Acta Cardiol Sin 2023;39:135–143
Original Article
doi: 10.6515/ACS.202301_39(1).20220623A

Basic Science

Effect of the Ligation and Reperfusion Timeframe on Maximal Ischemia-Reperfusion Injury in Diverse Rat Models

Shih-Tai Chang,[1] Chi-Ming Chu,[2] Chia-Shiang Cheng,[3] Kuo-Li Pan,[1] Yu-Sheng Lin,[1] Chang-Min Chung[1] and Ming-Shyan Lin[1,4]

Background: Little is known about the effect that different time sequences for coronary ligation and reperfusion have on ischemic-reperfusion (IR) injury.

Objective: To investigate the relationship between the extent of IR injury and the timeframe for coronary ligation/reperfusion in three animal models.

Methods: Three rat models were used: normal Sprague-Dawley rats, diabetes mellitus (DM) rats, and fat rats. The rats in each model were divided into four groups based on the coronary ligation period (L): 30, 60, 120, and 180 min, and then divided into seven sub-groups based on the reperfusion period (R): 0, 30, 60, 120, 180, 270, and 360 min. R0 was the IR injury baseline for each sub-group. The hearts were harvested and stained with Evans blue and 2,3,5-triphenyl tetrazolium chloride dye to distinguish the different myocardial injury areas: area at risk (AAR) and myocardial necrosis. The difference between each subgroup and baseline (R0) for the necrotic area/AAR was calculated.

Results: In the normal rats, the highest IR injury differences compared with the baseline group occurred at L120, with a reperfusion time of > 180 min. The highest IR injury difference compared to the baseline group occurred at L30, with a reperfusion time of > 180 min in the DM rats and at L60R270, L120R180 in the fat rats.

Conclusions: IR injury, as induced by different coronary ligation and reperfusion time intervals, had diverse expression profiles in the different animal models. Optimal animal models with optimal coronary ligation/reperfusion protocols to achieve maximal IR injury will affect the results and interpretation of future studies.

Key Words: Acute myocardial infarction • Coronary artery disease • Ischemic-reperfusion injury • Percutaneous coronary intervention

Abbreviations

AAR	Area at risk
AMI	Acute myocardial infarction
CAD	Coronary artery disease
DIO	diet-induced obesity
DM	Diabetes mellitus
IR	Ischemic-reperfusion
L	Ligation period
LAD	Left anterior descending
NIH	National Institutes of Health
PCI	Percutaneous coronary intervention
R	Reperfusion period
TTC	2,3,5-triphenyl tetrazolium chloride

Received: November 21, 2021 Accepted: June 23, 2022
[1]Division of Cardiology, Chiayi Chang Gung Memorial Hospital; Chiayi School, Chang Gung Institute of Technology, Chaiyi Hsien; College of Medicine, Chang Gung University, Taoyuan; [2]Section of Health Informatics, Institute of Public Health; [3]Graduate Institute of Life Sciences, National Defense Medical Center and University, Taipei; [4]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan.
Corresponding author: Dr. Ming-Shyan Lin, Department of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital, No. 6, Sec. West, Chai-Pu Road, Putz City, Chiayi 61363, Taiwan. Tel: 886-5-362-1000 ext. 2854; Fax: 886-5-362-3005; E-mail: mingshyan@gmail.com

INTRODUCTION

Re-establishing blocked coronary blood flow using thrombolysis or primary coronary intervention (PCI) is essential to reduce myocardial damage and necrosis in patients with acute myocardial infarction (AMI). However, coronary reperfusion can result in advanced myocardial injury and life-threatening arrhythmia, which clinically means ischemic-reperfusion (IR) injury.[1] After decades of investigation, the exact mechanism of myocardial IR injury remains unclear.

