Novel Rat Model of Ischemic Cardiomyopathy Induced by Repetitive Myocardial Ischemia/Reperfusion Injury While Conscious

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Background  A rodent model of ischemic cardiomyopathy (ICM) induced by repetitive brief ischemia/reperfusion (I/R) injury while conscious has not been previously established.

Methods and Results  A newly developed coronary occluder was implanted in male Wistar rats. A repetitive I/R protocol (20s, 2 min, followed by main 30min−ischemia, every 48h, for 4 weeks) was introduced while the animals were conscious. The I/R protocol did not induce transmural scar formation but induced (1) residual myocytes with scattered infiltration of fibrosis (Masson trichrome stain), (2) coronary hypoperfusion (201Tl-Cl autoradiogram), (3) reduced coronary microvascular volume fraction (microCT), and (4) gradually progressive left ventricular (LV) dilation (echocardiography). These parameters of ICM showed interindividual variation; however, the percent increase in LV diastolic area on day 3 was significantly correlated with LV dilation (r=0.91, p<0.0001), fibrosis (r=0.77, p=0.0034), and reduction in microvessels (r=0.67, p=0.040) at week 4. The LV dilatory response on day 3 also correlated with inducible nitric oxide synthase expression (immunohistochemistry, day 3) in the LV (r=0.92, p=0.028).

Conclusions  A novel rat model of ICM induced by repetitive I/R while conscious showed interindividual variation in the severity of ICM in the advanced stage, but this was predictable non-invasively (by LV dilatory response) during the initial stage of repetitive I/R. (Circ J 2007; 71: 788–795)

Key Words: Interindivdual variation; Ischemic cardiomyopathy; Noninvasive assessment; Repetitive ischemia/reperfusion

Multiple episodes of myocardial ischemia/reperfusion (I/R) injury occurring downstream of a coronary stenosis is considered to be a critical event in the development of cardiac dysfunction associated with ischemic cardiomyopathy (ICM). In the lesion the myocardium shows pathological changes, such as interstitial fibrosis, residual myocytes, matrix remodeling, and inflammatory activity. Reproduction of this sequence has been established in models of repetitive I/R injury in closed-chest mice and rats. However, the technical limitation in those animal models is the possible adverse effects of general anesthesia during I/R stimuli, because the alteration of the autonomic nervous system under general anesthesia is considered to exaggerate and jeopardize the I/R injury. Therefore, remaining conscious during repetitive I/R stimuli is required as the more appropriate animal model, so the primary purpose of the present study was to establish a novel rat model of ICM induced by repetitive I/R while conscious using an implanted coronary arterial occluder.

In the clinical setting ICM varies in the grade of severity of the morphological alterations and in the time course of progression to cardiac dysfunction. Even in animal experiments under identical I/R protocols, the severity of the morphological alterations and cardiac dysfunction of ICM is variable among different species and strains. Variability in severity may still occur, even in the same strain, probably because of heterogeneity of genetic background etc. We hypothesized that the severity of ICM in rats following an identical surgical procedure and protocol of repetitive I/R would show a wide range of variability among the subjects. If this hypothesis is true, it is important to predict the severity of the ICM by non-invasive parameters during the initial phase of I/R injury. The second purpose of the present study was to establish an echocardiographic parameter during the initial stage of repetitive I/R induced-ICM, to investigate any association with echocardiographic parameters.

Methods

Animal Preparation

Experimental procedures and protocol were conducted according to the institutional guidelines approved by the Animal Research Committee of Kawasaki Medical School (#06-028). Male Wistar rats (Charles River Laboratories Japan, 325–415 g, n=22) were premedicated (ketamine:
90 mg/kg, xylazine: 10 mg/kg), then orally intubated (16G polyethylene tubing) using an otoscope. Ventilation was mechanically controlled under general anesthesia (sevoflurane inhalation, 1.0–3.0%, with 100% oxygen, Anesthesia WorkStation, Hallowell EMC). Body temperature was controlled by an electric heating pad. Surgery was performed using aseptic technique. The carotid artery was cannulated to maintain systemic hemodynamics and blood volume during the surgery. The jugular vein was cannulated with venous tubing (MRE040, Braintree Scientific), filled with heparinized saline, for drug administration during the protocol. The heart was exposed by left thoracotomy and a mini-pneumatic coronary occluder was placed around the mid-portion of the left anterior descending artery (LAD). The efficacy of the occluder was confirmed by myocardial blanching and hypokinesis during LAD occlusion. After instrumentation the chest was closed under positive end-expiratory pressure following evacuation of the air inside the chest. The occluder-catheter and venous tubing were tunneled subcutaneously, exteriorized between the scapulae and passed through a tethering sheath sutured at both scapulae. After the surgery the animals were placed in a recovery cage for 2 h, then transferred to the animal care facility. Five days after the surgery, the I/R protocol was started. After completing the I/R protocol and data sampling, the rats were killed by extracting the heart under anesthesia.

