CHAPTER 2

Myocardial contrast echocardiography in mice: Technical and physiological aspects

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Abstract

Myocardial contrast echocardiography (MCE) offers the opportunity to study myocardial perfusion defects in mice in detail. The value of MCE compared with single-photon emission computed tomography, positron emission tomography, and computed tomography consists of high spatial resolution, the possibility of quantification of blood volume, and relatively low costs. Nevertheless, a number of technical and physiological aspects should be considered to ensure reproducibility among research groups. The aim of this overview is to describe technical aspects of MCE and the physiological parameters that influence myocardial perfusion data obtained with this technique. First, technical aspects of MCE discussed in this technical review are logarithmic compression of ultrasound data by ultrasound systems, saturation of the contrast signal, and acquisition of images during different phases of the cardiac cycle. Second, physiological aspects of myocardial perfusion that are affected by the experimental design are discussed, including the anesthesia regimen, systemic cardiovascular effects of vasoactive agents used, and fluctuations in body temperature that alter myocardial perfusion. When these technical and physiological aspects of MCE are taken into account and adequately standardized, MCE is an easily accessible technique for mice that can be used to study the control of myocardial perfusion by a wide range of factors.
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Introduction

Myocardial contrast echocardiography (MCE) has proven a highly valuable technique for determining myocardial perfusion in patients with coronary artery disease and healthy subjects at risk of developing this condition. The MCE technique uses echogenic contrast agents that consist of gas-filled microbubbles, thus enabling visualization of the vasculature, including myocardial arterioles, capillaries, and venules. Microbubbles have the unique capability to reflect ultrasound at specific ultrasound frequencies and to burst when exposed to high-intensity ultrasound. These nonlinear oscillations of microbubbles result in the generation of harmonic frequencies that can be distinguished from signal reflected by the surrounding tissue. Because microbubbles have sizes between 1 and 10 μm with a mean diameter of 2–3 μm, they are able to pass the microcirculation without being trapped and thus can be used to visualize the total perfused myocardial microvasculature. This makes MCE the only technique presently available that allows real-time quantification of the total volume of blood and an estimation of the speed of the blood flow through a region of interest within the myocardium. Experimental studies have shown the occurrence of microvascular leakage, capillary rupture, cardiomyocyte killing, inflammatory cell infiltration, and premature ventricular contractions when ultrasound is used in combination with microbubbles. However, these undesired bioeffects have only been observed at low ultrasound frequencies and high mechanical indexes (MIs), which are typically not used anymore during MCE. No tissue damage has been noted when ultrasound or microbubbles are not used in combination, indicating that microbubbles by themselves are not harmful to the vasculature.

Myocardial blood flow ensures sufficient oxygen and nutrient supply to the cardiac tissue. Perfusion of the myocardium is directly regulated by myogenic, endothelial, autoregulatory, and pharmacological influences and indirectly by neural, humoral, and metabolic factors and myocardial mechanical forces. Coronary arterioles are the main regulators of flow through the myocardium and are able to increase myocardial blood flow in response to increased demand. Failure of the myocardial vasculature to respond to increased demand will reduce perfusion and will eventually lead to cardiac hypoxia and dysfunction. Therefore, research on myocardial perfusion derangements is essential in understanding cardiac ischemia and failure.

Mice are often used in cardiovascular research, predominantly because of the availability of conditional and inducible transgenic models suitable for molecular and physiological studies. The main challenge of MCE in mice is their small size. MCE was initially developed for application in humans, yet experimental techniques have developed fast to make the MCE technique available for mouse research. Other techniques to measure myocardial perfusion beside MCE include singlephoton emission computed tomography (SPECT), positron emission tomography (PET), and cardiac MRI (CMR) (see Table 1).
The use of SPECT to assess myocardial perfusion has been extensively validated across species, including mice. The advantage of this technique is that it is compatible with stress stimuli to measure myocardial flow reserve, and, because the imaging does not occur during first pass of a contrast agent, there is less demand for high temporal resolution. On the other hand, limitations of this technique include long acquisition protocols, poor spatial resolution, and motion artifacts, although this technique is under continuous development to improve accuracy. PET has a better spatial resolution compared with SPECT, and attenuation artifacts, resulting from breast or subdiaphragmatic attenuation, are less of an issue. Also, PET in combination with CT, e.g., PET/CT, makes it possible to combine perfusion data with anatomical imaging. On the other hand, high costs and the need for a cyclotron for most radionuclide productions makes this technique less suitable for mouse studies. CMR, often used to examine stress perfusion, has a high spatial resolution and the ability to perform absolute quantification of perfusion, and studies that can be performed in a short time period. On the other hand, the contrast agent gadolinium is not optimal to assess myocardial perfusion because it has intermediate extraction fraction out of capillaries into the surrounding tissue during first-pass imaging. CMR can also be used without the use of a contrast agent. Spin labeling techniques use the labeling of the nuclear magnetization of water protons, but this technique is challenged by a poor contrast-to-noise ratio and only practically relevant for cardiac studies at 4.7 T or higher field strengths to increase spatial resolution. Also, blood oxygen level-dependent (BOLD) imaging is used to assess myocardial perfusion by detecting deoxygenated hemoglobin changes and can be used in a quantitative manner, has reasonable signal-to-noise ratio, and is less prone to magnetic susceptibility artifacts. On the other hand, significant changes in perfusion result in relatively small changes in signal, and the acquisition time is relatively long.

