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TITLE:

Evaluation of Coronary Flow Reserve after Myocardial Ischemia Reperfusion in Rats

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KEYWORDS:

Coronary flow reserve, Ischemia/reperfusion, Left anterior descending artery, Coronary blood flow, Microcirculation, Coronary artery disease

SUMMARY:

Coronary flow reserve (CFR), is defined as the ratio of maximal coronary blood flow to the resting coronary blood flow. We present a protocol for evaluating CFR in rats via ultrasound, which offers the opportunity to predict cardiovascular risk factors in the absence of obstructive coronary disease.

ABSTRACT:

Coronary artery disease is the leading cause of death worldwide. After an acute myocardial infarction, early and successful myocardial intervention via recanalization of the coronary artery is the most effective strategy for reducing the size of ischemic myocardium. The coronary microvasculature cannot be visualized and imaged *in vivo*, but there are several invasive and noninvasive techniques that can be used to assess parameters which depend directly on coronary microvascular function. The endothelial function after ischemia reperfusion can be assessed also at the level of the coronary circulation via the coronary flow reserve (CFR). In this study, peak velocity of left anterior descending (LAD) coronary arteries was measured in rats *in vivo* via Transthoracic Doppler Echocardiography during resting and stress challenge (induced by Dobutamine). A normal heart can increase its coronary blood flow up to four times above the resting values during stress induction. Following ischemia reperfusion, we found a significantly diminished CFR, which can be used as a marker of coronary microvascular dysfunction. CFR has opened a window on the importance of microvascular dysfunction and has been shown to predict cardiovascular risk independent of whether the severe obstructive disease is present.

INTRODUCTION:

Myocardial ischemia reperfusion (IR) is a condition where blood supply is restricted to the heart followed by the restoration of perfusion and simultaneous reoxygenation[1]. Occlusion of coronary arteries can be caused by an embolus or cholesterol plaque rupture, which results in a severe imbalance of metabolic supply and demand, causing tissue hypoxia. Salvage of jeopardized myocardium, improve left ventricular function, and enhance survival in patients with acute myocardial infarction have been observed by the reperfusion therapy. However, after recanalization of the coronary artery, functional abnormalities of small coronary vessels may occur[2-5]. A significant proportion of patients, perhaps as many as 40%, do not regain microvascular and myocardial perfusion despite the restoration of coronary flow. Visualization and evaluation of the coronary microvasculature can be difficult *in vivo,* but there are a number of invasive and noninvasive techniques that can be used to assess parameters directly depending on the coronary microvascular function[6,7]. Also, the endothelial function can be assessed at the level of the coronary circulation via the CFR[5].

Transthoracic Doppler echocardiography is a noninvasive tool which allows us to study coronary artery flow velocity and CFR[5]. CFR represents the ratio of maximal coronary blood flow to the resting coronary blood flow[8]. During the stress challenge, a normal heart increases coronary blood flow up to four times above the resting value. Cardiovascular risk increases when CFR is diminished[9]. Ishihara et al showed that the CFR was severely impaired immediately after the coronary angioplasty[5]. In the absence of coronary artery stenosis, CFR decreases during the coronary microvascular dysfunction and is present in about half of the patients with stable coronary artery disease[10].

The overall goal of this method is noninvasive visualization of left anterior descending coronary artery (LAD) function in rats via echocardiography, which may be used to calculate CFR. This offers an important assessment tool for diagnosing microvascular dysfunction and evaluating potential therapeutic treatments.

PROTOCOL:

All procedures were performed in accordance with protocols approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC-approved protocol 18223) and the NIH Guide for the Care and Use of Laboratory Animals[11].

1 Animals

1.1. Use 4-month-old female Fisher 344 rats (BW~150-180 g) for the study.

2 Ultrasound Imaging before IR surgery

2.1 Anesthetize the rat with isoflurane - induction chamber at 5% with 1.5–2.0 L/min O_2 flow followed by 1.5–2.0% with 1.5–2.0 L/min O_2 flow. This anesthesia is maintained throughout the experiment, during the resting and stress stages.

2.2 Place the animal in a supine position and shave the thorax. Maintain the body temperature at 37-38 °C using the built-in warming platform. Monitor the heart rate using the software.

