogenic growth factors are used. The most substantially studied angiogenic growth factors are the vascular endothelial growth factors (VEGFs) that mediate their angiogenic signaling through vascular endothelial growth factor receptors (VEGFRs) and their co-receptors[5]. Several clinical trials have proved the benefit and safety of cardiac gene therapy and made this novel treatment method a realistic option for treating ischemic heart diseases[6,7]. However, this concept still needs enhancement of the therapeutic genes and viral vectors put to the test in large animal models before entering the clinics. The pig has been frequently used as a laboratory animal since its heart is very similar to the human heart. The size of the cardiovascular system of a pig allows the usage of similar catheter inventions as used in humans. All imaging modalities available for humans can be used in pigs[8].

There are several large animal models for chronic ischemia. The most commonly used is the ameroid constrictor model[9–11]. The downside of this method is the invasiveness since thoracotomy is needed to access the coronary vasculature. Previously in our group, a mini-invasive bottleneck stent model for chronic myocardial ischemia has been developed[12]. This method is also used in this manuscript to induce myocardial ischemia.

The usability of ultrasound imaging has evolved substantially despite the age of the imaging modality. For example, myocardial strain is still mainly in research usage due to its novelty. Myocardial strain reflects changes in the contractile function of the heart better than the traditional M-mode ejection fraction measurement[13]. Thus, here in the large animal model, myocardial strain measurement is utilized. To evaluate the function of the heart, cardiac output is also measured by cine imaging of the left ventricle during angiography. Cardiac output is measured both at rest and under dobutamine-induced stress to evaluate myocardial function under stress.

In addition to the measurements of the heart function, information on myocardial perfusion is essential in gene therapy studies aiming at therapeutic angiogenesis. In this animal model, animals are imaged with ^{15}O-labeled radiowater positron emission tomography ($^{15}H_2O$-PET) as this is the golden standard for measuring myocardial perfusion. $^{15}H_2O$-PET has been previously validated for measuring perfusion of ischemic porcine heart[14].

Thus, the methods and modalities mentioned above constitute an excellent perspective for evaluating the efficacy of gene therapy in the ischemic heart.

PROTOCOL:

The experiments presented here are performed using about 10-week-old female domestic pigs and are approved by the Animal Experiment Board in Finland. Animals weigh 30–40 kg at the beginning of the protocol, allowing the same procedural equipment and imaging modalities as possible for humans. Chronic ischemia is induced 14 days before the gene transfer, and the

follow-up time after the gene transfer depends on the viral vector used. The study protocol is shown in **Figure 1**. This protocol can be used to perform adenoviral or AAV-mediated gene therapy injections. The time of sample collection has to be adjusted to the transgene expression peak, which depends on the viral vector used. For example, when performing adenoviral gene transfers, the time of the sample collection is set to 6 days after the gene transfer.

1. Medication

1.1. Administer a daily dose of 200 mg of amiodarone and 2.5 mg of bisoprolol to prevent fatal ventricular arrhythmias. The medication begins 1 week before the ischemia operation and is continued daily until the follow-up.

1.2. In addition, administer peroral doses of clopidogrel (300 mg) and acetylsalicylic acid (300 mg) to the animals 1 day before the ischemia operation to prevent acute in-stent thrombosis after the stent placement.

1.3. Administer 100 mg of lidocaine and 2.5 mL of $MgSO_4$ intravenously to the animals at the beginning of the ischemia operation to prevent ventricular arrhythmias.

1.4. Administer intramuscular injection of cefuroxime (500 mg) at the beginning of each operation for infection prophylaxis.

1.5. Administer 30 mg of enoxaparin intravenously at the beginning of the ischemia operations and subcutaneously after the operation procedure for thrombosis prevention.

2. Transthoracic echocardiography

2.1. Perform transthoracic echocardiography before ischemia operation, gene transfer, and euthanasia to evaluate any detectable pericardial fluid and determine the myocardial strain.

2.2. Place the transducer in the third or fourth intercostal space under the armpit of the pig to access parasternal short-axis views at the mitral valve level, papillary muscle, and apical levels (**Video 1**). The marker of the transducer should point to the sternum of the pig. To save a clip, press **Acquire**.