Importantly, most scanners have a relatively slow acquisition frame rate compared with the rapid heart rate of mice. The advantage of MCE over SPECT, PET, and CT is the absence of ionizing radiation, use of contrast agents that remain in the vasculature, high spatial resolution over SPECT, and relative low costs. As a result, this technique is accessible and valuable for perfusion imaging in mice.

The first paper on myocardial perfusion in mice describing the use of MCE was published in 1999. The technique was not immediately picked up by others, and it took until the late 2000s for other groups to report on MCE in mice. Besides studies assessing myocardial perfusion in mouse models of cardiac ischemia, effects of therapy that target vascular endothelial growth factor on myocardial perfusion in cancer research have also been reported. Only a few studies discussed technical aspects of MCE in detail. The first paper discussing MCE in mice by Scherrer-Crosbie et al. validated this technique by assessing video intensity in different parts of the left ventricular wall before and after coronary ligation. Importantly, a close correlation between perfusion defects measured by
Table 1. Comparison between PET, SPECT, MRI, CT and MCE for myocardial perfusion measurements.

<table>
<thead>
<tr>
<th></th>
<th>PET</th>
<th>SPECT</th>
<th>MRI</th>
<th>CT</th>
<th>MCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acquisition time</strong></td>
<td>Intermediate acquisition time (minutes)</td>
<td>Intermediate acquisition time (minutes)</td>
<td>Long acquisition time (minutes to hours)</td>
<td>Short acquisition time (seconds to minutes)</td>
<td>Short acquisition time (seconds to minutes)</td>
</tr>
<tr>
<td><strong>Precision</strong></td>
<td>Very high sensitivity</td>
<td>High sensitivity</td>
<td>Medium sensitivity</td>
<td>Low sensitivity</td>
<td>High sensitivity</td>
</tr>
<tr>
<td><strong>Spatial resolution</strong></td>
<td>± 1mm</td>
<td>0.4 - 0.9 mm</td>
<td>0.1-0.2mm</td>
<td>± 0.1mm</td>
<td>&lt;0.1mm</td>
</tr>
<tr>
<td><strong>Type of tracer</strong></td>
<td>Radionuclides</td>
<td>Radionuclides</td>
<td>Contrast agent</td>
<td>None</td>
<td>Contrast agents</td>
</tr>
<tr>
<td><strong>Pros</strong></td>
<td>Most sensitive technique, absolute quantification of perfusion, compatible with stress stimuli</td>
<td>Relatively long half-lives of tracers, simultaneous use of multiple radiopharmaceuticals, compatibility with stress stimuli</td>
<td>Relatively safer in use than radiation applications, good soft tissue contrast resolution to distinguish between normal and diseased tissue, absolute quantification of perfusion, tracers not confined to the vasculature</td>
<td>High anatomic resolution, rapid data acquisition, can be combined with anatomic and functional information</td>
<td>High spatial resolution, real-time imaging, portable, no radiation, compatible with multiple stress modalities, low cost</td>
</tr>
<tr>
<td><strong>Cons</strong></td>
<td>Need for a cyclotron, need for radioactive facilities, short half-life of radioactive isotopes, low spatial resolution, motion artifacts, high cost tracers</td>
<td>Need for a cyclotron, need for radioactive facilities, sensitivity and spatial resolution are greatly influenced by design of the collimator (filters a stream of rays), long acquisition protocols, motion artefacts</td>
<td>High cost, difficult to measure in real time, tissue movement limiting high spatial resolution, long acquisition times</td>
<td>High radiation dose needed, less sensitive than PET and SPECT, image quality inversely related to heart rate</td>
<td>As frequency increases (and so does resolution), maximum imaging depth decreases, short half-life of contrast agents, reproducibility dependent on technical and physiological variables</td>
</tr>
</tbody>
</table>