NOTE: Proper anesthesia is crucial for maintaining the heart rate at normal physiological rates (between 295-350 beats/min).

2.3 Apply ophthalmic vet ointment to prevent the dryness of eyes prior to the imaging.

2.4 Perform echocardiography using 13–24 MHz linear probe (e.g., MS250) (**Figure 1A**).

2.5 Place the animal in the supine position on the heated platform. Ensure that the anesthesia is controlled via the nose cone. Then position the probe to obtain the parasternal short axis view (PSAX) using the rail system (**Figure 1B**).

2.6 Moving the probe in the rostral direction from the PSAX, locate the pulmonary artery (**Figure 1B**).

2.7 Move the probe slightly in the direction caudal from the pulmonary artery to view the LAD and store the image.

NOTE: The LAD is difficult to find without Color Doppler, so B-Mode images of the LAD are not always possible.

2.8 When individual differences make it difficult to locate the LAD coronary artery, follow the technique mentioned below:

2.8.1. Move the probe lateral to the pulmonary artery.

2.8.2. Angle the platform so that the animal is inclined, inverted, or slightly towards the right side so that the left ventricle is more readily visible with the probe.

2.9 Once the image in B Mode is captured or cine-stored, click the **Color Doppler** on the touch screen (**Figure 1C**). Visualize the coronary artery (white arrow indicates LAD) in the short axis (**Figure 1C**). The red color, as seen in real time, is indicative of the direction of the flow (i.e., blood flow is towards the probe).

2.10 After visualizing LAD on a color-Doppler mode, change the mode to the **pulse wave** (PW)-mode. Look for the presence of a yellow-indicator line on the coronary artery (**Figure 2A**).

2.11 Place the yellow PW-line in the middle of the coronary artery. Ensure that the angle is parallel to the direction of the flow.

NOTE: Velocity of the flow is highly dependent on the angle of the PW line, so be sure to match the angle of the on-screen probe with the angle of the LAD. Use the touch screen to adjust the angle; PW angle should be less than 60°.

2.12 Use **cine store** to capture the velocity of the resting LAD coronary flow at the peak diastole as wave forms (**Figure 2B**).

2.13 After obtaining of resting LAD flow velocity, measure the maximum flow velocity of LAD during the stress to calculate CFR. To measure the maximum flow velocity during the stress, infuse Dobutamine at a dose of 20 µg/kg/min via tail vein[8] (**Figure 2C**).

NOTE: Dobutamine infusion should not be more than 8 min. Use an infusion pump and set the diameter of the infusion pump to 14.43 for BD 10 mL syringe.

2.13.1 Use 25-G infusion butterfly set for the tail vein cannulation. To place the infusion needle, place a small strip of gauze around the base of the rat's tail, then grab with hemostats and twist the tourniquet to apply pressure and cause the vein to enlarge.

2.13.2 Place the needle while directly connected to Dobutamine syringe.

NOTE: Take extra care not to accidentally introduce drug when drawing up blood to ensure proper placement of the needle in the tail vein. Also, be sure to avoid introducing an air bubble into the vein, as an embolism may be fatal to the animal.

2.13.3 Once the needle is placed, stabilize it with a glue and a piece of surgical tape, securing the infusion line to the tail.

2.13.4 Remove the hemostats and tourniquet to recover the flow.

2.13.5 Place Dobutamine syringe in the infusion pump and set it to inject 20 µg/kg/min.

2.13.6 During Dobutamine infusion, carefully monitor the LAD peak and heart rate. Periodically record LAD PW peaks in the Doppler mode, especially whenever it increases in response to Dobutamine.

NOTE: The stress challenge induced by Dobutamine causes the heart to work harder; this often results in the movement of the heart and the LAD. Be prepared to move the animal, the probe, or both in order to keep the LAD in view.

2.13.7 After LAD peaks and heart rate have plateaued during the challenge, stop the Dobutamine infusion, remove the tail vein infusion set and remove the animal from the platform. Allow the animal to recover in its home cage.