3. Endovascular operations under fluoroscopic guidance

3.1. Preparation of the operation

3.1.1. Prepare for the operations by sedating the pigs with an intramuscular injection of 1.5 mL of atropine and 6 mL of azaperone.

3.1.2. After the sedation, induce general propofol and fentanyl anesthesia for the angiographic procedures to the pigs with doses of 15 mg/kg/h and 10 µg/kg/h, respectively.

NOTE: The pigs are anesthetized for the entire procedure.

3.1.3. Support the ventilation by intubation and ventilator and monitor the vital physiological parameters, such as ECG and respiratory parameters.

3.2. Introducer sheath placement

3.2.1. Place an introducer sheath into the right femoral artery for all the operations as standard practice in cardiology. Use ultrasound to track the femoral artery and pierce it with an entry needle (18 G).

NOTE: Use an 8F introducer sheath for intramyocardial gene transfers and a 6F sheath for all other operations. Introduce the guidewire of the sheath through the needle to thread the artery and hold the guidewire still while removing the needle.

3.2.2. Insert the introducer sheath along the guidewire, and when placed, remove the guidewire and administer 1.25 mg of sublingual dinitrate to the pig to induce coronary vasodilation.

3.3. Coronary angiography

3.3.1. Perform coronary angiography directly before ischemia operation, gene transfer and euthanasia, and tissue collection. The machinery needed for the angiograms is shown in **Figure 2**.

3.3.2. Use a 6F catheter under fluoroscopic guidance with an iodine contrast agent to image the right coronary artery and the left ascending coronary artery (**Video 2**).

3.4. Ischemia operation

3.4.1. Place a bottleneck stent into the left coronary artery (LAD) 14 days before the gene transfer to induce chronic myocardial ischemia. After the bottleneck stent placement, check whether the bottleneck stent is placed correctly, restricting the coronary blood flow.

NOTE: The bottleneck stent is placed on a dilation catheter and consists of a bare-metal stent covered by a polytetrafluoroethylene tube formed in a bottleneck shape to reduce coronary blood flow[12].

3.5. Defining the stent size

3.5.1. Choose the size of the stent, either 3.0/3.5/4.0 x 8 mm, according to the size of the left ascending coronary artery in the angiogram by using the automatic measurement software in the angiographic workstation (**Video 3**)[12].

3.6. Stent placement

3.6.1. Place a coil to the left coronary artery and glide the bottleneck stent to the LAD, placing it distally to the first diagonal.

3.6.2. Inflate the stent to nominal pressure in the artery using an in-deflator with a stent-to-lumen ratio of 1.3, anchoring the bottleneck in place. After an additional 15 s, deflate the stent and retract the equipment from the artery.

NOTE: Confirm the correct placement of the bottleneck stent by angiogram.

3.7. Left ventricle cine imaging under rest and dobutamine stress

3.7.1. Administer a 21 mL bolus of iodine contrast agent into the left ventricle via a 5F pigtail catheter using an auto-injector. First, set the bolus duration at 3 s and the total volume for 21 mL. Then, press **Single** and **Yes**.

3.7.2. Calculate the ejection fraction by the measurement software of the angiographic workstation. To perform the calculation, select **Ventricular Analysis** of the image in question. Scroll the image to select a time frame, one in diastole and one in systole. Select a tool to draw ventricular outlines of each time frame.

NOTE: The software now calculates the ejection fraction and stroke volume by the Simpson's method. The ejection fraction measurement is performed during rest and under dobutamine-induced stress.

3.8. Stress imaging

3.8.1. Dose dobutamine intravenously in escalating doses from 10 µg/kg/min to 20 µg/kg/min for the dobutamine-induced stress imaging until the target heart rate of 160 bpm is reached. Then, perform the cine imaging.

4. PET imaging

NOTE: One day before the gene transfer, perform rest and stress ^{15}O-labeled radiowater PET/CT scans (requires hospital environment and radiological technicians).

4.1. Reference imaging

4.1.1. Perform computed tomography (CT) scans before rest and stress imaging. Use the CT information for attenuation correction.

4.2. ^{15}O-labeled radiowater imaging

4.2.1. Perform rest and stress imaging using an 800 MBq $^{15}H_2O$ bolus.

4.3. Stress imaging

4.3.1. Perform stress imaging with a further 800 MBq ^{15}O-water bolus after a suitable radioactive decay of 12 min.

NOTE: Hyperemia is induced by adenosine (200 µg/kg/min intravenous), as described previously[15].