Acquisition time, precision, spatial resolution, type of tracer, and pros and cons are shown for positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), computed tomography (CT), and myocardial contrast echocardiography (MCE) techniques to measure myocardial perfusion in mice.
MCE and postmortem dye exclusion with Evans blue after coronary ligation was observed, showing that MCE is suitable for measuring perfusion defects in the mouse myocardium.

In line with this, Raher et al.\textsuperscript{24} compared myocardial blood flow assessed by MCE with myocardial blood flow assessed by fluorescent microspheres and showed that the data obtained with these techniques correlate well ($r^2=0.7, P<0.0001$). In addition, the adenosine-induced increase in myocardial blood flow was comparable when determined using both techniques. French et al.\textsuperscript{25} described their methodology regarding MCE, thereby focusing on imaging procedures and monitoring of physiological parameters, including ultrasound modalities on the scanner, transducer positioning relative to the mouse heart, microbubble preparation and infusion, animal body temperature, and heart rate. This study mainly focused on three-dimensional mapping of perfusion defects in myocardium but lacks information on cardiovascular hemodynamics when using this technique.

The aim of this review is to highlight the technical aspects that determine myocardial perfusion results obtained using MCE and highlight factors that influence cardiovascular hemodynamics that should be taken into account when using MCE in mice (see appendix for an example protocol). By optimizing technical and physiological parameters, MCE can serve as a widely applicable technique in research on myocardial perfusion defects causing cardiac disease.

**Technical Aspects of MCE**

**Effect of Ultrasound Scanner Settings on Myocardial Perfusion Data**

The characteristics of the ultrasound beam generated by ultrasound systems have a critical influence on the data. First, the MI is essential in microbubble behavior. Microbubbles are disrupted when high-intensity ultrasound is used, and, therefore, the transmitting power of an ultrasound scanner is usually kept between MIs of 0.05 and 0.40 to avoid this. When different MIs are used, just normalizing the microbubble signal for MI is not applicable because microbubble behavior is nonlinearly related to ultrasound pressures and thus MI\textsuperscript{26-28}. Therefore, to circumvent corrections for different MIs, it is advisable to keep the MI of the scanner constant between experiments.

Another setting that can be adjusted on the scanner and influences the ultrasound beam is focal depth. Changes in focal depth alter the shape of the ultrasound beam, and, consequently, ultrasound pressure amplitudes can be higher or lower in the region of interest (ROI)\textsuperscript{28}. Microbubbles within this ROI might therefore be sonicated differently depending on the relative position of the ROI and focal depth\textsuperscript{28}. Again, a constant focal depth during experiments will prevent alterations in ultrasound beams.

Linear data are log compressed to generate data files that are small enough to be handled by the user, which will be discussed in more detail below. For this purpose, the dynamic
range is reduced but may also lead to pixels outside the dynamic range and will therefore not be correctly linearized. An experimental study showed that a dynamic range of 50 dB is optimal, and lower dynamic ranges result in more signal saturation. In addition, this dynamic range in combination with a gain of 55 dB resulted in the lowest percent error.

Finally, transducers with a frequency higher than 10 MHz are generally selected for mouse echocardiography because of their small body size and high heart rate. A change in ultrasound frequency alters the harmonic scattering of microbubbles, resulting in less microbubble destruction at higher frequencies, and should therefore also be kept constant between experiments. On the other hand, higher transducer ultrasound frequencies also result in lower signals. This is caused by a greater difference between the transducer ultrasound frequency (e.g., 30 – 40 MHz) and the resonating frequency of the microbubbles (usually around 2–5 MHz). This explains why the intensity of the contrast signal is higher when using 10 – 13 MHz as opposed to 30 – 40 MHz.

