Submission ID #: 61503
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18758463

Title: Predicting Amputation Using Local Circulating Mononuclear Progenitor Cells in Angioplasty-treated Patients with Critical Limb Ischemia

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

Authors provided still images. Please process missing shots with "missing footage" place card.

3. **Interview statements:** Considering the covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **50**

Introduction

1. Introductory Interview Statements

1.1. **Ignacio Escotto-Sánchez**: Although angioplasty improves blood flow in critical limb ischemia, some patients will still progress to limb amputation **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Juan Antonio Suárez-Cuenca**: Compared to prognoses based on traditional risk factors and intrinsic vascular features, the number of local MPCs can be more predictive of the clinical outcome regarding endothelial dysfunction and limb amputation **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Rebeca Perez Cabeza De Vaca:** The number of local MPCs represents a promising indicator of the prognosis of vascular events, such as ischemic heart disease and lower limb ischemia, with potential application in risk stratification and prevention enforcement **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.4. **Juan Antonio Suárez-Cuenca**: Demonstrating the procedure will be Eduardo Vera-Gómez, Alejandro Hernández-Patricio, Carolina Aranda-Rodríguez, and Mario Antonio Téllez-González, PhDs from the laboratories of Experimental Metabolism and Tissue Regeneration, and Juan Miguel Rodríguez-Trejo, Gabriel Hernández-De Rubin, and Oscar Antonio Lomán-Zúñiga, MDs from Vascular Surgery Department **[1][2]**.

1.4.1. INTERVIEW: Author saying the above

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Ethics Title Card

1.5. Procedures involving human subjects have been approved by the institutional research ethics committee from Centro Médico Nacional “20 de Noviembre” ISSSTE.

Protocol

2. Flow-Mediated Dilation (FMD) Evaluation

2.1. To determine the pre- and post-angioplasty FMD (F-M-D) **[1]**, use a vascular linear transducer to measure the diameter of the brachial artery of the Patient **[2]**.

2.1.1. WIDE: Talent measuring artery *Videographer: Important step*
2.1.2. LAB MEDIA: Step 2.1 USG Brachial artery before vascular stress

2.2. Next, place the cuff of a sphygmomanometer above the measurement site in the forearm **[1]** and insufflate the cuff 50 millimeters of mercury above the systolic blood pressure for 5 minutes **[2]** before deflating **[3]**.

2.2.1. Talent placing cuff onto arm
2.2.2. Talent inflating cuff
2.2.3. Shot of inflated cuff, then cuff being deflated

2.3. Within 60 seconds of deflating, measure the brachial artery diameter again **[1]** and use the equation to estimate the FMD **[2]**.

2.3.1. ~~Talent measuring artery~~ Note: Recommend using LAB MEDIA: Step 2.3. Brachial Artery measure after vascular stress instead of videographer capture
2.3.2. BLACK TEXT ON WHITE BACKGROUND: FMD (%) = (maximum diameter after transient ischemia - basal diameter) × 100/basal diameter

3. Lower Limb Vascular Blood Evaluation, Blood Sampling, and Balloon Angioplasty

3.1. To evaluate the clinical severity of limb ischemia according to the Rutherford classification, place an 18-gauge needle into the blood vessel at the selected groin site **[1]** and place an introducer over the needle **[2]**.

3.1.1. WIDE: Talent inserting needle
3.1.2. Introducer being placed

3.2. Advance a flexible guide wire into the introducer **[1]** and replace the guide with a 6-French introducer **[2]**.

3.2.1. Wire being inserted

3.2.2. Guide being replaced

3.3. Using fluoroscopic guidance **[1]**, inject contrast medium to identify the artery trajectory and blocked vascular sites **[2-TXT]**.

3.3.1. Talent placing probe
3.3.2. LAB MEDIA: Note: Please use "Missing footage" place card **TEXT: Alternative: Inject CO_2**

3.4. Introduce two 0.014-French navigation guidewires **[1]** and two 0.014-French support guidewires into the vessel **[2]** and advance the guidewires to the blocked site **[3]**.

3.4.1. Navigation guidewire(s) being inserted
3.4.2. Support guidewire(s) being inserted
3.4.3. LAB MEDIA: Note: Please use "Missing footage" place card Guidewire(s) being advanced

3.5. Next, introduce one 5-French catheter **[1]** and then one 3-French catheters **[2]** and collect 10 milliliters of blood from the site closest to the vascular obstruction **[3]**.

3.5.1. 5-French catheter being inserted *Videographer: Important/difficult step*
3.5.2. 3-French catheter being inserted *Videographer: Important/difficult step*
3.5.3. Blood being collected *Videographer: Important/difficult step*

3.6. Place the sample on ice **[1-TXT]** and advance a guidewire again **[2]**.

3.6.1. Talent placing sample on ice **TEXT: Maintain all blood samples on ice**
3.6.2. LAB MEDIA: Note: Please use "Missing footage" place card

3.7. When the guidewire is in place, introduce an angioplasty balloon catheter containing an inflatable balloon located at the end of the catheter **[1]** and advance the angioplasty balloon catheter to the site of lesion **[2]**.

3.7.1. Catheter being introduced
3.7.2. LAB MEDIA: Note: Please use "Missing footage" place card

3.8. Inflate the balloon against the blocking plaque located at the vascular wall **[1]** and verify restoration of the blood flow **[2]**.

3.8.1. LAB MEDIA: Note: Please use "Missing footage" place card
3.8.2. LAB MEDIA: Note: Please use "Missing footage" place card

3.9. Thirty minutes after performing the angioplasty, advance a catheter to the site closest to the vascular block **[1]** and collect 10 milliliters of blood **[2]**.