5. Gene transfer

5.1. Electroanatomical mapping

5.1.1. Proceed to electroanatomical mapping after a coronary angiogram and functional measurements (echocardiography, LV cine imaging).

5.1.2. Introduce a mapping catheter to the left ventricle via femoral sheath in fluoroscopic guidance.

NOTE: Register about 100–150 points around the left ventricle with the mapping catheter to create the electroanatomical map.

5.2. Finishing the electroanatomical map

5.2.1. Delete the outlier points to ensure a more reliable electroanatomical map of the left ventricle.

5.2.2. Do this by selecting **Clip Planes** of the map and delete the points that differ from the points forming the ventricular shape. Next, select **Trajectories** for the map view and delete the points that have traveled horizontally during point registering.

NOTE: Make sure the remaining points cover the left ventricle and register more points if needed.

5.3. Gene transfer injections

5.3.1. Introduce an intramyocardial injection catheter to the left ventricle via the femoral sheath under fluoroscopic guidance. Set the injection needle length to 3 mm.

5.4. Criteria for intramyocardial injections

5.4.1. Guide the gene transfers by electroanatomical mapping system and target the injections into viable but hypokinetic areas of the left ventricle.

NOTE: For viability, use a unipolar voltage over 5 mV as a criterion. For hypokinesia, select a local linear shortening (LLS) as low as available, at least below 12% but preferably below 6%[16].

5.5. Intramyocardial injections

5.5.1. During 30 s, inject the vector material into the point of selection (step 5.4) and keep the injection needle inside the myocardium for an additional 5 s before retracting to prevent backflow to the left ventricle.

6. Euthanasia and sample collection

NOTE: After the coronary angiogram and ejection fraction measurements described in steps 3.3 and 3.7, administer the pig 50 mL of saturated potassium chloride intravenously.

6.1. Perfusion fixation of the heart

6.1.1. Harvest the heart from the thoracic cavity. Rinse with water. Place an 18 G needle above the aortic valve and attach the needle to a perfusion pump. Perfuse the heart with 750 mL of 1% paraformaldehyde (PFA).

6.2. Sample collection

6.2.1. Slice the heart into 1 cm thick slices using a sharp kitchen knife. Collect the samples from the gene transfer area into 4% PFA and liquid nitrogen.

NOTE: To harvest negative controls, collect a control sample from the posterior wall of the left ventricle.

6.3. Safety tissue collection

6.3.1. Harvest samples from remote tissues, such as the lung, liver, kidney, spleen, and ovaries. Take samples into 4% PFA and liquid nitrogen.

7. Sample storing

7.1. Store the samples for staining in 4% PFA for 48 h at 4 °C.

NOTE: Replace the PFA daily with a fresh liquid.

7.1.1. After 48 h, replace the PFA with 15% sucrose in deionized water. Store for at least 24 h before embedding the samples into paraffin blocks. Snap frozen samples are stored at -70 °C.

REPRESENTATIVE RESULTS:

The success of the ischemia operation can be confirmed with this protocol by coronary angiogram

and by determining the hypokinetic area by transthoracic ultrasound (**Figure 1**) before proceeding to the gene delivery. The state of the coronary occlusion can be evaluated by coronary angiogram, and the electroanatomical mapping ensures the ischemic and hibernating areas.

The efficacy of the gene therapy can be analyzed by measuring the circumferential strain, ejection fraction, and myocardial perfusion by $^{15}H_2O$-PET (**Figure 3**). The tissue samples can be collected directly from the gene transfer area by comparing the heart to the electroanatomical map. The transgene expression and therapeutic angiogenesis (**Figure 4**) can be evaluated through immunohistological analysis by analyzing the number of positive cells after beta-galactosidase staining and by analyzing the myocardial capillary area after CD31 staining. In addition, the safety of the gene therapy may be assessed by diagnostic imaging (pericardium effusion assessment by echocardiography), immunohistology, and distribution analysis.