Therapies to lessen the degree of IR injury have proven elusive and disappointing. Many experimental trials for IR injury have shown mixed results and conclusions even when the same pharmacological therapy was used.[2-6] One possible explanation for these controversial conclusions may be the lack of a specific animal model with an optimal coronary ligation/reperfusion period protocol designed for the study of IR injury. Although Reimer et al. reported the wavefront phenomenon of ischemic death in dogs, no follow-up study has explored the relationship between IR injury and the time sequence for coronary ligation/reperfusion intervals in different animal models.[7] In experimental animal studies used to explore the benefits of pharmacological therapy, the optimal setting for the coronary ligation/reperfusion period might affect the interpretation of the same drug because of diverse differences in IR injury. In addition, diabetes mellitus (DM) and obesity are thought to be potential risk factors for coronary artery disease (CAD). However, little attention has been paid to DM and obese groups in IR injury studies.

In this study, we investigated the relationship between the extent of IR injury and the timeframe of coronary ligation/reperfusion in three animal models. An optimized animal model may be needed for future clinical experiments to accurately assess the benefits of IR injury therapies. In addition, we also analyzed the optimal coronary ligation and reperfusion time intervals, which could affect the extent of IR injury in these three animal models.

METHODS

Animal preparation

The Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital reviewed and approved the animal protocol (Institutional Review Board number: 2015121703). Institutional guidelines for the care and use of laboratory animals were followed according to the "ARRIVE Guidelines for Reporting Animal Research".[8] Three kinds of rats (obtained commercially from BioLASCO Taiwan Co., Ltd) were used in this study: (1) Normal Sprague-Dawley rats (8 weeks old, body weight: about 250-300 g) fed with a standard diet (normal rat group); (2) Sprague-Dawley rats with diet-induced obesity (DIO) (8 weeks old, body weight: about 300-400 g) fed with a diet containing 45% fructose and 40% fat for 4 weeks before the experiments were performed (fat rat group); and (3) Sprague-Dawley rats with STZ-induced diabetes (8 weeks old, body weight: about 250-300 g) fed with a standard diet (DM rat group). The rats were acclimated in a quarantine room for 7 to 10 days before the experiments were performed. R0 was the IR injury baseline for each sub-group. A mixture of Rumpon (6 μg/g body weight) and Zoletil (50 μg/g body weight) was used to anesthetize the rats via intramuscular injection. If the rats showed a response to pain or appeared to have an irritable status during the experimental period, an additional dose of anesthetic was given. The rats were then placed in a supine position with their paws taped and fixed to the table. A PE-70 endotracheal tube was placed into the trachea for airway maintenance. The rats were placed on a ventilator and oxygen saturation was maintained at > 95% throughout the experiment. The rodent ventilator was set at a rate of 66-120 beats/min with a tidal volume of 0.7-1.25 ml/min (SAR-830/AP, CWE Inc., USA). A heparinized central catheter was implanted to monitor hemodynamic status by puncturing through the right femoral artery. All rats received heparinization with a loading dose of 200 units/kg before the experimental protocol progressed.

Induction of ischemia

The rats received a left vertical thoracotomy and pericardiectomy to expose the heart. The thoracotomy was implemented 2 mm to the left of the midline of the chest. Regional ischemia was induced by tying the left anterior descending (LAD) coronary artery with a 6-0 silk suture. The LAD snaring position was lower than the bottom of the left atrium to avoid a large ventricular infarct, which would increase the mortality of the rats during

the experimental procedure. A grossly visual evaluation and assessment of myocardial infarction and dyskinesis was considered to indicate myocardial ischemia. Due to the high number of lethal complications that occurred before and after LAD ligation and reperfusion, 100% oxygenation was given to the rats for 5 min, and a drop of 0.5% xylocaine was dripped onto the heart, just before LAD ligation and reperfusion. During the experimental period of heart exposure, the open wound was kept moist, and a parafilm covering was used to avoid dehydration. Additionally, 1 ml normal saline was infused through a suitable femoral artery catheter every 30 min to prevent hypotension caused by dehydration. No cardioversion was performed, and the rats were taken out of the study if their systolic blood pressure was < 50 mmHg.

Animal groups

Based on the ligation period, the animals were divided into four groups: 30 min (L30), 60 min (L60), 120 min (L120), and 180 min (L180). Every ligation group was then divided into seven reperfusion sub-groups: 0 min (R0, baseline group), 30 min (R30), 60 min (R60), 120 min (R120), 180 min (R180), 270 min (R270), and 360 min (R360). In this study, every sub-group contained five rats.