**Mini-Pneumatic Coronary Occluder**

The mini-pneumatic coronary occluder (Fig 1) was modified according to the prototype described in the previous paper. It consists of a balloon, a basket sheath, a suture, and a catheter. The balloon (7 mm length) is made of soft latex membrane, which is pliable and puts negligible physical force on the LAD during balloon deflation. The balloon is mounted within the basket sheath (3.2 mm diameter, 12 mm length with 10 longitudinal slits). The suture (Prolene 5-0) is securely notched at 2 holes in the mid-part of the sheath and passed through over the balloon. After the surgery the animals were placed in a recovery cage for 2 h, then transferred to the animal care facility. Five days after the surgery, the I/R protocol was started. After completing the I/R protocol and data sampling, the rats were killed by extracting the heart under anesthesia.

“crimping” the LAD upward/outward and (2) compression by the expanded balloon/sheath. The base of the balloon is connected to the polyethylene catheter, which is exteriorized. Inflation/deflation can be performed by the operator from outside of the cage while the rats are conscious.

Four days after the surgery (1 day before starting the I/R protocol), the effect of the occluder under closed-chest conditions was evaluated under anesthesia using echocardiography (Sequoia 8.5 MHz, Acuson). Myocardial ischemia induced by balloon inflation was confirmed by reduced left ventricular (LV) wall motion and LV dilation during inflation for approximately 20 s. If this duration was insufficiently effective, the rat was excluded from the experiment.

**I/R Protocol**

Five days after the surgery the repetitive I/R protocol was started. The order was: (1) occlusion for 20 s once, (2) release for 5 min, (3) occlusion for 2 min once, (4) release for 5 min, and then (5) the main occlusion for 30 min. This conditioning prior to the 30-min occlusion was required to avoid cardiac death. Our preliminary study had demonstrated that the rats consistently died after continuous occlusion for approximately 15 min if the preconditioning was not performed (see Discussion). We assume that the 2-min occlusion is mainly effective in prohibiting ventricular fibrillation during sustained ischemia (personal communication Dr Hiroyuki Yaoita, Fukushima University). The protocol was repeated every 48 h and continued for 4 weeks. If the tethering tubing at the back was disconnected or the tissue became seriously infected before completing the I/R protocol, the affected rats were excluded from the experiment.

As a preliminary experiment a single 90-min occlusion of the LAD followed by reperfusion was performed while the animals were conscious as a comparison with the repetitive I/R protocol.

**Cardiac Function**

The time course of cardiac function during the I/R protocol was evaluated by echocardiography (Sequoia, 8.5 MHz, Acuson) under general anesthesia (thiopental sodium 30 mg/kg, through IV tubing) on the day before starting the...
I/R protocol (Pre), on the 3rd day (D-3), in the 2nd week (W-2), and in the 4th week (W-4) of the I/R protocol. The LV short-axis view was recorded. LV diastolic and systolic areas, defined as the maximum and minimum LV cavity areas during the cardiac phase, respectively, were analyzed (Nahri, Nexis). The percent change in LV area compared with baseline (Pre) was analyzed: 100 \times \frac{LV \text{ size on the protocol date} - LV \text{ size at Pre}}{LV \text{ size at Pre}} (%). The measurement of LV area was performed by 2 independent observers. In the preliminary study, the coefficient of correlation of the measured LV cavity area between observers was excellent (r=0.83, p<0.0001).