FIGURE LEGENDS:

Figure 1: Study protocol. Ischemia is induced 14 days before the gene transfer. $^{15}H_2O$-PET imaging is performed 1 day before gene transfer and before euthanasia and sample collection. The time of the sample collection depends on the viral vector and therapeutic gene used. When using adenoviral vectors, the second $^{15}H_2O$-PET is on the 5th day, and the time of sample collection is on the 6th day, respectively.

Figure 2: **Angiolaboratory setup.** The machines needed for coronary interventions: ultrasound machine, ventilator, and angiographic station, from left to right.

Figure 3: **Representative image of circumferential strain, and $^{15}H_2O$-PET and an electroanatomical map from the ischemic heart.** $^{15}H_2O$-PET: red color represents the area of maximal perfusion, and blue indicates the area of hypoperfusion. Electroanatomical map: Brown dots in the electroanatomical map represent the injection sites. The red color indicates hypokinetic areas of the left ventricle, whereas purple indicates the area of normal contractability.

Figure 4: Representative image of β-galactosidase and PECAM-1 stainings. β-galactosidase is expressed in AdLacZ transduced hearts and can be used to show transgene expression. PECAM-1 staining is used to detect myocardial capillaries and to analyze the capillary area. The lower row represents the area distant from the gene transfer. Scale bar in β-galactosidase stainings: 200 μm. Scale bar in PECAM-1 stainings: 100 μm.

Video 1: **Transthoracic echocardiography short-axis view.**

Video 2: Coronary angiogram of LAD before gene transfer.

Video 3: Left anterior descending artery diameter measurement.

DISCUSSION:

The timepoints of this protocol may be modified according to the viral vector used. Also, the immunohistological analyses may be selected according to the therapeutic gene. It is also possible to add more timepoints and endpoints to the protocol if needed.

This protocol comprises stages, which are essential to succeed and impossible to correct afterward. First, if one fails to induce appropriate ischemia, the animal must be excluded from further procedures and analyses. Standardization of the methods and imaging is crucial so that the results are comparable between the timepoints and animals. Second, the samples must be collected from the exact gene transfer area and processed successfully to perform further analyses. Also, this protocol requires profound familiarity with angiographic procedures and different imaging modalities. For example, coronary angiogram and viral injections to a beating heart require extensive training as well as performing correct transthoracic echocardiography. Nevertheless, these imaging modalities measure myocardial function and perfusion to provide essential information for further studies.

The cardiovascular system of a pig resembles the human one due to its anatomical and physiological similarities, and therefore, pigs are often used to model cardiovascular disease mechanics and procedures. However, the follow-up time is restricted to approximately 6 months due to the rapid growth of the animal. After 6 months, the handling of the animal becomes challenging, and the imaging quality deteriorates.

Also, pigs are considerably resistant to atherosclerosis, making diet-induced atherosclerosis complicated to model in pigs[17]. However, chronic ischemia models have been developed to mimic the original disease. The significant advantage of the bottleneck stent ischemia model used in this protocol is that the gradual occlusion of the stent represents coronary artery disease better than sudden occlusion. Compared to the ameroid constrictor model, this method is less invasive. Secondly, percutaneous bottleneck stent placement is a quick procedure to perform. Utilizing the electroanatomical mapping system enables targeting the gene transfer to the hibernating myocardium, not to the infarct area, which is a possible outcome when ultrasound guidance is used to target the injections. However, the downside of the electroanatomical mapping is the length of the procedure. Furthermore, since the pig heart is highly sensitive to ventricular arrhythmias, mapping can induce ventricular fibrillation during the mapping procedure. However, these arrhythmias are easily defibrillated.

The endpoints used in this large animal model identify those used in clinical trials, enhancing the transition to the clinics. In addition, these methods are applicable for large animal studies evaluating the efficacy of myocardial gene therapy with different follow-up times and other adjunct endpoints in addition to the ones described in this model. This protocol has been standardized after a vast experience of large animal experiments. In the future, this protocol applies to evaluating the safety and efficacy of myocardial gene therapy before translation to the clinics.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