Assessment of infarction size and area at risk (AAR) of the left ventricle

The LAD ligature was retightened when the study protocol was accomplished in each sub-group. Then 1 ml of Evans blue dye (2% solution) was administered into the beating left atrium to stain the myocardium, which was perfused by the patent coronary arteries for 5 to 10 mins, and the unstained myocardium was confirmed as the AAR of the left ventricle. The heart was then harvested and subsequently removed from the atria, right ventricle, and major blood vessels. The gathered left ventricle was then sliced into 4 to 5 myocardial slices of around 1 mm thickness, parallel to the atrioventricular groove. The AAR was separated from the stained parts of the myocardium in each myocardial slice and then incubated in 1.0% 2,3,5-triphenyl tetrazolium chloride (TTC) for 5 min at 37 °C and fixed in 10% formalin. After this procedure, the TTC unstained portion was defined as the myocardial necrotic area. These serial slices were scanned using an Epson AL-CX11 flatbed scanner (Epson, Long Beach, CA). The general area of the left ventricle, necrotic portion, and the AAR of each slice were measured using National Institutes of Health (NIH) Image J software (computer-assisted planimetry with ImageJ-1.37 software). The necrotic area/AAR percentages were then averaged to calculate a summation value for each heart.[9,10] The difference in IR injury from baseline was defined as the necrotic area/AAR difference between each reperfusion sub-group and baseline (R0).

Euthanasia/sacrifice methods

All rats were euthanized through immediate decapitation after deep anesthesia, and their bodies were incinerated after freezing.

Statistical analysis

All data are presented as the mean ± standard error of the mean. The upper and lower limits were the 95% confidence intervals. The Kruskal-Wallis test and post hoc tests with Bonferroni correction were used to analyze statistical differences. Significance was set at $p < 0.05$.

RESULTS

Time effects of IR injury on ligation and reperfusion

Table 1 shows the impact of different ligation/reperfusion timeframes on myocardial infarct size for the left ventricle in the three different rat models. The extent of IR injury varied according to the different ligation and reperfusion time periods. Figure 1 shows images of the left ventricular cross-section in different ligation/reperfusion timeframes for the three animal models. The timeframes for L120 are representative of normal (Figure 1A) and fat rats (Figure 1C). The timeframe for L30 is representative of DM rats (Figure 1B).

Figure 2 shows the relative IR injury percentage change compared to R0 depending on the different ligation time points. There was a significant difference in the relative IR injury percentage change across the 30 min, 60 min, 120 min, and 180 min ligation groups in the normal rats (relative IR injury 10.4 ± 1.1%, 8.4 ± 2.6%, 19.2 ± 7.4%, and 2.9 ± 3.0%, respectively; $p < 0.001$, Figure 2A), while the peak elevation was in the L120 group. In the DM rats, there was also a significant difference in the relative IR injury percentage change among the different ligation groups (relative IR injury 6.4 ± 3.1%, 2.4 ±

Table 1. Effects of different ligation/reperfusion timeframe on myocardial infarct size with respect to the left ventricle in different models