Coronary Flow Distribution and 3-Dimensional (3-D) Visualization of Coronary Microvasculature

After completing the I/R protocol (W-4) coronary blood flow (CBF) distribution was evaluated by autoradiography and the coronary microvasculature was visualized by X-ray microcomputed tomography (microCT). The $^{201}$Tl-Cl (11 MBq) and heparin (300 U) were administered intravenously, the heart was excised, connected to a Langendorff perfusion system and contrast medium was injected under controlled perfusion pressure (85 mmHg). This procedure was modified to that described in the previous studies. The whole heart was enclosed in tubing and observed by microCT (ELE-SCAN, NittetsuElex) under 28-μm spatial resolution. The coronary microvasculature was visualized 3-dimensionally (Voxblast v2.2, Vaytek) and the coronary microvascular volume fraction was calculated (NIHimage1.62) as described in our previous study. The vascular volume fraction in the region of interest (ROI) was analyzed in the I/R target region (ie, LAD area) and in the control LV region referring to the image of the histological specimen (see below). Large vessels were excluded from the ROI. The ratio of the microvascular volume fraction between both regions was calculated by (volume fraction in the I/R targeted region)/(volume fraction in the control region).

LV Fibrosis

Using the identical specimen for microCT and autoradiography, infiltration of fibrotic tissue and residual myocytes in W-4 was observed by Masson trichrome staining (3-μm thickness). The stereoscopic image (×7) of the specimen was recorded (DiMega, KonicaMinolta) and the percentage of fibrotic tissue infiltration in LV was calculated as follows: (fibrotic tissue area)/(fibrotic tissue area + myocyte area) \times 100 (%, IPLab, v3.6, BD Biosciences). Detailed configuration was observed at high magnification (×100–200).

Immunohistochemistry

In a separate experiment iNOS expression in the initial stage of repetitive I/R-induced-ICM was examined to evaluate its association with the echocardiographic parameters. After echocardiography on D-3 (the day after second I/R procedure), the rat (n=5) were killed and their hearts were frozen in OCT compound. Samples were sliced (6-μm) and fixed in cold acetone (4°C) for 10 min. MorphoSave® was applied to preserve configuration of the tissue and decrease background noise. Hydrogen peroxide was applied. Sections were stained immunohistochemically with polyclonal antibody of iNOS (Chemicon International, 1:5,000, 32 min) and anti-rabbit secondary antibody (Jackson ImmunoResearch laboratory, 1:2,000, 16 min). Staining was performed using a peroxidase-based technique using Discovery® XT system (Ventana Japan). Each section was scanned at low magnification (×7), and the percentage of iNOS-positive area in the LV was calculated as follows: (iNOS-positive area in LV)/(total LV area) \times 100 (%), IPLab, v3.6, BD Biosciences). Details of distribution and localization of iNOS were evaluated at high magnification (×100–200).
Statistical Analysis
Data are expressed as the mean±SD. Correlation between 2 variables were analyzed by single linear regression analysis. A value of p<0.05 was considered statistically significant.

Results
Success Rate of Completing the I/R Protocol
A total of 22 rats were enrolled for the I/R protocol and 12 completed the 4-week procedure (success rate: 55%). Three rats had died by the 4th day of the protocol, and 7...
rats were excluded because of insufficient effect of the occluder (n=2) or disconnection of the tethering tubing (n=5), both of which mainly occurred after the second week of the protocol.

**Time Course of LV Size**

Figs 2A, B shows the time course of LV size during the repetitive I/R protocol. The percent change in LV area in diastole and systole showed interindividual variability (3–173%, -12–200%, respectively, in W-4).

Interestingly, the percent change in the LV diastolic area on D-3 (after second I/R procedure, Fig 2C) significantly correlated with that in both W-2 (r=0.77, p=0.0034) and W-4 (r=0.91, p<0.0001). The percent change in the LV systolic area on D-3 (Fig 2D) also correlated with that in W-2 (r=0.83, p=0.0007) and W-4 (r=0.90, p=0.0001). Therefore, a detectable increase in the LV size on D-3 was an excellent parameter for predicting progression of LV dilation in the advanced stage.