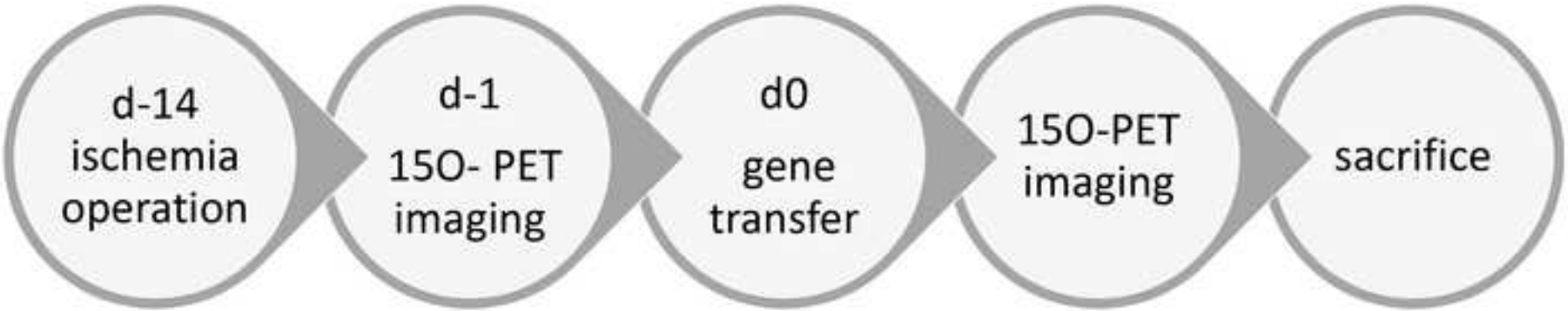

Figure 2

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Figure 