2.14 Select **Peak Vel** tool to obtain the peak diastolic velocities from the images shown in **Figure 2B and C.**

2.15 Calculate the CFR index as the ratio of the LAD stress (Dobutamine) peak diastolic flow velocity to the resting LAD peak diastolic flow velocity (**Figure 2A**).

3. Ischemia Reperfusion Injury

3.1 Anesthetize rat with isoflurane - induction chamber at 5% with 1.5–2.0 L/min O_2 flow followed by 1.5–2.0% with 1.5–2.0 L/min O_2 flow.

3.2 Confirm the depth of anesthesia by the lack of the pedal withdrawal reflex. Use ophthalmic vet ointment on the eyes to prevent dryness. Maintain the proper body temperature of 37-38 °C using a heating pad or an animal temperature controller.

3.3 Administer pre-surgical Meloxicam, 5 mg/kg intramuscularly, 15 min before the surgery, followed by 2 mL subcutaneous bolus of 0.9% saline to prevent dehydration during the surgery. Using the fiber-optic light source, intubate rat using 18-gauge IV catheter and connect to the ventilator.

3.4 Ligate LAD using 8-0 monofilament suture through 15 mm opening at the 5th intercostal space. Tie a plain knot and leave for 30 min. Visually confirm ischemia in all rats via discoloration of the heart surface[12]. Release the ligature after 30 min and verify reperfusion by the reddening of the previously discolored area of heart muscle for 1-2 min[12].

3.5 Close the rib cage using 4-0 absorbable suture with an interrupted suture pattern, then close the skin using 5-0 non-absorbable silk suture with continuous suture pattern.

3.6 Remove the animal from anesthesia. Allow the animal to recover in the home cage.

4. Ultrasound imaging after IR surgery

4.1. 72 h after IR surgery, measure the coronary flow and CFR again. Compare the measurements to before IR measurements. Repeat steps 2.1 to 2.15 as described above.

REPRESENTATIVE RESULTS:

For this study, we used 12 female Fisher 344 rats. We performed a stress test with Dobutamine and measured LAD coronary artery velocity before and 72 hours after the IR surgery. Before the IR surgery, resting LAD coronary artery velocity was measured as 423 ± 59 mm/s, which was increased after Dobutamine infusion (1005 ± 77mm/s) (**Figure 3A**). After 72 h of ischemia

reperfusion, resting LAD coronary artery velocity was significantly higher compared to the resting LAD coronary artery velocity before the IR surgery (743 ± 40mm/s vs 423 ± 59 mm/s) **(Figure 3A**). Stress response to Dobutamine test after the IR surgery was reduced compared to the before IR surgery responses (937 ± 67ms/s vs 1005 ± 77mm/s) (**Figure 3A**).

CFR is calculated as the ratio of the peak flow velocity during stress (Dobutamine) to the resting flow velocity (measured prior to Dobutamine infusion)[8]. CFR was 2.5 ± 0.35 in young rats before the IR surgery (**Figure 3B**) but significantly reduced (1.1 ± 0.25) after 72 h of the IR surgery, even though the resting LAD coronary artery velocity was higher in these rats compared to data obtained before IR surgery (**Figure 3C**). In addition, there were no significant changes in the systolic function of the left ventricle in rats after 72 h of the IR surgery (**Figure 3C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Coronary artery location. A. Probe position on the rat while obtaining LAD coronary artery velocity. **B.** Short axis anatomical representation of the pulmonary artery, aorta, and LAD coronary artery. **C.** Anatomical visualization of LAD coronary artery on echocardiography.

Figure 2: The Pulse Wave Velocity imaging of the LAD coronary artery. A. Representation of the pulse wave velocity sensor placement on LAD coronary artery. **B.** LAD coronary artery pulse wave image during the rest condition. **C.** LAD coronary artery pulse wave image during the stress (Dobutamine) condition.

Figure 3. Measurement of the coronary flow using Doppler echocardiography. A. Pulse wave velocity measured in Control during the rest (Cont B) and during Dobutamine infusion (Cont D) and in animals after 72 h of IR surgery during the rest (IR B) and during Dobutamine challenge (IR D), $p < 0.05$ Cont B vs. ischemia reperfusion B (*). **B.** CFR was calculated from pulse wave in the experimental animals (n=9), $p < 0.05$ Cont vs. IR (*). **C**. Fractional shortening assessment from the experimental groups (n=9). Data are presented as mean ± SD, analyzed with one-way ANOVA.