	R0	R30	R60	R120	R180	R270	R360
Normal rat model							
L30	37.1 ± 1.6	41.3 ± 2.1	41.2 ± 1.6	39.7 ± 0.7	40.0 ± 1.2	42.0 ± 1.3	42.0 ± 1.5
L60	41.1 ± 1.3	41.6 ± 1.3	41.8 ± 1.2	47.4 ± 2.3	46.7 ± 1.9	46.7 ± 2.3	43.5 ± 1.6
L120	43.8 ± 1.4	42.4 ± 1.7	44.5 ± 1.6	50.1 ± 3.3	63.1 ± 1.2	55.8 ± 2.5	57.5 ± 1.9
L180	54.2 ± 2.4	50.0 ± 1.1	55.5 ± 6.3	55.3 ± 3.8	60.6 ± 1.1	59.8 ± 1.4	53.4 ± 2.8
DM rat model							
L30	48.6 ± 1.3	45.8 ± 2.0	49.7 ± 3.0	50.9 ± 1.7	54.7 ± 2.5	54.5 ± 2.1	55.2 ± 4.1
L60	54.8 ± 0.5	57.7 ± 2.4	54.5 ± 1.8	56.3 ± 1.4	55.7 ± 3.5	58.6 ± 0.4	53.8 ± 3.0
L120	57.2 ± 1.2	53.3 ± 1.7	59.9 ± 2.4	59.4 ± 1.7	61.3 ± 1.3	60.0 ± 2.7	53.9 ± 1.5
L180	60.5 ± 1.0	59.2 ± 1.1	65.5 ± 1.2	59.5 ± 1.5	56.9 ± 2.5	60.7 ± 1.4	60.3 ± 1.5
Fat rat model							
L30	51.7 ± 5.4	56.9 ± 1.8	64.9 ± 2.3	57.8 ± 3.4	51.9 ± 4.6	47.2 ± 2.9	51.9 ± 2.7
L60	47.2 ± 4.4	52.9 ± 0.6	57.5 ± 1.8	59.7 ± 2.1	60.3 ± 3.6	62.0 ± 1.8	57.1 ± 3.0
L120	53.6 ± 5.0	53.5 ± 6.4	65.4 ± 3.9	64.1 ± 1.7	71.4 ± 1.9	67.2 ± 3.6	66.7 ± 2.4
L180	57.5 ± 1.7	63.9 ± 3.0	48.6 ± 5.9	60.5 ± 2.8	62.8 ± 4.1	62.9 ± 1.6	61.5 ± 2.3

The data presented as mean ± standard error of the ratio of myocardial infarct size to the left ventricle (%).
N = 5 in each subgroup, total were 28 (4 × 7) subgroups. DM, diabetes mellitus; L, ligation; R, reperfusion.

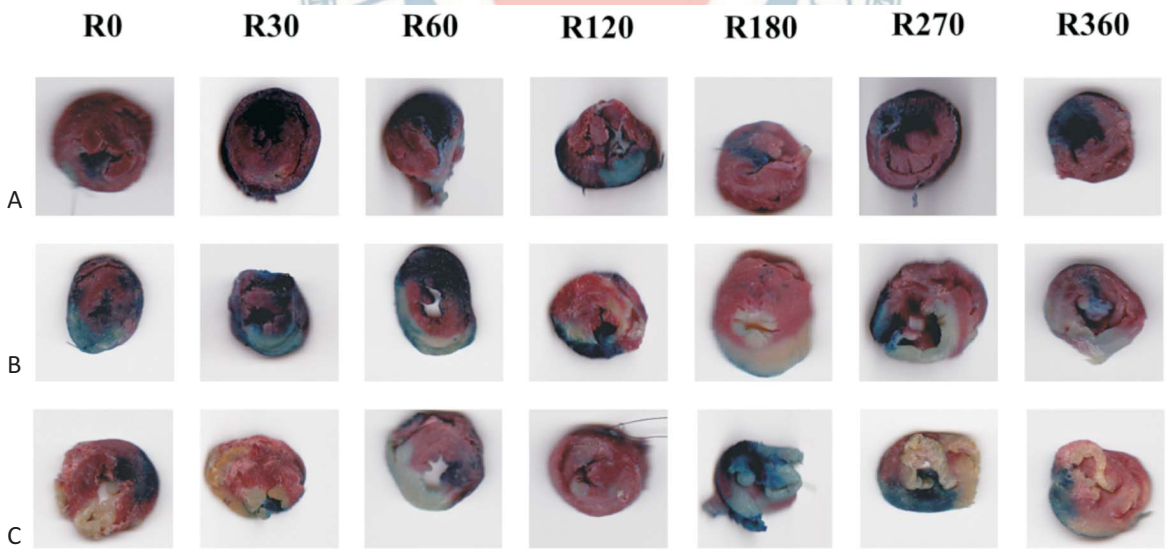

Figure 1. *Gross picture of myocardial infarct size with respect to the left ventricle in different ligation/reperfusion time courses. Blue color represented as Evans blue-stained myocardium (survival myocardium) and the other unstained myocardium was defined as the area at risk (AAR). White color represented the area not stained with 2,3,5-triphenyl tetrazolium chloride (TTC) dye and was defined as the myocardial necrotic area. (A) Normal rats at L120; (B) DM rats at L30; (C) Fat rats at L120. DM, diabetes mellitus; L, ligation; R, reperfusion.*