3

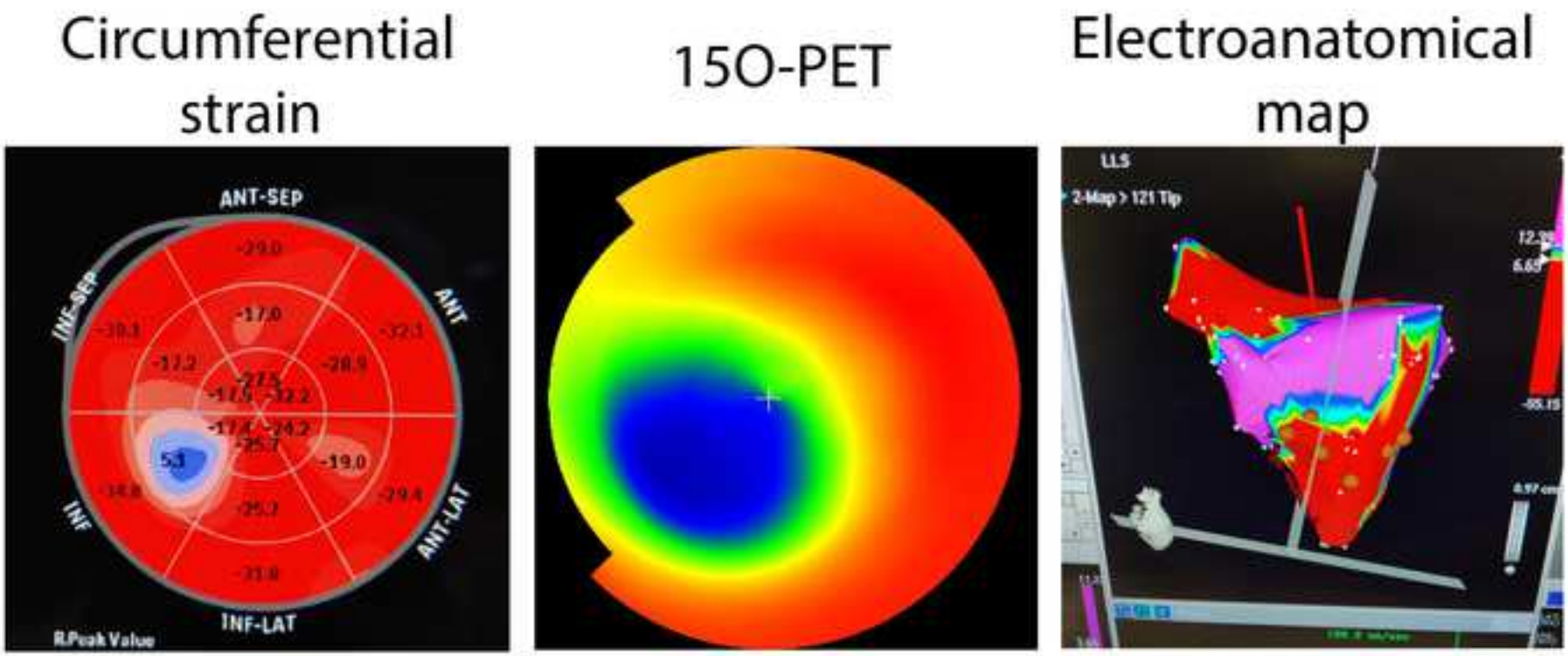
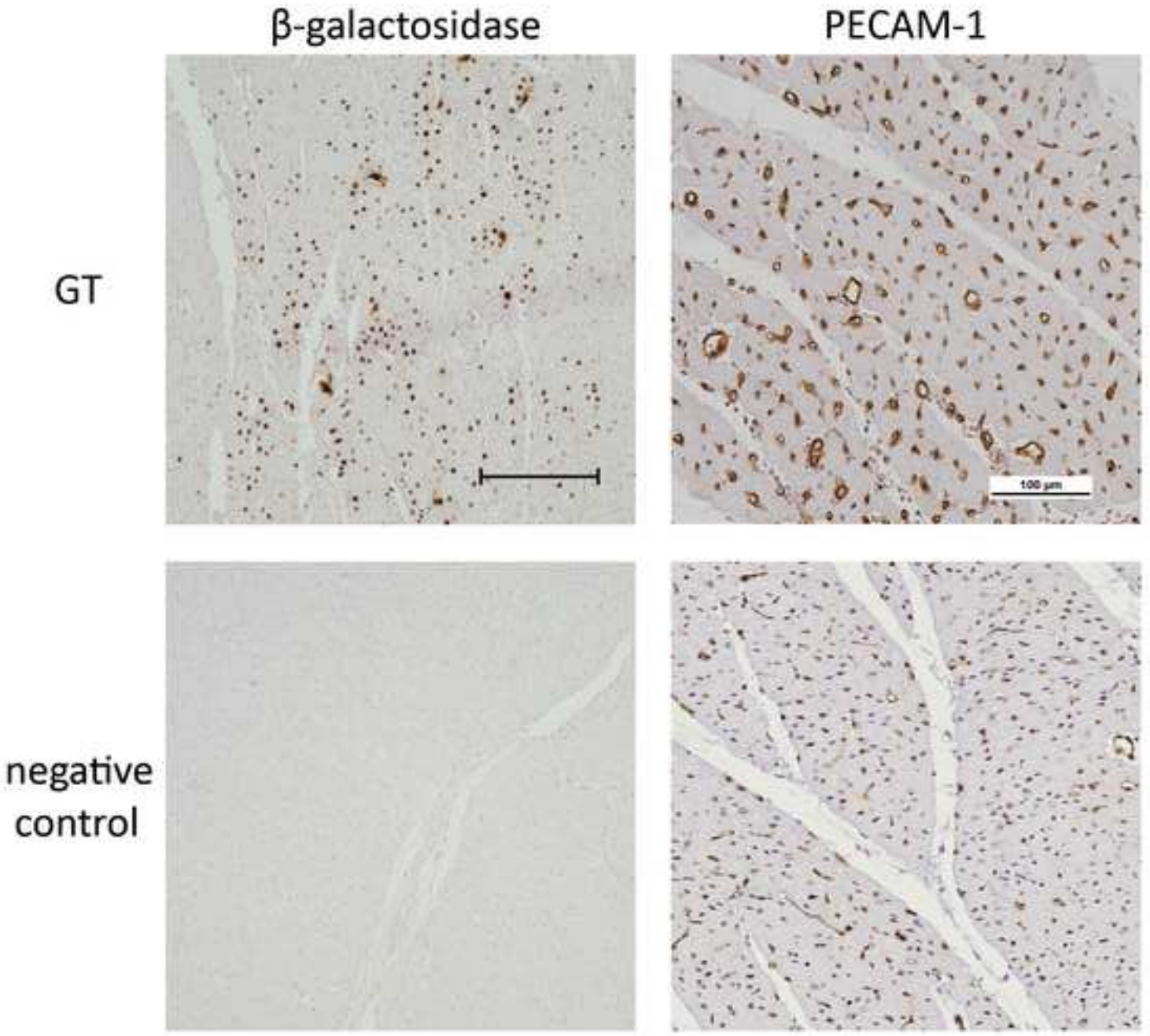