**Histological Alteration and LV Dilation**

Representative images for W-4 are shown in Fig 3. Fibrotic tissue had extensively infiltrated the I/R target region, where myocytes remained scattered. The thickness of the LV wall was preserved. Neither transmural/extensive scar formation with massive loss of myocytes, nor LV wall thinning was observed. This histological characteristic contrasted with that for the single long occlusion (90 min) in the preliminary experiment, shown in Fig 4A.

At the end of the repetitive I/R protocol the percentage of fibrotic tissue infiltration showed interindividual variability (10–64%); however, it significantly correlated with the percent increase in LV diastolic area (r=0.83, p=0.0016, data not shown).

Furthermore, the percent change in the LV diastolic area on D-3 correlated with the percentage of fibrotic tissue in-
filtration at the end of the I/R protocol (r=0.77, p=0.0034, Fig 5A). Thus, the LV dilatory response on D-3 was a predictor of the severity of fibrotic infiltration in the advanced stage of repetitive I/R-induced ICM.

**Coronary Flow Distribution and Coronary Microvasculature**

Fig 3 shows the $^{201}$Tl-Cl autoradiogram and corresponding histological images. Coronary perfusion (radioactivity) in the I/R target region was 70.4±15.0% lower than in the control region (n=11). The 3D architecture of the coronary microvessels is shown in Fig 4D. Note that the coronary microvasculature (28-μm resolution) that was preserved is in contrast with the avascular zone observed after single 90-min ischemia in the preliminary experiment (Fig 4C). Taken together, coronary perfusion of the I/R target region showed no defects after the repetitive I/R protocol.

The ratio of microvascular volume fraction at the end of the repetitive I/R protocol (W-4) showed interindividual variability (0.01–1.42), but correlated with the percent change in the LV diastolic area on D-3 (r=0.67, p=0.040, Fig 5B). Thus, the LV dilatory response on D-3 was a predictor of a reduction in the coronary microvascular volume in the advanced stage of repetitive I/R-induced ICM.

**iNOS Expression in the Initial Stage of Repetitive I/R-Induced ICM**

Immunohistochemistry of iNOS was evaluated in the heart on D-3 (Fig 6). iNOS was observed over the target I/R region, mainly in the myocardium, infiltrating cells and interstitial space. The coronary endothelium in the I/R region also expressed iNOS. The percentage of iNOS-positive area in the LV on D-3 showed significant correlation with the LV dilatory response on the same date.

**Discussion**

In the present study we were able to establish a novel rat model of ICM induced by repetitive I/R injury while conscious using an implanted mini-pneumatic coronary occluder. Typical cases following the repetitive I/R protocol revealed the characteristics of morphological alteration and cardiac dysfunction observed in patients with ICM. However, interindividual variation in the severity of ICM was observed among the rats, despite the identical surgical procedure and I/R protocol. Interestingly, the LV dilatory response on D-3 of the I/R protocol (after second I/R procedure) was an excellent parameter for predicting the severity of the ICM in the later phase of the repetitive I/R protocol. Therefore, predicting the severity of ICM in the advanced stage was practical using non-invasive assessment by echocardiography in the initial phase of the repetitive I/R procedure. Furthermore, the expression of iNOS in the LV on D-3 significantly correlated with the LV dilatory response on the same date. Taking these findings together, the iNOS expression in the initial stage of repetitive I/R procedure was considered to be associated with the severity of repetitive I/R induced-ICM in the advanced stage.

The rat’s heart in a typical case following repetitive I/R protocol while conscious was characterized by morphological alterations similar to those in biopsy specimens obtained from patients with sustained myocardial ischemia:3–6 that is, residual myocytes and infiltration of fibrotic tissue scattered over the I/R target region. Neither replacement by transmural/extensive scar nor LV wall thinning was observed. Myocardial perfusion ($^{201}$Tl-Cl) was low but not defected. An avascular zone was not observed by 3-D microvascular visualization (28-μm resolution). These characteristics were obviously different from those in the experimental heart under long-period occlusion of the coronary artery.