**Analysis of Microbubble Inflow Curves and Linearization of Video Intensity Data**

As described for the first time in vivo by Wei et al. and validated by Vogel et al., the inflow of microbubbles after the local destruction in ROI, where the destruction of microbubbles should be complete to accurately estimate the number of microbubbles in view, can be described by the following formula: video intensity = MBV X (1 - e^-βt), where MBV is the total microvascular blood volume in the ROI analyzed, β is the rate constant of contrast agent inflow into the ROI, i.e., microvascular blood flow velocity, and t is time.

Ultrasound devices apply logarithmic compression on the recorded radiofrequency data to generate data files that are small enough to be handled by the user. For quantitative purposes, this is not optimal because these log-compressed video intensities consequently increase nonlinearly with the number of microbubbles in the image. We tested this nonlinearity by in vitro analysis of log-compressed video intensities with different microbubble concentrations (Fig. 1A). Nonlinearity of video intensity is most pronounced at high-intensity contrast signals, where saturation occurs. After linearization of the video intensity using antilog transformation (y = 10^x), the relationship between microbubble number and microbubble signal indeed showed a more linear relationship in vitro (Fig. 1, A and B). Doubling of the infusion velocity in vivo resulted in almost a doubling of the video intensity only when data were linearized (Fig. 1, C and D). Therefore, all data obtained from ultrasound devices should first be linearized before analysis.
Figure 1. Linearization of log-compressed data improves correlation of microbubble concentration with video signal intensity.

(A) Video intensity of noncorrected radiofrequency-data showing a nonlinear curve, profoundly in the upper intensity signals. (B) Video intensity of linearized radiofrequency data showing a linear curve. (C) Doubling of the infusion velocity of the same concentration of microbubbles in vivo did not double the video intensity. (D) After linearization of the radiofrequency data, video intensity almost doubled after doubling the infusion velocity of microbubble infusion in vivo.

Data are mean ± SE. N = 2 per group for A and B and 5 per group for C and D.

Signal Saturation and Routes of Microbubble Administration

With a continuous infusion, video intensity signal will reach a plateau after microbubble destruction (Fig. 2A). When microbubble concentrations or perfusion rates are too high, the video signal intensity will reach its maximum. This is due to saturation of the contrast signal, and the resulting perfusion increments will not be detected. Also, hemodilution can influence myocardial perfusion by a reduction in the number of perfused capillaries, increased inflammatory responses, reduced blood viscosity, and reduced erythrocyte deformability. Therefore, the optimal microbubble concentration and infusion rate should be determined before use, as has been previously suggested. The contrast infusion rate should be chosen such that the contrast signal is within the linear part of the concentration–signal intensity curve (Fig. 2B). Whereas commercially available microbubbles are used in
human research, in animal experiments microbubbles can be produced in the laboratory \(^{35}\) and thus can be varied in concentration to avoid signal saturation.

Microbubbles are delicate, and both their number and oscillating quality can be significantly altered by factors like pressure and temperature \(^{28, 36}\). In vivo, microbubbles are exposed to high pressures when passing the heart. In addition, intravenous and intra-arterial injections of microbubbles can alter bubble size and distribution in blood by the influence of lung filtration of larger microbubbles \(^{37}\). It is important to take this into account during experiments by constantly using the same injection route and site.

**Figure 2. Video signal intensity reaches a maximum high microbubble infusion rate.**

(A) Typical example of an inflow curve of microbubbles in a certain region of interest after microbubble destruction.

(B) Saturation of the contrast signal was observed at high microbubble infusion rates. Typically the infusion rate is chosen within the linear part of the concentration signal intensity curve. Average heart rate was 626 ± 8 beat/min (means ± SE, data not shown). Data are means ± SE; n = 4 per group.

**Correction for Plasma Microbubble Concentration**

During constant intravenous infusion of microbubbles, the steady-state concentration of microbubbles reached in the blood depends on the microbubble infusion rate, total circulating volume, and microbubble clearance rate. Microbubbles are cleared in part via phagocytosis by macrophages but mainly by gas exchange in the lung \(^{38}\). Total pulmonary flow determines the microbubble clearance rate and depends on cardiac output. Any intervention that affects cardiac output changes microbubble clearance and thus steady-state microbubble concentration.