Figure 4



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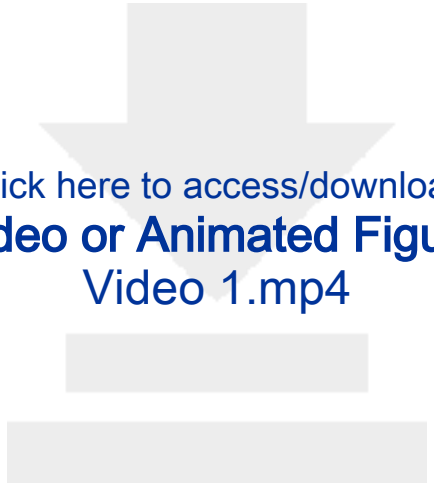

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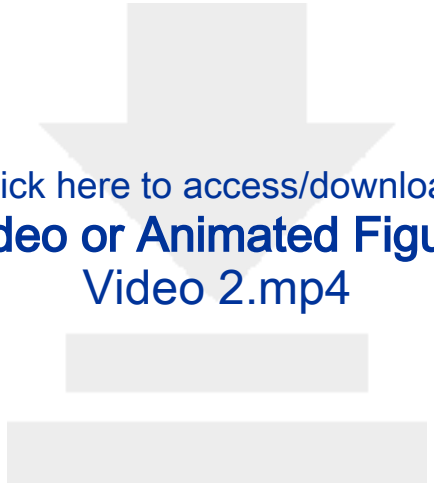

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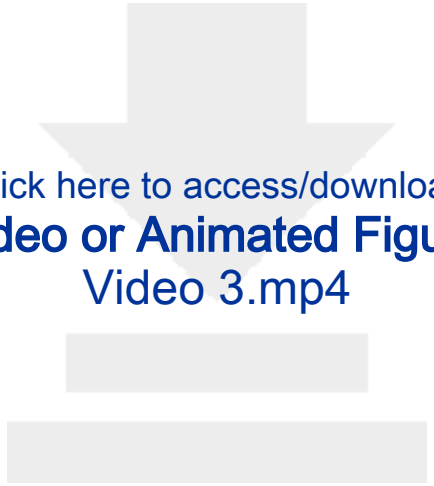

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6/11/2021

Dear Editors,

Thank you for taking our manuscript under review. We have now modified the manuscript considering the insightful comments from the editorial and the reviewers. Every comment or concern has been notified, and the manuscript has been supplemented systematically.

We have fixed the manuscript formatting to respond better to the guidelines of JoVE as asked by the editorial. We have done all the corrections asked by the editorial. The rest of the comments are addressed in the following.

Reviewer #1:
Comment 1: The videos were helpful, but an additional video showing the injection of the gene therapy with the Myostar intramyocardial injection catheter would also be useful. This is a key procedure. A figure/photo of this catheter would also be informative.
Reply: We agree that intramyocardial injections are the key procedure. Unfortunately, we did not have a possibility to film intramyocardial injections during the given time for revision. However, we are happy to show the gene transfers in the final video.

Comment 2: A brief note to explain the selection of female pigs would be helpful. I assume this is related to size. Inclusion of the approximate body weight of the pigs would also be helpful.
Reply: Female pigs are preferred over males due to less aggressive behavior and easier handling. The body weight of the pigs has been added to the manuscript.

Comment 3: Section 1.1- Medication. Please insert the route of administration for all medications on lines 75-78.
Reply: We have now added the route of administration for all medications.

Comment 4: Section 3.3, line 131. It is noted a bolus of iodine contrast agent is administered. An approximate volume would be helpful to insert here
Reply: The approximate volume (21 ml) of the iodine contrast agent has been added to the manuscript.

Comment 5: Section 3.3.1, line 137. It would be helpful to note the approximate doses of dobutamine.
Reply: The approximate dose of dobutamine (10 µg/kg/min) has been added to the manuscript.

Comment 6: Figure 1 could be more informative with an accurate experimental time line in regard to days. The time line currently does not reflect how long after gene therapy the 150-PET imaging and tissue collection occurs. I would recommend changing "sacrifice" to "euthanasia and tissue collection".
Reply: The term "sacrifice" has been changed to "euthanasia and tissue collection". The second ^{15}O-PET imaging and tissue collection time points depend on the viral vector used and thus are not included in Figure 1.