1.4%, 1.4 ± 2.4%, and -0.2 ± 1.9%, respectively; p < 0.001, Figure 2B). The maximal IR injury percentage change was observed in the 30 min group, and then the IR injury percentage change decreased as the ligation period increased. In the fat rats, there was also a significant difference in relative IR injury percentage change among the groups (relative IR injury 6.5 ± 4.9%, 22.9 ± 2.9%, 20.7 ± 4.6%, and 4.4 ± 4.0%, respectively; p = 0.002, Figure 2C); the highest IR injury percentage change occurred in the L60 and L120 groups.

Figure 3 shows the IR injury relative percentage change compared with R0 and the different reperfusion time groups. In the normal rats, the relative IR injury percentage change showed a significant difference ac-

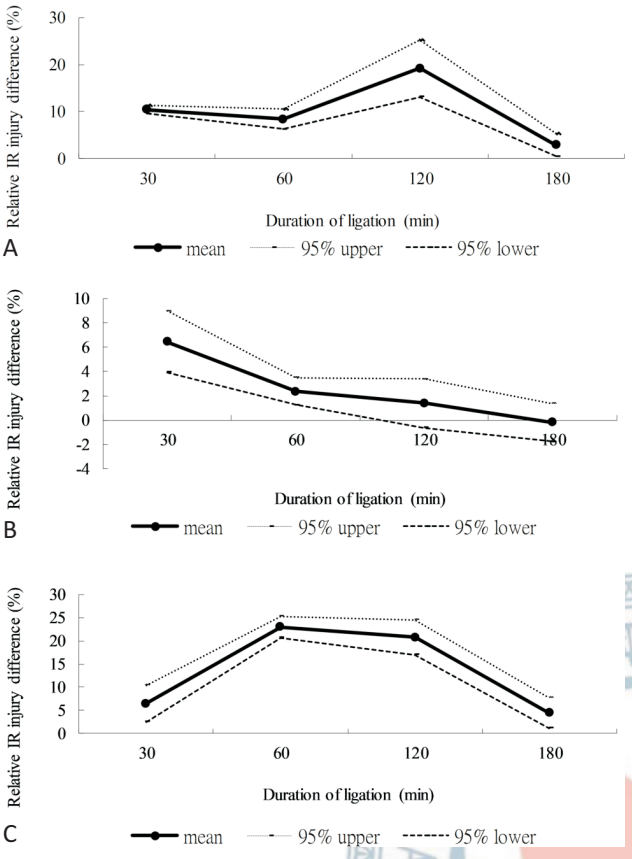

Figure 2. Time effects in ligation for reperfusion injury in 3 animal models. (A) Normal rats; (B) DM rats; (C) Fat rats. DM, diabetes mellitus.

