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Original article

Comparison of cumulative planimetry versus manual dissection to assess experimental infarct size in isolated hearts

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ABSTRACT

Introduction: Infarct size (IS) is an important variable to estimate cardiac ischemia/reperfusion injury in animal models. Triphenyltetrazolium chloride (TTC) stains viable cells red while leaving infarcted cells unstained. To quantify IS, infarcted and non-infarcted tissue is often manually dissected and weighed (IS-DW). An alternative is to measure infarcted areas by cumulative planimetry (IS-CP). **Methods:** We prospectively compared these two methods in 141 Langendorff-prepared guinea pig hearts (1.44 ± 0.02 g) that were part of different studies on mechanisms of cardioprotection. Hearts were perfused with Krebs–Ringer's and subjected to 30 min global ischemia after various cardioprotective treatments. Two hours after reperfusion hearts were cut into 6–7 transverse sections (3 mm) and stained for 5 min in 1% TTC and 0.1 M KH_2PO_4 buffer (pH 7.4, 38 °C). Each slice was first scanned and its infarcted area measured with Image 1.62 software (NIH). Infarctions in individual slices of each heart were averaged (IS-CP) on the basis of their weight. After scanning, IS-DW was determined by careful manual dissection of infarcted from non-infarcted tissue and measuring their respective total weight. **Results:** We found limited tissue permeation of TTC in relation to the slice thickness leaving tissue in the center unstained, as well as significant cross-contamination of stained vs. unstained tissue after manual dissection. IS-CP and IS-DW ranged from 6.0 to 73.1% and 19.4 to 70.5%, respectively, and correlated as follows: IS-DW $= (27.6 \pm 1.4) + (0.518 \pm 0.038) \cdot$ IS-CP; $r = 0.75$ (Pearson), $p < 0.001$. In addition, IS-CP correlated better with return of function after reperfusion like developed left ventricular pressure, contractility and relaxation, and myocardial oxygen consumption. **Discussion:** Despite a good correlation between both methods, limited tissue permeation by TTC diffusion and limited precision in the ability to manually dissect stained from unstained tissue leads to an overestimation of infarct size by dissection and weighing compared to cumulative planimetry.

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1. Introduction

Considerable investigative efforts over recent decades have focused on the study of ischemia/reperfusion (I/R) injury in general, and cardioprotective strategies in particular. Indeed, the study of pre- and post-conditioning alone now leads to several hundred articles per year in the peer-reviewed literature. The vast majority of these investigations have relied on isolated hearts or intact animal models (Hausenloy & Yellon, 2007). I/R is usually applied before or after one or more interventions, and the degree of cardioprotection is subsequently assessed by measuring and comparing the various degrees of myocardial infarction.

Therefore, infarct size (IS) measurement has become pivotal in several areas of cardiac research, to the extent that many investigations are ultimately based mainly or solely on this variable. However, use of different methods by various investigators makes comparisons among laboratories challenging. 2,3,5-triphenyltetrazolium chloride (TTC) staining is a widely used and accepted method to delineate infarcted from non-infarcted tissue and is well correlated with more traditional staining procedures (Fishbein et al., 1981; Khalil et al., 2006). TTC itself is colorless in solution, but is reduced by dehydrogenases of functioning mitochondria to yield a brick red formazan (Altman, 1976). Infarcted tissue remains unstained and can be recognized without microscopic examination. Nevertheless, there is considerable variation in how TTC is used to stain the tissue – perfusion into the coronaries before slicing or diffusion from the

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surface after slicing – and in how the staining results are converted to quantify IS. Some investigators, for example, manually dissect the tissue and separately weigh infarcted and non-infarcted tissue (Chiari et al., 2004; Stowe, Camara, Heisner, Aldakkak, & Harder, 2007). Others use a computer-based method, in which cross-sectional images of heart slices are made, and IS is measured by color analysis using specialized software (Fishbein et al., 1981). This latter method, however, only measures two-dimensional surface staining in a finite number of slices, and measurement of infarction in the third dimension is solely a factor of the number of slices. In addition, tissue penetration by TTC diffusion may be incomplete, thus it is possible that the planimetric method misses valuable information.

To our knowledge, no effort has been made to compare the image-based planimetric technique to the physical dissection and weighing of the tissue after TTC staining. We used Langendorff-prepared guinea pig hearts that were part of different studies on mechanisms of cardioprotection where hearts were subjected to various cardioprotective strategies followed by I/R. These hearts were studied prospectively to contrast the results of the two different methods in discriminating less infarcted from more infarcted myocardium. In each heart, IS was measured first by cumulative planimetry, then by manual dissection.

2. Methods

2.1. Statement on use and care of animals

All investigations conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee (Medical College of Wisconsin, Milwaukee, WI, U.S.A.). Thirty mg of ketamine and 1000 units of heparin were injected intraperitoneally into 143 albino English short-haired guinea pigs (weight 250–300 g) of either sex that were, with the exception of two hearts, part of different studies on cardioprotection (Riess et al., 2002; Riess, Camara, Kevin, An, & Stowe, 2004; Riess et al., 2003). Animals were decapitated 15 min later when unresponsive to noxious stimulation.