Comment 7: It would be helpful to label the machines/equipment in Figure 2.
Reply: Machines are now listed in the figure legend of Figure 2.

Comment 8: Figure 3: interpretation of the different colors would be helpful.
Reply: The interpretation of different colors is now detailed in the figure legend (Figure 3).

Comment 9: Figure 4: The scale bars are different sizes. Do they both reflect 100um?
Reply: The scale bar in β-galactosidase stainings is 200 µm, and the scale bar in PECAM-1 stainings is 100 µm. This information has been added to the figure legend.

Comment 10: At the end of the study, how is the heart collected, fixed etc for staining as shown in Fig 4?
Reply: These steps have been added to the manuscript (see 6.1 and 7.1).

Name of Material/Equipment: Some longer entries have been truncated.
Reply: This problem has been corrected.

Comment 12: Success rate of particular procedures and information on trouble shooting would also be helpful. Some aspects of the procedure are more difficult than others and may not always be successful on the first attempt. There are no comments regarding difficulties of gene transfer injection with a beating heart etc.
Reply: Thank you for the insightful comments! We have now discussed the difficulties of the procedures more extensively in the Discussion section.

Reviewer #2:
Comment 1: Some statements in Introduction and Discussion should be supported by references. For instance, statements on line 33 (refractory angina), line 36 (viral vectors. Maybe the authors can cite a good review on this topic,) and line 201 (resistance of pigs to atherosclerosis).
Reply: Thank you for this valuable comment. We have now added references to support these statements.

Comment 2. The text contains many stylistic imperfections.
Reply: To our best knowledge, we have fixed these stylistic imperfections.

Reviewer #3:
Comment 1: In the protocol age and size of the pigs at beginning and end of the experiment are missing and a short discussion on the imaging quality and possibility in relation to pig size would be helpful for the reader.
Reply: The body weight of the pigs has been added to the manuscript, and a short discussion of the limitations of imaging concerning the pig size has been added to the Discussion section.

Comment 2: In figure 4 ß-galactosidase and PECAM-1 staining is shown, however it would be nice to show the staining for the target region and a non treated region or non-treated animals, to show the success of the NOGA guided, selective gene delivery/experession
Reply: Thank you for this good idea. We have now added pictures of negative controls to the Figure 4.

Comment 3: Reference 7 and 10 are the same publication
Reply: This error in the references has been corrected.

- The protocol is now written in the imperative tense.
- We also edited Figure 3 by removing the word “NOGA” and changing it to “Electroanatomical”.
- Negative controls were added to Figure 4 to make the figure more informative.
- Company names and commercial language have been removed from the products in the List of Materials document.

On behalf of all authors, I hereby submit a revised version of our manuscript.

Sincerely,

Henna Korpela, MD

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9/4/2021

Dear Editors,

Thank you for taking our manuscript under review. We have now further modified the manuscript considering the comments from the editorial. Please find the edits in the revised manuscript.

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

"2. The manuscript is formatted to match the journal style. Please review. "
We have reviewed and accepted the edits made by the editorial.

"3. Highlighting has been adjusted to fit the 3-page limit, including headings and spacings. Please check/edit. However, if you perform any changes, please ensure that the highlighting does not exceed the 3-page limit, including headings and spacings, and is making a cohesive story for the script. Also, please ensure that it is in line with the title of the manuscript."
We have checked that the highlighting does not exceed the 3-page limit.

"4. Please include the explanation for Figure 2 somewhere in the protocol or in the result section."
This has been added under title 3.3. Coronary angiography.

5. Please rewrite the result section to include all the observations and conclusions you can derive from the figures.
We have added more details to the Results section.

6. Please revise the Table of Materials. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. In the present table, the catalog numbers are missing. Please sort the Materials Table alphabetically by the name of the material.
Table of Materials has been revised and sorted and the catalog numbers have been added all the relevant materials.

7. Additional comments are in the attached manuscript.
The manuscript has been edited to better answer the comments by the editorial.

On behalf of all authors, I hereby submit a revised version of our manuscript.

Sincerely,

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