DISCUSSION:

The major findings from the present study are that IR increases the resting LAD coronary artery velocity and impairs CFR, even in the absence of any residual angiographic stenosis.

Understanding the coronary physiology is an essential part of the clinical decision-making for cardiologists to treat coronary artery disease. CFR is one of the important functional parameters in understanding the pathophysiology of coronary microcirculation[7,13]. CFR is a noninvasive method to assess both coronary artery stenosis and coronary microvascular circulation and is an indicator of myocardial blood supply, explicitly the ability of the coronaries to increase blood flow under stress conditions[7]. A normal CFR ($\geq$2.0) often reflects a good prognosis, while CFR less than 1.90 provides incremental diagnostic information for the identification of high-risk coronary artery disease[14-16].

Our results show that, even though the systolic function was preserved after ischemia

reperfusion (**Figure 3C**), CFR was significantly lower (**Figure 3B**). Thus, recanalization of stenotic coronary arteries does not improve microvascular perfusion. Decreased CFR enables detection of impaired microvascular vasodilation after ischemia reperfusion.

This study demonstrates the serial CFR evaluations to explore the effect of various pharmacological therapies using noninvasive transthoracic Doppler echocardiography. This method of coronary functional assessment can be used in small animal research as a feasible and viable clinical diagnostic tool. This will lead to minimizing the requirement of animal use, euthanasia, or necropsy in the small animal models. Critical steps in this protocol are visualizing the coronary artery and obtaining the PW velocity images of good quality. Another critical step is to maintain LAD visualization during the stress state. During Dobutamine challenge, the heart rate increases and the LAD may move from the field of view; researchers should be prepared to move the field in order to follow the coronary artery. Limitations in the current study include the relatively small sample size, the lack of correlation between CFR and the coronary artery lumen diameter *in vivo* in rats, due to the difficulty in obtaining accurate visualization for the size measurement of the coronary artery. However, the methods described here are reliable, reproducible, and offer insightful information on the damage inflicted on the cardiac microvasculature following ischemia reperfusion.

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DISCLOSURES:

The authors have nothing to disclose

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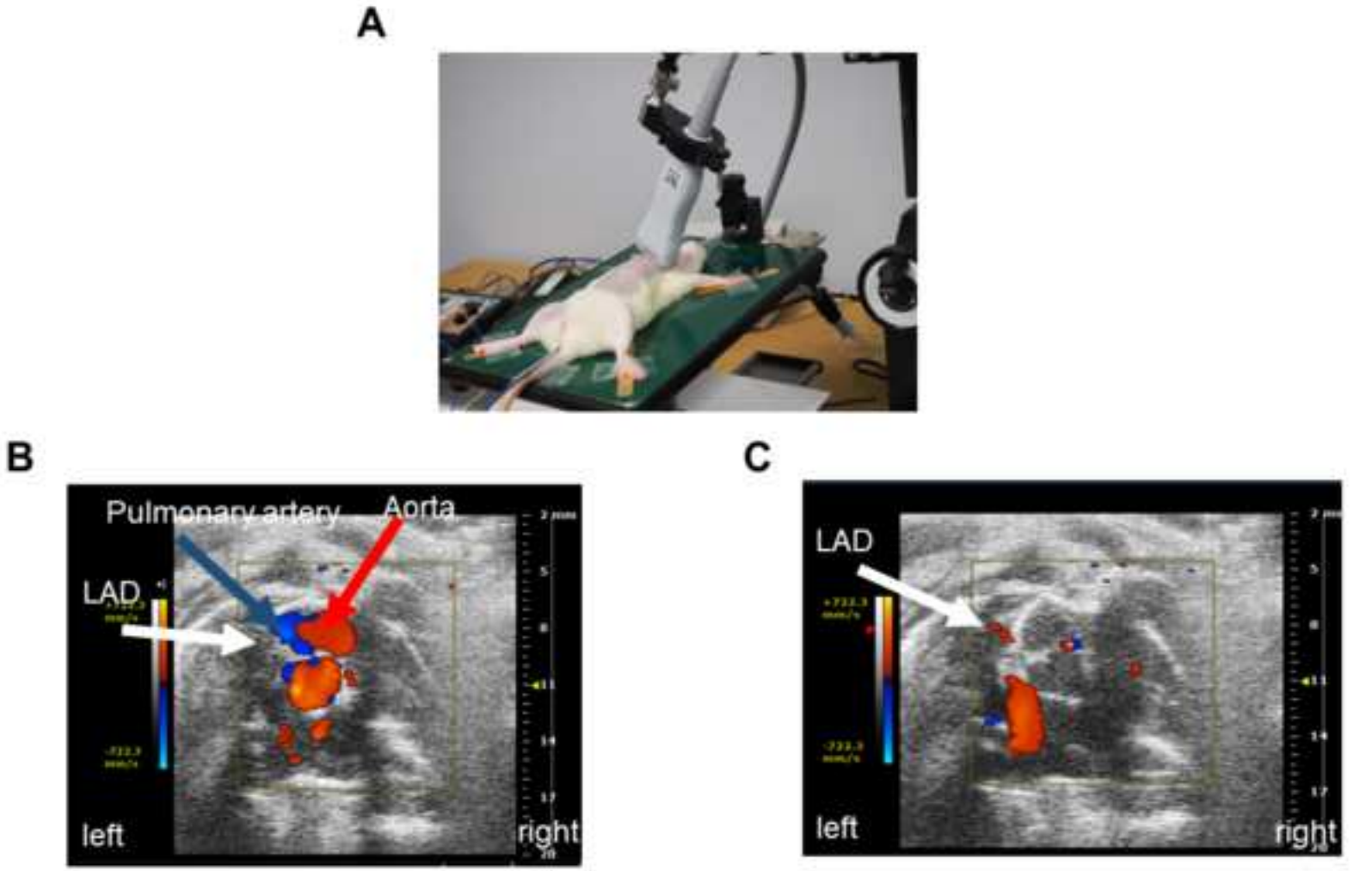