3.9.1. LAB MEDIA: Note: Please use "Missing footage" place card Blood being collected

3.10. Then remove all of the guidewires **[1]** and provide the appropriate post-operative care **[2-TXT]**.

3.10.1. LAB MEDIA: Note: Please use "Missing footage" place card
3.10.2. Talent placing compress onto puncture site **TEXT: See text for full post-operative care details**

3.11. Two weeks after the angioplasty, evaluate the clinical severity of the limb ischemia, the resolving rest pain, lower ischemia, and functional foot preservation according to the Rutherford classification **[1]** and identify those cases requiring a major amputation due to an unfavorable outcome **[2]**.

3.11.1. LAB MEDIA: Supplementary Table 1 *Video Editor: please emphasize Characteristic column*
3.11.2. Talent looking at patient chart

4. Circulating Mononuclear Progenitor Cell (MPC) Isolation and Antibody Staining

4.1. Within one hour of collection, transfer 6 milliliters of each collected blood sample into new 15-milliliters tubes **[1]** and dilute the samples at a 1:1 ratio in PBS **[2]**.

4.1.1. WIDE: Talent adding blood to tube(s)
4.1.2. Talent adding PBS to tube(s), with PBS container visible in frame

4.2. Add 2 milliliters of density gradient medium to each of three test tubes **[1]** and add 3 equal volume aliquots of the diluted blood to each tube to no more than 3/4 full **[2]**.

4.2.1. Talent adding density gradient to tube(s)
4.2.2. Talent adding blood to tube(s)

4.3. Separate the blood cells by density gradient centrifugation **[1-TXT]** and use a pipette to collect the cells at the interface of the resulting layers **[2]**.

4.3.1. Talent placing tube(s) into centrifuge **TEXT: 30 min, 1800 x g, 4 °C**
4.3.2. Shot of layers, then cells being collected at interface *Videographer: Important/difficult step*

4.4. Pool the cells from each sample into a single 15-milliliter tube **[1]** and wash each sample six times with 2 milliliters of PBS per wash **[2-TXT]**.

4.4.1. Talent adding cells to tube(s)
4.4.2. Talent adding PBS to tube(s), with PBS container visible in frame **TEXT: 6 min, 1800 x g, 4 °C, x6**

4.5. After the last wash, resuspend the MPC-containing pellets in 1 milliliter of PBS per sample for counting **[1]** and add 1 x 10^6 cells per tube to the appropriate number 5-milliliter flow cytometry tubes per sample **[2]**.

4.5.1. Shot of pellet if visible, with PBS container visible in frame *Videographer: Important step*
4.5.2. Talent adding cells to tube(s) *Videographer: Important step*

4.6. Pellet the cells by centrifugation **[1]** and resuspend each cell sample in 100 microliters of the antibody cocktail of interest for 10 seconds **[2-TXT]** before incubating the cells for 20 minutes at 4 degrees Celsius protected from light **[3]**.

4.6.1. Talent placing tube(s) into centrifuge
4.6.2. Cells being resuspended **TEXT: See text for all antibody suggestion and labeling concentration details**
4.6.3. Talent placing tube(s) at 4 °C

5. MPC Quantification

5.1. At the end of the incubation, use isotype-matched control antibodies to set up the appropriate forward and side scatter gates for analysis **[1]** and use a tube containing a high number of CD45 (C-D-forty-five)- and CD34-positive cells to gate the MPCs **[2]**.

5.1.1. WIDE: Talent loading tube onto cytometer *Videographer: Important step*
5.1.2. SCREEN: Note: Please use “Missing footage” place card

5.2. To select for double positive immunophenotypes, create new plots and gate identify the KDR (K-D-R)-, CD133 (C-D-one-thirty-three), and CD184-positive CD34-positive CD45-positive cells. Then gate to identify the main MPC subpopulations **[1-TXT]**.

5.2.1. SCREEN: Note: Please use “Missing footage” place card **TEXT: KDR: kinase insert domain receptor**

5.3. The number of MPCs can be then correlated with the baseline FMD value and post-angioplasty delta of FMD **[1]** and the proportion of patients requiring major amputation of the lower limb **[2]**.

5.3.1. LAB MEDIA: Figures 3B and 3C left graphs
5.3.2. LAB MEDIA: Figures 4B and 4C

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.1., 3.6., 4.3., 4.5., 5.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

3.6., 4.3.

Results

6. Results: Representative Relationship Between MPC and Hemodynamic Indicators and Lower Limb Amputation Prognosis After Angioplasty

6.1. In this representative analysis, 30 days after lower limb angioplasty, **[1]** the baseline number of CD45-positive CD34-positive KDR-positive MPCs negatively correlated with the FMD **[2-TXT]**, whereas the change in CD45-positive CD34-positive CD133-positive CD184-positive MPCs after angioplasty significantly correlated with FMD improvement **[3]**.

6.1.1. LAB MEDIA: Figures 3B and 3C

6.1.2. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize dashed line in Figure 3B left graph*

6.1.3. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize dashed line in Figure 3C right graph*

6.2. An increased baseline number of MPC CD45-positive CD34-positive KDR-positive subpopulations **[1]** as well as a post-angioplasty reduction in MPC CD45-positive CD34-positive CD133-positive CD184-positive cells were also observed in patients who evolved to limb amputation **[2]**.

6.2.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize green data box in Figure 4B left graph*

6.2.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize red data box in Figure 4C right graph*

Conclusion

7. Conclusion Interview Statements

7.1. **Ignacio Escotto-Sánchez**: During the vascular approach, be sure to collect the blood from the site closest to the vascular obstruction **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.6.)