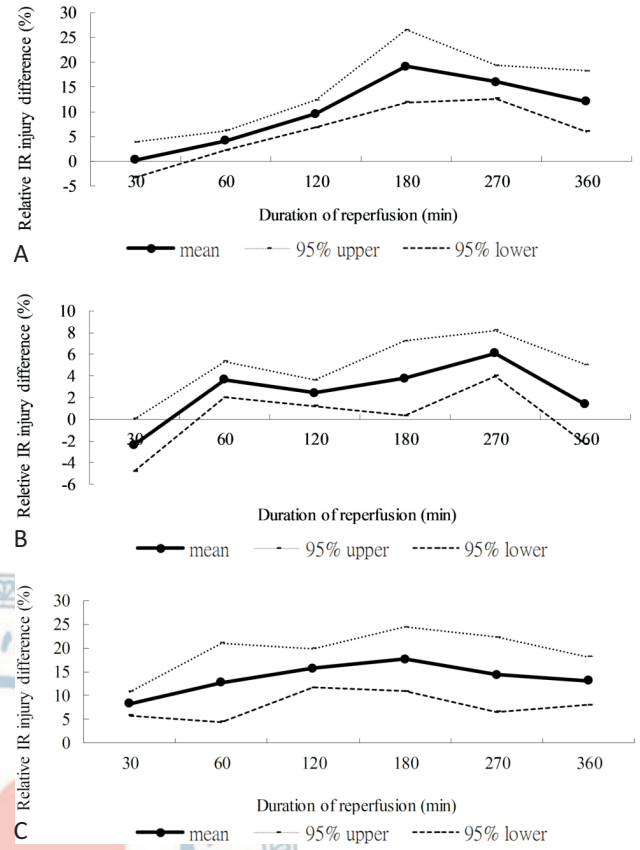

Figure 3. Time effects in reperfusion for reperfusion injury in 3 animal models. (A) Normal rats; (B) DM rats; (C) Fat rats. DM, diabetes mellitus.

ross the different time periods (30 min, 60 min, 120 min, 180 min, 270 min, and 360 min; relative IR injury 0.3 ± 4.0%, 4.2 ± 2.3%, 9.6 ± 3.2%, 19.2 ± 8.4%, 16.0 ± 3.8%, and 12.1 ± 7.0% respectively; p = 0.001, Figure 3A). The IR injury percentage change showed a gradual increase until R180, and then a decrease at R270 and R360. In the DM rats, there was no significant difference among the different reperfusion time groups (relative IR injury -2.4 ± 2.8%, 3.7 ± 1.9%, 2.4 ± 1.4%, 3.8 ± 4.0%, 6.1 ± 2.4%, and 1.4 ± 4.2% respectively; p = 0.242, Figure 3B). In the fat rats, there was a significant IR injury percentage change among the different groups (relative IR injury 8.3 ± 2.9%, 12.7 ± 9.5%, 15.8 ± 4.7%, 17.6 ± 7.8%, 14.4 ± 9.0%, and 13.1 ± 5.8% respectively; p = 0.033, Figure 3C). The IR injury percentage change in the fat rats showed a gradual increase until R180 and then a decrease at R270 and R360.

Effects of different time courses on IR injury

Figure 4 shows the effects of different ligation/reperfusion timeframes on IR injury percentage changes in the different animal models. In the normal rats, the L120R180, L120R270, and L120R360 groups had high mean IR injury differences compared with baseline (R0) (Figure 4A). In the DM rats, the maximal IR injury change was observed at L30R180, L30R270, and L30R360 (Figure 4B). In the fat rats, the L60R270 and L120R180 groups had high mean IR injury differences compared with baseline (Figure 4C).

DISCUSSION

High rates of life-threatening complications or cardiac disability occur if CAD progresses to myocardial infarction. Furthermore, IR injury may exacerbate and limit the prognosis of patients who survive an AMI event. In some cases, it may cause acute or chronic heart failure, life-threatening cardiac arrhythmia, or even death. Our study showed the differences in maximal IR injury

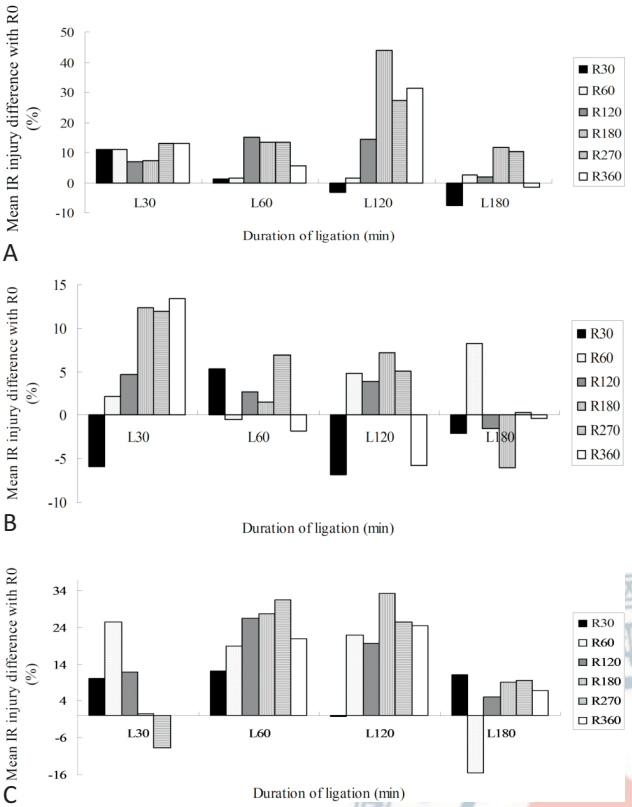

Figure 4. *Impacts of different ligation/reperfusion time courses on reperfusion injury in 3 animal models. (A) Normal rats; (B) DM rats; (C) Fat rats. DM, diabetes mellitus; L, ligation; R, reperfusion.*