Figure 1

Figure 2

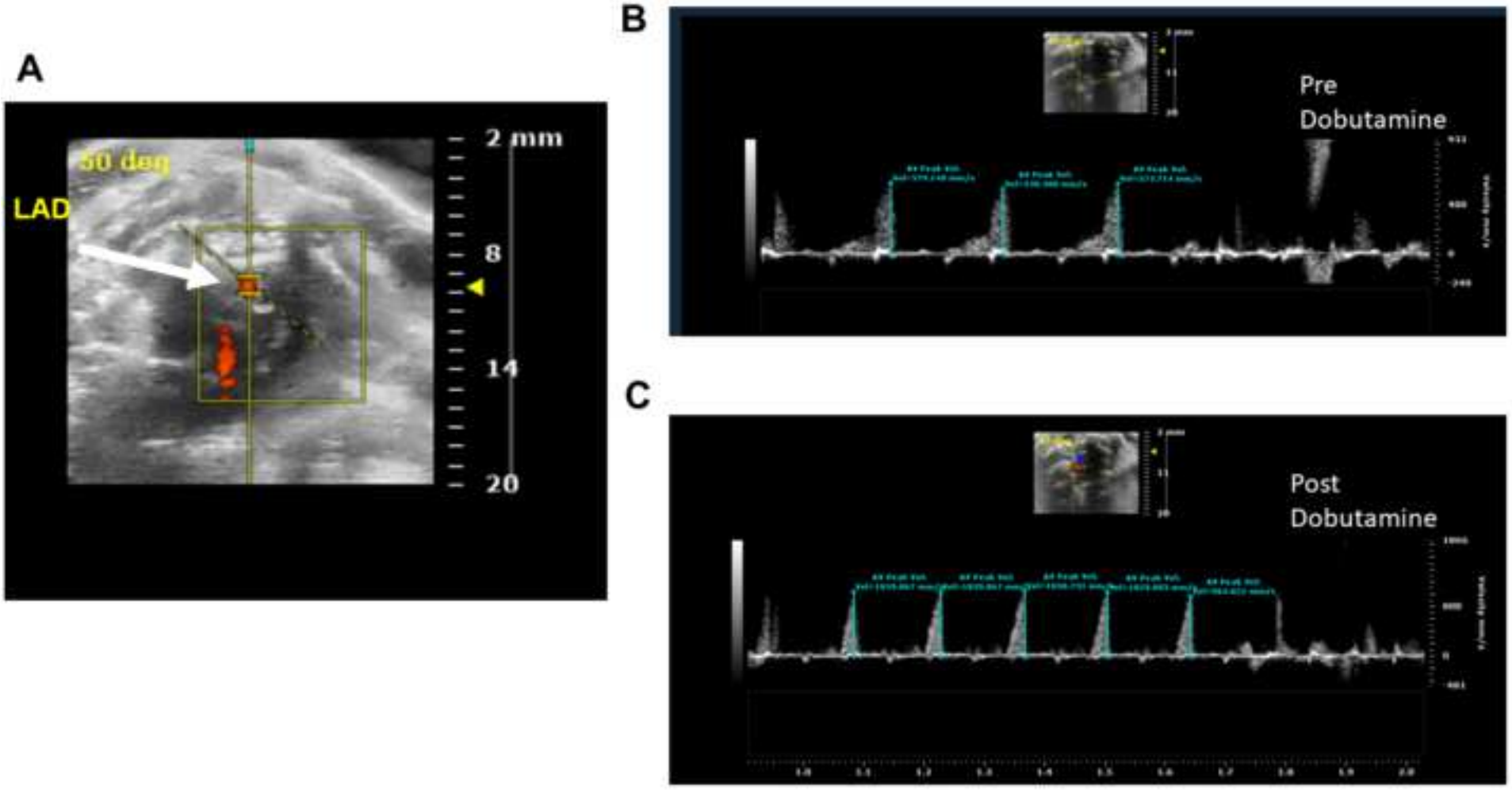

Figure 2

Figure 3

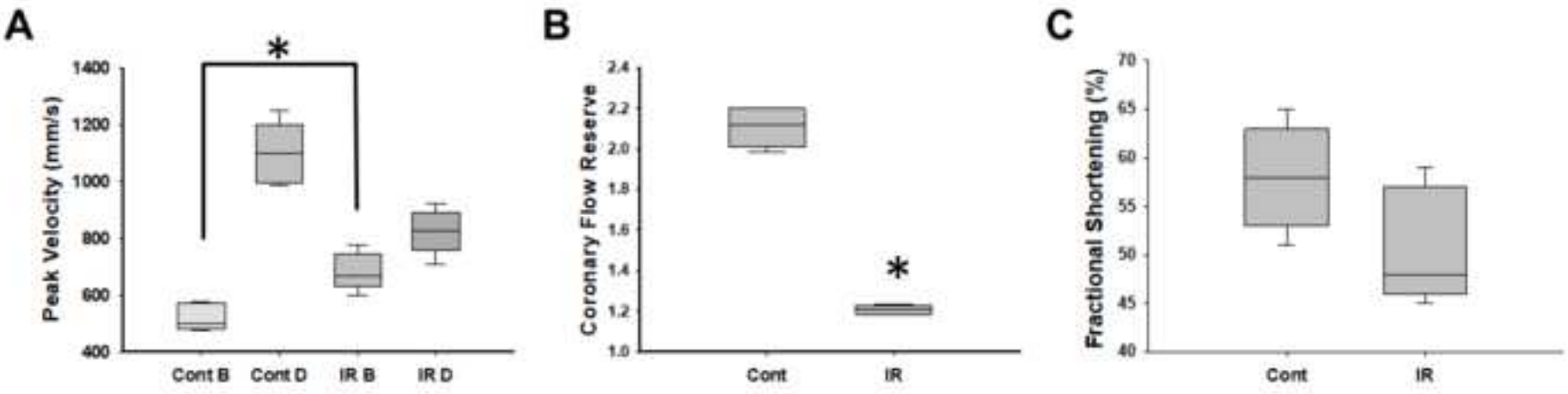

Figure 3

Name of Material/ Equipment	Company	Catalog Number
10 mL syringe	BD Syringe	302995
250S 13–24 MHz linear probe	FUJIFILM VisualSonics Inc	
Dobutamine hydrochloride	Sigma	D0676-10mg
Isoflurane	RRC	27376
Legato 100 Syringe pump	KD Scientific	788100
Vevo 3100	FUJIFILM VisualSonics Inc	
Winged infusion set, 27G x 1/2",	Medline.com	TMOSV27ELZ

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Dear editors and reviews,

Thank you for the insightful comments. We have revised this paper based on the suggestions. The revision to the manuscript may be found in red text. Please find our responses to each reviewer comment below

Editorial comments:

Q1: citations?