2.2. Langendorff heart preparation

Our methods have been described previously (Riess et al., 2002, 2004, 2003). After thoracotomy, the aorta was cannulated distal to the aortic valve, and the heart was immediately perfused retrograde with 4 °C cold oxygenated Krebs–Ringer solution. The inferior and superior venae cavae were ligated, and the heart was rapidly excised (wet weight 1.44 ± 0.02 g). After cannulation of the pulmonary artery to collect the coronary effluent, the heart was placed in the support system and perfused at 55 mmHg at 37 °C. The Krebs–Ringer perfusate was equilibrated with ~97% O_2 and ~3% CO_2 to maintain a constant perfusion pH of 7.40 ± 0.01 with a carbon dioxide partial pressure (pCO_2) of 25 ± 2 mmHg and an oxygen partial pressure (pO_2) of 570 ± 10 mmHg. The perfusate was filtered (5-µm pore size) in-line and had the following calculated composition (nonionized): 138 mM Na^+, 4.5 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 134 mM Cl^-, 14.5 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 11.5 mM glucose, 2 mM pyruvate, 16 mM mannitol, 0.1 mM probenecid, 0.05 mM EDTA, and 5 U/l insulin.

Left ventricular pressure (LVP) was measured isovolumetrically with a saline-filled latex balloon inserted into the left ventricle through a cut in the left atrium. At the beginning of the experiment the balloon volume was adjusted to achieve a diastolic LVP of 0 mmHg, so that any subsequent increase in diastolic LVP reflected an increase in left ventricular wall stiffness, or diastolic contracture. Characteristic data from LVP were as follows: developed (systolic–diastolic) LVP, and the maximal and minimal first derivatives of LVP ($dLVP/dt_{max}$ and $dLVP/dt_{min}$) as indices of contractility and relaxation, respectively. Coronary flow (CF) was measured at constant temperature and perfusion pressure by an ultrasonic flowmeter (Transonic T106X, Ithaca, NY) placed directly into the aortic inflow line. Coronary inflow (a) and venous (v) pO_2, pH, and pCO_2 were measured off-line with an intermittently self-calibrating analyzer system (Radiometer Copenhagen ABL 505, Copenhagen, Denmark). pO_2v tension was also measured continuously on-line with an O_2 Clark type electrode (model 203B; Instech, Plymouth Meeting, PA). Myocardial oxygen consumption (MVO_2) was calculated as CF • heart wet weight^{-1} • (pO_2a − pO_2v) • 24 µl O_2/ml at 760 mmHg. The degree of functional cardioprotection was assessed as % return of LVP, $dLVP/dt_{max}$, $dLVP/dt_{min}$ and MVO_2, respectively, at 120 min reperfusion compared to their respected baseline values before I/R.

2.3. Cardioprotective strategies

Different levels of myocardial infarction were obtained by applying various strategies of cardioprotection such as ischemic preconditioning (IPC) (Murry, Jennings, & Reimer, 1986), anesthetic preconditioning (APC) (Kersten, Schmeling, Pagel, Gross & Warltier, 1997) or hypothermia (Camara, Riess, Kevin, Novalija & Stowe, 2004). Time control hearts (CON; $n = 20$) were perfused for 190 min and not subjected to I/R. All other hearts underwent 30 min of global no-flow ischemia followed by 120 min of reperfusion. Ischemic control hearts (ISC; $n = 68$) were not subjected to cardioprotection. IPC ($n = 8$) was achieved by two times 5 min of global ischemia with 5 min reperfusion between the two IPC episodes and 20 min between the second IPC episode and index ischemia (Riess et al., 2002). APC was achieved by exposure to 0.5 ± 0.1 mM ($n = 8$) or 1.3 ± 0.1 mM ($n = 22$) sevoflurane (Abbott Laboratories, Chicago, IL) for 15 min ending 20 min before index ischemia (Riess et al., 2002, 2003). Hypothermic cardioprotection ($n = 15$) was achieved by cooling from 37 to 17 °C 20 min before index ischemia and rewarming on reperfusion (Riess et al., 2004). At the end of each experiment, hearts were removed from the perfusion apparatus and IS was determined as described below. Two additional hearts were used to specifically investigate the depth of TTC permeation by diffusion from the slices' surface immediately after removal from the chest.

2.4. Assessment of infarct size

2.4.1. TTC staining

At the end of 120 min of reperfusion, hearts were quickly removed and weighed. Atria were discarded and ventricles were cut into 6 to 7 uniform transverse slices of 3 mm thickness using a rat heart matrix. Slices were immediately stained by diffusion of 1% TTC (Sigma; St. Louis, MO) in 0.1 M KH_2PO_4 buffer (pH 7.4, 38 °C) for 5 min. TTC stains viable tissue red, indicating the presence of a formazan precipitate that results from the reduction of TTC by dehydrogenases present in viable tissue only (Altman, 1976; Fishbein et al., 1981).

2.4.2. Scanner-based image acquisition

Slices were individually weighed and positioned on a transparent sheet protector with their apical surface facing upwards. The sheet and slices were then placed on the scanning surface of a color scanner (ScanJet 6300C, Hewlett-Packard, Palo Alto, CA) connected to a personal computer (Dimension L866r, Dell, Round Rock, TX) and controlled with the appropriate software (HP Precision Scan Pro, Hewlett-Packard). The scanner was set to acquire 24-bit color images at 1200 pixels per inch image resolution. The resulting images were saved as uncompressed tagged image files. Fig. 1A and B show examples from two different types of hearts representing different magnitudes of TTC staining.

2.4.3. Infarct size assessment by cumulative planimetry

Images were analyzed manually with a computer mouse by a person blinded to the experimental protocol using public domain image