To address this issue dependent on the physiological condition investigated, a correction of signal from a cavity that is filled with blood, e.g., the ventricular cavity or the femoral artery in the hindlimb, eliminates the signal variation caused by differences in microbubble clearance rate. Importantly, blood signal in a certain cavity, e.g., the left ventricle, should not reach saturation because this would then overestimate the calculated blood volume in an ROI. However, to obtain an optimal signal in the myocardium, the infused microbubble
concentration is relatively high compared with body weight, and therefore the left ventricular cavity signal is likely to be on the saturated portion of the dose-signal curve. Also, microbubbles are exposed to high pressures in the left ventricle and could therefore produce a variable signal. It is therefore not advised to use signal intensity in the left ventricle to correct for overall blood signal, as the signal intensity in the cavity is highly dependent on intraventricular pressure. Instead, when all variables that could influence the contrast signal are kept constant between experiments, e.g., microbubble concentration, microbubble infusion rate, and cardiac output, correction for microbubble blood signal is not necessary.

**MCE in Different Phases of the Cardiac Cycle**

It is known that there is hardly any forward flow through the coronary circulation occurs during systole due to compression of vessels. Therefore, it would be reasonable to measure myocardial perfusion during the diastolic phase of the cardiac cycle. However, the small ROI in the murine myocardium may increase the variation of the signal during diastole. We compared microvascular blood volume increments measured during diastole (Fig. 3B) with measurements during systole (Fig. 3C) by ECG triggering after administration of acetylcholine. Increments in microvascular blood volume during diastole correlated well with increments during systole ($R^2 = 0.92$, $P = 0.0005$; Fig. 3D), although the intensity of contrast signals during diastole was ~26% higher than during systole, which is consistent with earlier observations. Increments in microvascular blood flow during diastole correlated less well with increments during systole ($R^2 = 0.38$, $P = 0.1429$; Fig. 3E) and were ~16% lower in diastole. In addition, myocardial perfusion during systole was also correlated to myocardial perfusion during diastole, although borderline significant ($R^2 = 0.54$, $P = 0.0590$; Fig. 3F) and was also ~15% lower in diastole. In conclusion, because of small differences in measurements between systole and diastole, perfusion of the mouse myocardium can be measured during systole to reduce variation in MCE data, enabling reliable determination of perfusion increments after administration of vasodilators.
Figure 3. Measurement of myocardial perfusion during systole is preferable over measurement during diastole.

(A) The yellow/green/blue dots indicate microbubbles in the cardiac lumen and vasculature. Black regions are nonperfused or poorly perfused regions or shadowing from highly perfused regions. In systole, only a small part of the left ventricular (LV) lumen is observed (white line), and the LV myocardium comprises a larger part of the image (orange line). (B) Region of interest (red dots and line) drawn in the myocardium of the LV during systole. Only the anterior part of the myocardium is used to draw a region of interest to avoid shadowing. (C) During diastole, a region of interest is drawn in the myocardium of the LV. Again, only the upper part of the image was used to draw a region of interest due to shadowing of the contrast signal intensity. (D) Myocardial blood volume was measured during diastole and systole at baseline and after administration of a vasodilator. Data are represented as increased myocardial blood volume upon the vasodilator relative to baseline. (E) Myocardial filling velocity was measured during diastole and systole at baseline and after administration of a vasodilator. Data are represented as increased myocardial filling velocity relative to baseline. (F) Myocardial blood flow (the product of myocardial blood volume and myocardial filling velocity) was calculated during diastole and systole at baseline and after administration of a vasodilator. Data are represented as increased myocardial blood flow relative to baseline. Average heart rate before administration of vasodilator was 588 ± 9 beats/min and after administration of vasodilator 608 ± 26 beats/min (means ± SE, data not shown).

Bolus Versus Continuous Infusion of Contrast Agent

As discussed by Porter and Xie, a bolus injection or continuous infusion of microbubbles can be chosen for MCE. A bolus injection can be administered relatively easily and results in a brief period of high contrast because the half-life of microbubbles is in the minutes range, and hence administered microbubbles remain in the circulation in the order of several minutes. One can imagine that, with an intravenous injection of a certain volume of contrast agent, blood pressure will increase and heart rate will subsequently change.
Therefore, in case of a bolus injection, the lowest possible volume of contrast agent should be chosen. As discussed above, the video intensity becomes saturated briefly after the bolus injection and might therefore yield falsely positive myocardial perfusion data in regions where perfusion is reduced. Only during the washout period are perfusion defects revealed. Also, myocardial blood flow cannot be measured by a bolus injection because clearance of microbubbles from blood limits the calculation of the mean transit time. In contrast, when continuous infusion is used, saturation of the signal can be avoided, and perfusion defects in the myocardium can be detected more easily. This continuous infusion permits the calculation of mean microbubble filling velocity in a certain myocardial area and in addition the mean myocardial blood volume in that area. Myocardial blood flow is estimated by multiplying these two variables. In addition, myocardial blood flow reserve can be measured only by continuous infusion, as was commented by others. Finally, when a continuous infusion is used, images from different scanning planes can be acquired, providing a better estimation of local perfusion defects.