in different animal models with diverse ligation-reperfusion timeframes. We used a shorter ligation time in the DM rat model to achieve the maximal effect of IR injury compared with the normal and fat rat models.

Many previous reports have applied different coronary ligation and reperfusion periods in animal models. For example, Viehman et al. completed a coronary ligation trial in a cat model, but no reperfusion was allowed. They found that significant myocardial necrosis did not occur until 360 min of coronary ligation.[10] The degree of myocardial necrosis was only 18 ± 4% of the AAR with this long period of myocardial ischemia. Tsao et al. also conducted an IR injury trial with 90 min of coronary ligation in a cat model. The found that myocardial necrosis occurred after 180 min of coronary reperfusion, and that the necrosis increased from 10% to 18% when the reperfusion period was prolonged to 270 min.[9,11] Mulch et al. reported that, in an isolated heart model, a lack of recovery ability in heart function was observed after ischemic periods extending beyond 30 min, despite reperfusion periods.[12] Furthermore, two studies on dogs demonstrated that nearly 50% of the myocardial infarct size may be attributable to IR injury.[13,14] Previous studies have even shown contrary and opposite conclusions in the same pharmacological trials.[4-6,15-20]

There were some drawbacks in the previous studies that investigated IR injury therapy. First, the use of different animal models could result in diverse conclusions. Second, variations in the coronary ligation and reperfusion time intervals could also affect the study results. Third, whether experimental medications were used or whether interventional therapy was performed at the time point as maximal IR injury will also impact the therapeutic effectson IR injury. Our findings showed that different degrees of IR injury can occur in the same animal model (rat) with different health statuses (normal, DM or fat), ligation and reperfusion times.

In this study, we found that relative IR injury change was significantly elevated at L120 in the normal rats (Figure 2A). Moreover, the IR injury percentage change showed a gradual increase at different reperfusion periods until R180, and then a decrease at R270 and R360 (Figure 3A). This result may be due to cardiac myocyte death at R180. To explore the timeframe for ligation and reperfusion intervals, and to establish the maximal IR injury extent, multivariate ANOVA tests were performed between groups for the time effects of ligation and reperfusion. We found that the L120R180, L120R270, and L120R360 groups had significantly higher mean IR injury differences compared with baseline (R0) (Figure 4A). However, a longer reperfusion time led to more animal deaths due to vital cardiac arrhythmia and heart failure. The animal death rate was around 20% at R180, but rose to 30% to 50% at R270 and R360. Based on these results, we suggest that L120R180 seems to be a reasonable choice as the optimal IR injury therapy experimental model for normal rats.

DM is thought to be equivalent to CAD in clinical practice, and many studies have reported that CAD severity is higher in diabetic patients compared with non-diabetic patients.[21,22] DM has also been associated with increased premature atherosclerosis, subsequently leading to an increase in heart failure and cardiac death at a younger age.[23,24] In the Framingham population, a 16-year follow-up study showed that DM patients had a 2.5-fold greater risk of developing cardiovascular dis-

ease and a 2 to 4-fold increased risk of mortality compared with non-diabetic patients.[25] Similarly, in DM patients, obesity has also been associated with premature death, and it has been shown to be a potential risk factor for myocardial infarction. In obese patients who have a myocardial infarction, a higher risk of cardiac arrhythmia and sudden death has also been reported.[26,27] In addition, many previous studies have reported that pharmacological interventions could limit the extent of IR injury in obese-insulin-resistant models.[28,29] Therefore, even though DM and obesity are prominent risk factors for CAD and act as significant prognostic factors for CAD, a lack of attention has been paid to IR injury experimental study design in humans and animals. In this study, we used the same protocol to survey IR injury percentage differences in normal, DM and fat rat models, with diverse phenotypes and clinical impact.