Another characteristic of the ICM model in the present study was the large interindividual variation in the severity of the myocardial damage despite an identical surgical procedure and I/R protocol. The true reasons for this variation are unclear; however, anatomical variation of the coronary arterial branch and genetic heterogeneity related to susceptibility to I/R insult are candidates. First, the coronary artery is located below the myocardial surface in the rat, so is not clearly observed. We sutured around the coronary artery (without isolation) at the same section in the same manner. The inflation volume was the same. Native collaterals are reported to be less in the rats1,2,25 but possible variation in this may affect the results. Second, variation in myocardial vulnerability/tolerance against I/R insults based on genetic heterogeneity should be considered2 but remains to be resolved in the future.25 We consider that the demonstrated interindividual variability is not unnatural and probably caused by several mechanisms.

In the present study we demonstrated that (1) iNOS was expressed in the myocardium and the endothelium of the coronary microvasculature on D-3 of a repetitive I/R protocol and (2) the percentage of iNOS-expressing area in the LV on D-3 was significantly correlated with the LV dilatory response. Furthermore, the dilatory response of LV on D-3 significantly correlated with the parameters of severity of the ICM in the later phase (W-2 and W-4) of the repetitive I/R protocol. Taking all these findings together, we consider that the interindividual variation in the severity of ICM may be associated with interindividual variation in the level of iNOS expression during the initial phase of repetitive I/R injury may be associated with interindividual variation in the severity of ICM in the advanced stage. iNOS is considered to be associated with the grade of myocardial injury caused by ischemia19,20 and in the patient with ICM the level of myocardial iNOS is related to functional recovery after revascularization.26 I/NOS is upregulated by I/R injury7 through myocardial inflammation.28 An iNOS-induced high concentration of nitric oxyde may induce myocyte apoptosis29 and contractile dysfunction.18 However, the pathophysiological significance of INOS in the ICM remains to be resolved.30,31

**Critique and Limitations of the Present Study**

ICM is considered to advance the manifest irreversible dysfunction by recurrently occurred I/R although the mechanisms remain to be resolved.32,33 This situation is often observed in patients with residual coronary stenosis (ie, in whom therapeutic revascularization is expected to improve cardiac dysfunction34 but is impossible to perform because of technical reasons; the so-called “no-option” patients). These patients are not simulated by animal models using conventional methodology (ie, ligation/single long period of occlusion of coronary artery that induces transmural/extensive scar formation with massive myocyte loss). Therefore, our rat model is a breakthrough in methodology. Massive scar formation with LV wall thinning was not induced, but residual myocytes, patchy infiltration of fibrosis, and LV dilation were induced in typical cases. Unfortunately, this model still cannot precisely reproduce ICM with stunning and hibernation1 caused by multiple persistent high-grade coronary stenoses.
The 30-min occlusion of the coronary artery is known to induce massive infarction in anesthetized rats,12 however, massive scar formation with LV wall thinning was not observed in the present study. We emphasize that the present study is the first developed rat model with ICM induced by repetitive 30-min ischemic stimuli while conscious. The possible reason for the mild histological findings is considered to be the lack of adverse effects of general anesthesia13,14 and/or adaptation mechanisms against repeated stimuli. Finally, we observed patchy distribution of myocyte loss and infiltration of fibrosis instead of massive scar formation, but we cannot exclude that microscopic infarctions occurred heterogeneously15,16 and merged with each other.

The severity of ICM in the later phase (W-2 and W-4) was predicted individually by observing the LV dilatory response after the second I/R procedure (D-3). This is the first systematic study to grade the susceptibility of advancing to ICM by noninvasive assessment during the initial stage of repetitive I/R injury. We believe that grading by echocardiography in the initial stage is important, for example, for evaluating the efficacy of optional therapeutic intervention in ICM-animal models in future studies (eg, gene transfection and drug delivery systems). If after the therapeutic intervention the experimental animals exhibit mild cardiac damage, still there remains some difficulty in assessing whether it was related to the beneficial effects of the therapy or the animal’s own tolerance against I/R injury. Therefore, we believe that grading the susceptibility/tolerance to I/R before starting treatment is important.

In conclusion we have established a novel model of ICM induced by repetitive I/R protocol while conscious. Variability in the severity of ICM in the advanced stage was predicted using a noninvasive echocardiographic parameter in the initial stage of the repetitive I/R procedure. We believe that this model will give new insight into the genetic background of individual differences in the degree of cardiac dysfunction in ICM, and has application for diagnostic molecular imaging and therapeutic strategies of ICM in the future.

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