**Physiological aspects**

**Effects of Anesthetics on Myocardial Blood Flow and Cardiovascular Hemodynamics**

When assessing myocardial blood flow, an anesthetic regimen should be chosen carefully to minimize its effect on cardiovascular physiology. Only a few studies have assessed the effect of mostly volatile anesthetics on myocardial perfusion, whereas more studies have assessed the effect of anesthetics on cardiac physiological parameters. Isoflurane inhalation has variable effects on myocardial blood flow, which has been reported to be increased, decreased, or to remain unaltered. One study compared effects of isoflurane inhalation to that of propofol infusion in rats and showed similar myocardial blood flow values. Propofol induces coronary vasodilation but only at supratherapeutic concentrations. Low isoflurane concentrations (1.25%) showed a comparable myocardial blood flow compared with ketamine-xylazine, but higher isoflurane concentrations (2.00%) showed highly increased myocardial blood flow. When the effect of isoflurane was compared with that of pentobarbital, myocardial blood flow was higher in rats anaesthetized with isoflurane. Exposure of rats to sevoflurane increased the microvascular filling velocity without altering myocardial blood flow. In humans, sevoflurane also did not alter myocardial blood flow; rather, adenosine-induced increments in myocardial perfusion were decreased with sevoflurane. Isoflurane thus seems to have vasodilatory effects on coronary arteries, whereas xenon and sevoflurane have minimal effects on myocardial blood flow.

The effect of anesthetics on physiological parameters such as blood pressure and heart rate in mice is described in more detail. The effect of tribromoethanol, ketamine-xylazine, ketamine-midazolam, and isoflurane on cardiovascular parameters has been compared.
in mice. A ketamine-xylazine combination caused the greatest cardiac depression, i.e., low heart rate, decreased fractional shortening, and increased end-diastolic dimension, whereas hemodynamics were comparable between the other anesthetics. Isoflurane and tribromoethanol were recommended as anesthetics of choice because they cause less cardiac depression, show reproducible results, are easy to administer, and are rapid in onset and recovery of mice. Of note, within the first 20 min after the induction of anesthesia, mice were hemodynamically unstable. To avoid large variations in perfusion data, myocardial perfusion measurements should not be performed directly after anesthesia induction but rather when cardiovascular hemodynamics are stable. Another study compared fentanyl-ketamine-midazolam, fentanyl-midazolam-haldol, pentobarbital, fentanyl-fluanisone-midazolam, fentanyl-midazolam-acepromazine (FMA), ketamine-medetomidine-atropine (KMA), isoflurane, and propofol-fentanyl-midazolam and their effects on blood pressure and heart rate in mice. KMA was recommended for stabilization of arterial blood pressure, although this also induced bradycardia. Zuurbier et al. therefore suggested to use isoflurane when optimal hemodynamics are pursued.

We tested whether a combination of midazolam (6.25 mg/kg), acepromazine (6.25 mg/kg), and fentanyl (0.31 mg/kg) (FMA) by intraperitoneal injections contributes to cardiovascular changes in mice. Midazolam is a benzodiazepine, exerting sedative, hypnotic, muscle relaxant, and anxiolytic actions. Acepromazine is a phenothiazine, possessing tranquilizing and sedative effects. Fentanyl is an opioid, possessing analgesic effects. Mean arterial pressure (MAP) was measured in the left carotid artery. Indeed, intraperitoneal dosages between 30 and 50 I FMA decreased blood pressure on average by 38±13 mmHg (mean SD; Fig. 4A). This effect occurred within several minutes, and a stable blood pressure was reached after 10 min. Midazolam is known to lower blood pressure. Also, acepromazine, nowadays almost only used in animals, has been shown to lower blood pressure, as was observed in different animals. Fentanyl has minor effects on cardiovascular parameters. In agreement with the data described above, previous research has shown that different anesthetics influence cardiovascular parameters in mice and thus should be chosen carefully before measuring myocardial perfusion. To circumvent these bolus injections, continuous intravenous administration of anesthetics could be used in combination with monitoring of heart rate and blood pressure.
Myocardial contrast echocardiography in mice

Figure 4. Administration of anesthesia and vasodilators in vivo lower blood pressure and alter inflow curves.