A1: We added more citations where it was asked.

Q2: Please mention in the introduction that you are using LAD to measure the CFR and how?

A2: we added in introduction that CFR was calculated from LAD velocity measurement. Line 70

Q3 Also, is this the resting blood flow? Please bring out this clarity because both in the abstract and in the introduction, this is emphasized.

A3: resting blood flow is recorded when animal is anesthetized and resting on the platforms. In the protocol we made more clear when and how we were capturing the resting blood flow. Line 105, 147

Q4: Is this the maximal flow? Please explain in which step do you measure these two: maximal coronary blood flow and resting coronary blood flow
Also, the steps following this are converted to substeps since they describe what is being said here.
Also need a citation here to show that Dobutamine is acceptable for measuring maximal coronary blood flow.

A4: maximal blood flow measured during the stress is the peak blood flow during dobutamine infusion. Lines 150-152- step 2.13
resting blood flow is recorded when animal is anesthetized and resting on the platforms. In the protocol we made more clear when and how we were capturing the resting blood flow. Line 105, 147

Q5: Isn't there any isoflurane in this case?

A5: Anesthetize rat with isoflurane - induction chamber at 5% with 1.5–2.0 L/min O_2 flow followed by 1.5–2.0% with 1.5–2.0 L/min O_2 flow. We maintain this anesthesia throughout the experiment, during resting and stress stages. Step 2.1 lines 99-101

Q6: This is not clear, 72 hours later you would perform the baseline measurement, then after how many hours do you perform the Dobutamine infusion. Wil this not increase the time from reperfusion? How do you account for this.

Need result figure on pulse wave velocity imaging of LAD coronary artery after I/R for comparison.

A6: All the rats were scanned and CFR was obtained before IR surgery. After 72 hours of surgery coronary flow was obtained from resting and stress state and CFR was calculated.
LAD velocity images are present before and after surgery is in figure 3A

Q7: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

A7: we don’t use any figure which have been used in any other publications before.

Q8: Please do not make points in the Discussion section. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

A8: We have made the requested changes.

Dear editors and reviews,

Thank you for the insightful comments. We have revised this paper based on the suggestions. The revision to the manuscript may be found in red text. Please find our responses to each reviewer comment below

Editorial comments:
Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

A1. Thank you for the suggestion, proofreading was done.

Q2. Please check either the Standard Access or Open Access checkbox in the Author License Agreement (ALA). Please then scan and upload the signed ALA to your Editorial Manager account. Please note that in the Questionnaire Responses Standard Access is selected. Please be consistent.

A2. Thank you for your suggestion, Standard Access is marked and signed with new data.

Q3. Please revise lines 56-58 (84-85), 76-77, 132-134, 135-137, 146-147, 205-207 to avoid previously published text.

A3. We are unaware of any previous published text that matches our wording.

Q4. Keywords: Please provide at least 6 keywords or phrases.

A4. Additional keywords are provided

Q5. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

A5. Summary was rephrased:
Coronary flow reserve (CFR) is the ratio of maximal coronary blood flow to resting coronary blood flow. CFR demonstrates the importance of microvascular dysfunction. Here we present a protocol for evaluating CFR via ultrasound, which offers the opportunity to predict cardiovascular risk factors in the absence of obstructive coronary disease.

Q6. Please rephrase the Abstract to more clearly state the goal of the protocol. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate.

A6. Abstract was changed according to the comments. Please see lines 48-64

Q7. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

A7. We have made it more clear and changed introduction according to the comments.

Q8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.

A8 We have made the requested changes.

Q9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Vevo, FUJIFILM VisualSonics Inc., etc.