We found that the maximal IR injury percentage change occurred at L30, and then decreased as the ligation period was extended in the DM rat model. However, there was no significant difference in IR injury among the different reperfusion groups in the DM rat model, and it showed a different IR injury expression profile compared with the normal rat model. The effect of ligation time interval appeared to be more critical than reperfusion time interval on the extent of IR injury in the DM rats. In different ligation and reperfusion intervals (Figure 4B), the highest IR injury was obtained in the L30R180, L30R270, and L30R360 groups. Therefore, a shorter ligation interval of L30 may be sufficient to achieve the maximal IR injury extent in DM rats. Peak changes persisted with a prolonged reperfusion time (180-360 min), which indicates extensive infarction of IR injury in the DM model, and long-term observation (prolonged reperfusion time) might be needed in such high-risk IR injury models. Other assessment tools (echocardiography and cardiac magnetic resonance imaging) or biomarkers should be considered for late IR injury.

In the fat rat model, the extent of IR injury appeared to be significantly different among the different ligation and reperfusion groups. Maximal IR injury occurred in the L60R270 and L120R180 groups (Figure 4C). Given the lower reperfusion time, which can reduce the time cost and animal death rate, L120R180 may be the best choice for an IR injury therapy experimental model in fat rats. This finding is similar to the normal rat model.

Reimer et al. previously found that myocyte necrosis was restricted in the subendocardial region after 40 min of myocardial ischemia in a dog model.[7] They demonstrated that irreversible injury progressed as a wavefront towards the subepicardial layer during a prolonged ischemic insult period. However, to the best of our knowledge, no timeframe for different ligation and reperfusion periods in IR injury for different animal models has been explored in previous investigations. In addition, previous IR injury studies have reported controversial conclusions. Apaijai et al. concluded that the timing of pharmacological interventions might play a vital role in the inconsistent experimental results.[30] Our previously published study established and confirmed that the time sequence of coronary ligation (120 min) and reperfusion (180 min) achieved maximal IR injury in a normal adult rat model.[31] To the best of our knowledge, no previous experimental studies have used DM and fat rat models and the associated optimal time intervals for coronary ligation and reperfusion necessary to achieve maximal IR injury. Although the coronary anatomy of the rat is relatively easy to manipulate, it is different to human coronary anatomy; we could therefore establish more exact experimental results by using the most appropriate animal model settings for IR injury treatment studies. Our preliminary study results may then be more representative of the clinical efficacy of different therapies for IR injury in humans in the future.

Limitations

There were some limitations to this animal model study. The ligation level of the coronary artery could vary due to differences in the anatomy between rats. A longer interval of reperfusion, 60 min, could affect the extent of maximal IR injury. Hemodynamic status without significant shock was controlled throughout the experiment; however, we could not maintain the same blood pressure and saturation for all rats.

CONCLUSION

This study demonstrated maximal differences in IR injury to define the optimal time point for ligation and reperfusion intervals in three animal models. DM rats seemed to have a more significant change in the extent

of IR injury under shorter ligation (30 min) compared with normal and fat rats. These results could provide a reference for the design of future IR animal models in clinical research.

CONSENT FOR PUBLICATION

Not applicable (animal study).

DECLARATION OF CONFLICT OF INTEREST

All the authors declare no conflict of interest.

AVAILABILITY OF DATA AND MATERIAL

The data set used and/or analyzed during the current study is available from the corresponding author (Ming-Shyan Lin) on reasonable request.

FUNDING

The authors would like to thank Chang Gung Memorial Hospital for financially supporting this research under Contract No. CMRPG 6F0561, CMRPG 6H0081, and CMRPG 6I0021. The funder had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

AUTHORS' CONTRIBUTIONS

Conception: STC; Design and data collection; KLP and YSL; Revising the article with important intellectual content: CMC; Data analysis and interpretation: CMC and CSC; Final approval of the version to be published: MSL. All authors have read and approved the manuscript.

ACKNOWLEDGMENTS

Not applicable.

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