(A) Typical example of mean blood pressure after fentanyl-midazolam-acepromazine administration. Mean blood pressure before anesthesia administration was 87 mmHg. The dashed line indicates the time point of anesthesia administration. Mean blood pressure dropped to 49 mmHg within 10 min after administration. (B) Blood pressure drops significantly after administration of acetylcholine (ACh; 5 μg·kg⁻¹·min⁻¹) but is actively restored in time. Also, heart rate increases after administration of ACh (dashed line). (C) Mean arterial pressure was measured before and after administration of either ACh or sodium nitroprusside (SNP). Concentrations for ACh were 5 or 7.5 μg·kg⁻¹·min⁻¹ and for SNP were 3 or 6 μg·kg⁻¹·min⁻¹. Average heart rate before vasodilator administration was 540 ± 21 beats/min, after administration of ACh 581 ± 31 beats/min, and 565 ± 35 beat/min after SNP administration (means ± SE, data not shown). Data are means ± SE; n = 4-5 per group.
**Effects of Vasoactive Substances on Blood Pressure and Myocardial Perfusion**

To study the murine myocardial microcirculation, microbubbles are infused through a central venous line but also for possible administration of vasoactive substances to subsequently study their vascular effects on the coronary circulation. Differences in the response to these vasoactive agents between groups indicate alterations in vascular function. In disease, e.g., hyperlipidemia, the vasodilatory reserve in resistance arteries is decreased, consequently causing hypoperfusion of the microcirculation, resulting in myocardial ischemia, angina, or infarction. Indeed, several studies have shown that agonist-induced increments in flow, not basal myocardial blood flow, are most strongly impaired in heart failure. In one study, blood flow increment in the myocardium was significantly lower after infusion of adenosine in animals with left ventricular remodeling, in addition to features of chronic heart failure, compared with control animals. Also, patients with chronic heart failure had normal left ventricular perfusion at rest, but increments on the vasodilator dipyridamole were diminished compared with healthy volunteers. This diminished coronary flow reserve was correlated with a lower left ventricular ejection fraction. Even obese subjects and patients with diabetes mellitus, both without a history of cardiac disease, were shown to have a decreased coronary flow reserve, whereas baseline coronary flow velocity was not different among all groups. This highlights the importance of studying the vasodilatory capacity of the coronary circulation to assess myocardial perfusion defects. A possible explanation for this decreased coronary flow reserve may be endothelial dysfunction. Indeed, in the presence of atherosclerosis, the coronary vasculature shows impaired vasodilation. Also in heart failure, ischemia-reperfusion injury, and diabetes mellitus, endothelial dysfunction of the coronary circulation has been observed.

When vasoactive substances are used to assess changes in myocardial perfusion, systemic blood pressure also changes, as such possibly influencing myocardial perfusion pressure. We observed that acetylcholine administration (5 µg·kg⁻¹·min⁻¹) caused a sudden drop in blood pressure and subsequently increased heart rate (Fig. 4B), indicating a compensatory mechanism triggered by baroreceptors for the low blood pressure. Mean arterial blood pressure recovered soon after the initial decrease, although not fully, most probably attributable to the continuous infusion of acetylcholine. Indeed, prolonged acetylcholine administration induced a long-term MAP drop of 24 ± 3 mmHg at an infusion rate of 5 µg·kg⁻¹·min⁻¹ and a drop in MAP of 27 ± 2 mmHg at an infusion rate of 7.5 µg·kg⁻¹·min⁻¹ (Fig. 4C). Sodium nitroprusside, an endothelium-independent vasodilator, induced a long-term drop in MAP of 15 ± 3 mmHg at an infusion rate of 3 µg·kg⁻¹·min⁻¹ and 22 ± 4 mmHg at an infusion rate of 6 µg·kg⁻¹·min⁻¹ (Fig. 4B). Interestingly, Scherrer-Crosbie et al. reported only a minor change in blood pressure after acetylcholine infusion (2 µg·kg⁻¹·min⁻¹) but increased myocardial blood flow and blood volume.
These systemic alterations in hemodynamics in turn can affect perfusion in the region of interest, i.e., the myocardium. The optimal dose of vasodilators, or vasoconstrictors, should be determined before infusing in animals. Optimally, one would induce local vasodilation, without altering systemic cardiovascular parameters. Although vasodilators cause a sudden drop in blood pressure, this also activates baroreceptors to restore cardiac output and increases total peripheral resistance. Both actions will result in partial restoration of blood pressure. Depending on the concentration of the vasodilator used, the cardiovascular system can compensate for this drop in blood pressure, and a new equilibrium will be reached.