A9. We have removed all commercial languages from the manuscript

Q10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

A10. We have added the ethics statement before numbered protocol. Please see lines 94-96

Q11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

A11. Thank you for the comment. The numbering of the protocol was adjusted as indicated in JoVE instructions for Author.

Q12. Please order the steps properly so that the protocol can be followed in chronological order.

A12 Changes have been made as requested

Q13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

A13. We have added more details to the protocol.

14. Lines 99-101: Please describe how to perform the open heart ischemia/reperfusion surgery including details such as how the rats are anesthetized and how proper anesthetization is confirmed and specify all surgical tools used.

A14. Open heart ischemia/reperfusion was described detailed in lines 166 -188

Q15. Lines 106-107: Please mention how proper anesthetization is confirmed.

A15. We have added how proper anesthetization is confirmed. Lines –169 -170

Q16. Line 126: How to obtain a B Mode image?

A16. We are obtaining image in B-Mode as described in line 124

Q17. Line 139: How to infuse dobutamine via tail vein? Is a syringe used?

A17. We have added more detailed description of tail vein infusion. Please see lines 137- 152

18. Lines 213-214: JoVE articles do not have an Abbreviations section. Please remove it and define all abbreviations before use.

A18. Changes have been made as requested.

Q19. Figure 3: Please define error bars in the figure legend. Please define Cont B, Cont D, IR B, IR D in the figure legend.

A.19 Changes have been made as requested.

Q20. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

A20. Changes have been made as requested.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is interesting study, it will be more important if you can show some abnormalities of the coronary flow curve,
Some recent publication shows type of diagnostic parameters for coronary flow curve.

Defining Coronary Flow Patterns: Comprehensive Automation of Transthoracic Doppler Coronary Blood Flow.
Sunyecz IL1, McCallinhart PE1, Patel KU1, McDermott MR1, Trask AJ2,3

If you can show some specific changes during I/R it will be more interesting paper,

Response: Thank you very for sharing this interesting paper. Unfortunately in the paper offered by the reviewer, curve was evaluated using MATLAB program. We do not use this program to analyze our data. VEVO3100 analyzing software does not have ability to evaluate coronary flow curve.

Major Concerns:
1. For the ultrasound image it will be good to show witch is the left side and which is the right side of the heart,

Response: Thank you for your suggestion. We added the marker in the image.

2, Show the animals chest and the position of the probe.

Response: We have added additional figure to show the probe position. (**Fig. 1A**)

3. For the coronary artery orientation (left vs right) , you need to show short axis picture, move to probe towards the base of the heart and be close to LVOT. This step by step picture diagram will help readers to understand the method and reproduce,

Response: Thank you for your suggestions. We have different approach to find the coronary artery. We find pulmonary artery and move the probe toward the apex. We have found this method more consistent in locating the coronary artery. Please see the lines 109-135

4. In this paper you show only the flow velocity changes. Can you also show the coronary artery diameter and show blood flow reserve (which is more accurate the velocity ration). The citation for the CFR calculation is for human study. Is there any animal study?

Response: Obtaining the diameter of coronary arteries in small animals is limited and not consistent. Based on our experience, we cannot consistently visualize the full width of the coronary arteries clearly enough to obtain diameter measurement. The citation for the CFR calculation is for human study, but calculation is not different between the species. There are few studies using this method but there are no other papers where they are calculating CFR.

Minor Concerns:
1. Ultrasound imaging, point N5.
Ultrasound probe cannot have 250 MHz frequency,
This is the probe number
Please correct and mention the frequency you used for the imaging,

Response: Thank you for correction, you are right, probe name is MS250 -13–24 MHz linear probe, changes have been made.

Reviewer #2:
Manuscript Summary:
The authors report a study about evaluation of coronary flow reserve after myocardial ischemia reperfusion in 12 female rats.
This manuscript is generally well-written, and extends on an interesting area of research. Nonetheless, there are several issues that need to be addressed by the authors.

Minor Concerns:
-Abstract. The abstract does not explain what the authors have done. It should be explained at least the methods and results.

Response: Thank you for the insightful comment. Changes to the abstract have been made
-Limitations should be included like the small sample size etc

Response: Limitations have been added, please see lines 255-260