Therefore, one should first determine the optimal dose of a vasodilator, resulting in vasodilation in the ROI, without causing severe perfusion defects in vital organs. Prolonged periods of high concentrations of a vasodilator will cause a large drop in blood pressure, which cannot be compensated by the body and may therefore cause significant perfusion decreases in vital organs. It is therefore important to monitor systemic blood pressure throughout the experiment.

**Influence of Body Temperature on Myocardial Perfusion**

Core body temperature has been shown to be a valid determinant of myocardial blood flow. Clinical studies have shown that perioperative morbid cardiac events occurred less frequently in normothermic patients than in hypothermic patients undergoing noncardiac surgery. Also, patients with hypothermia had a threefold greater incidence of myocardial ischemia in the early postoperative period compared with normothermic patients.

Interestingly, a clinical study in which cold saline was infused in healthy subjects to lower the core body temperature did not trigger coronary vasoconstriction but rather triggered a -adrenoceptor-mediated increase in cardiac work and myocardial perfusion. It has been suggested that ischemia observed in patients with hypothermia may arise from increased myocardial O2 demand, which is not matched by a sufficient increase in myocardial blood flow, although atherosclerotic vessels might respond differently to cold. In conclusion, body core temperature is an important regulator of myocardial blood flow, and fluctuations should be avoided during myocardial perfusion measurements. This can be achieved in mice by homeothermic heating systems.

**Conclusions**

In summary, MCE is a promising technique for measuring myocardial perfusion in mice because the technique is easy to use and is relatively low in cost. A number of technical and physiological aspects of the technique should be considered when using MCE to reduce variation and increase reproducibility, including linearization of ultrasound data, obtaining a
dose-response curve of the contrast agent, and analysis of images during systole. In addition, vasoactive substances and anesthetic regimen should be chosen carefully with regard to cardiovascular hemodynamics, and body temperature should be closely monitored. When these technical and physiological issues are taken into account and optimized, MCE is an accessible and valuable technique to measure myocardial perfusion in mice.

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Author Contributions
Myocardial contrast echocardiography in mice

References


Myocardial contrast echocardiography in mice


Appendix: Example of Myocardial Contrast Echocardiography Experimental Protocol in Mice

Microbubble contrast agent was generated in the laboratory, and microbubble size distribution and concentration were determined using a coulter counter (Multisizer 3, Beckham Coulter, Woerden, The Netherlands), after which microbubbles were diluted in saline to a final concentration of $1 \times 10^9$ microbubbles/ml. Microbubbles were infused at a rate of 7.5 μl/min for 2 minutes to reach a systemic steady state. Four real-time inflow curves of 10 s each, in a long-axis view of the heart in the end-systolic phase of the cardiac cycle, were recorded after destruction of the microbubbles by a sequence of eight high-energy pulses (MI: 1.7). Thirty minutes after baseline measurements were acquired, acetylcholine (5 μg·kg⁻¹·min⁻¹, 15 mg/l) was infused intravenously to assess myocardial blood flow reserve. After 5 min of continuous infusion of the vasodilator and 2 min of infusion of microbubbles, four real-time inflow curves were obtained as described above. Microbubble inflow curves were analyzed offline using the Image Processing toolbox in MATLAB (Mathworks, Natick, MA). Average contrast intensity was quantified offline in a ROI manually drawn on the myocardial wall of the left ventricle. No corrections had to be made because microbubble concentrations were equal across all mice and measurements. Video intensities within the ROI were then fitted to the exponential function $y = A(1 - e^{-\beta t})$, whereby $y$ is the video intensity at any given time, $A$ is the plateau video intensity representing the microvascular blood volume, $\beta$ is the rate constant representing microvascular filling velocity, and $t$ is the time after microbubble destruction.