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Targeted temperature management by  
inhibiting HMGB1 release in myocardial  
ischemia / reperfusion injury

Jin Ho Beom

Department of Medicine

The Graduate School, Yonsei University

Targeted temperature management by  
inhibiting HMGB1 release in myocardial  
ischemia / reperfusion injury

Directed by Professor Hyun Soo Chung, M.D., Ph.D.

The Doctoral Dissertation submitted to the  
Department of Medicine  
the Graduate School of Yonsei University in partial  
fulfillment of the requirements for the degree of  
Doctor of Philosophy

Jin Ho Beom

December 2019

This certifies that the Doctoral Dissertation  
of Jin Ho Beom is approved.

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Thesis Supervisor : Hyun Soo Chung

-----  
Thesis Committee : Sungha Park

-----  
Thesis Committee : Jae-Kwang Shim

-----  
Thesis Committee : Kyungsoo Park

-----  
Thesis Committee : Jun Seok Park

The Graduate School  
Yonsei University

December 2019

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Although I feel like I have started my graduate school study just a few days ago, the time to complete my study for four years, bear a small fruit, and newly begin something is already coming close. While I am so sorry for not having done my best, I cannot but be delighted. I would like to extend my gratitude to those who have helped me so much despite that I was not so good.

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Finally, I dedicate this small fruit to my father and mother who gave birth to me and raised me devotedly.

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## **Abstract**

### **Targeted temperature management by inhibiting HMGB1 release in myocardial ischemia/reperfusion injury**

Jin Ho Beom

**Department of Medicine**

**The Graduate School, Yonsei University**

(Directed by Professor Hyun Soo Chung, M.D., Ph.D)

**Aims:** Acute myocardial infarction (AMI) is lethal and causes myocardial necrosis via time-dependent ischemia due to prolonged occlusion of the infarct-related artery. No effective therapy or potential therapeutic targets can prevent myocardial ischemia/reperfusion (I/R) injury. Targeted temperature management (TTM) may reduce peri-infarct regions by inhibiting the extracellular release of high mobility group box-1 (HMGB1). We used a rat left anterior descending (LAD) coronary artery ligation model to determine if TTM at 33°C and 36°C had similar myocardial protective effects.

**Methods and Results:** Rats were divided into sham, LAD I/R+ 37°C normothermia, LAD I/R + 33°C TTM, and LAD I/R + 36°C TTM groups (n = 5 per group). To verify the cardioprotective effect of TTM by specifically inhibiting HMGB1,

rats were assigned to the sham, LAD I/R, and LAD I/R after pre-treatment with glycyrrhizin groups (n = 5 per group). Different target temperatures of 33 °C and 36 °C caused equivalent reductions in infarct volume after myocardial I/R injury, inhibited the extracellular release of HMGB1 from infarct tissue, and suppressed the expression of inflammatory cytokines from peri-infarct regions. Plasma cardiac troponin T did not significantly differ at 33°C and 36°C TTM, indicating equivalent reductions in myocardial damage. Glycyrrhizin alleviated myocardial damage by suppressing the extracellular release of HMGB1. TTM at both 33°C and 36°C may reduce myocardial injury following acute myocardial I/R injury by suppressing the extracellular release of HMGB1. TTM attenuated the increase in infarct size, extracellular release of HMGB1, and pro-inflammatory effect against injury propagation in AMI rats. TTM at 33°C and 36°C significantly attenuated the elevation of cardiac troponin, a sensitive and specific marker of heart muscle damage, after myocardial I/R injury.

**Conclusions:** TTM at 33°C and 36°C had equivalent myocardial protective effects by similar inhibiting HMGB1 release against myocardial I/R injury in a rat AMI model.

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**Key words :** targeted temperature management, HMGB1, myocardial I/R injury

**Targeted temperature management by inhibiting HMGB1 release in  
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Jin Ho Beom

**Department of Medicine**

**The Graduate School, Yonsei University**

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I. Introduction

Coronary heart disease is the leading cause of death worldwide, and acute myocardial infarction (AMI) is the most severe manifestation of this disease<sup>1,2</sup>. In AMI, prolonged occlusion of the infarct-related artery leads to high levels of myocardial necrosis as a time-dependent ischemic process. To minimize myocardial necrosis, blood flow to the infarct-related artery must be restored by mechanical reperfusion using a coronary artery stent and thrombolytic therapy as rapidly as possible<sup>3,4</sup>. Although the door to balloon time has been significantly decreased, overall in-hospital

mortality has not significantly declined in patients with ST elevation myocardial infarction (STEMI) undergoing primary percutaneous coronary intervention (PCI) <sup>1,5</sup>. To achieve safe and effective therapeutic benefits, reperfusion therapy should be performed within 12 h of symptom onset as the therapeutic window <sup>6</sup>. Paradoxically, timely myocardial reperfusion is the cornerstone of therapy for acute STEMI <sup>7</sup>. However, this process leads to myocardial injury and cardiomyocyte death, known as myocardial reperfusion injury, which disrupts the therapeutic effects of reperfusion <sup>7,8</sup>. Currently, no effective therapies or potential therapeutic targets are available for preventing reperfusion injury in STEMI <sup>7,9</sup>. Therefore, the application of active adjunctive therapy to extend the critical therapeutic window and prevent reperfusion injury may improve clinical outcomes in patients with AMI. As the extent of myocardial salvage is an important determinant of the final infarct size in AMI, attenuation of ischemic/reperfusion (I/R) injury is critical for novel therapeutic strategies <sup>10</sup>.

Targeted temperature management (TTM, which involves therapy hypothermia (TH) or prophylactic controlled normothermia) has been widely used as a gold standard treatment for minimizing secondary brain damage and improving neurologic outcomes in survivors of sudden cardiac arrest <sup>11-14</sup>. Although mild therapeutic hypothermia of TTM at 32–34°C improves the survival and neurologic outcomes of patients who have been successfully resuscitated after cardiac arrest, a study comparing TTM at 33°C and 36°C after cardiac arrest showed that TTM at 33°C was not beneficial compared to TTM at 36°C in patients with out-of-hospital cardiac arrest of presumed cardiac

aetiology<sup>11,12</sup>. As a new concept regarding TTM, the 2015 American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care recommended selecting and maintaining a constant target temperature of 32–36°C over a duration of at least 24 h in patients with return of spontaneous circulation after cardiac arrest<sup>14</sup>. TTM may be a promising strategy for improving myocardial salvage and cardiac function<sup>15</sup>. Several studies showed that a core temperature of <35°C during reperfusion limits the infarct size. However, this goal core temperature is not always achieved<sup>16</sup>. Therapeutic hypothermia commonly induces harmful effects, including bradycardia, atrial and ventricular arrhythmia, decreased cardiac output, and mild diastolic dysfunction<sup>17</sup>. The optimal target temperature and duration are unknown in established post-cardiac arrest care<sup>14</sup>. Considering all of the expected benefits and disadvantages according to the target temperatures during TTM, determining the optimal target temperature that clinically improves the outcomes of patients with myocardial I/R injury remains challenging.

Although the pathophysiology of myocardial I/R injury is very complex and poorly understood, inflammatory response and apoptotic cell death are known to play an important role in the development of ischemic heart damage by myocardial I/R injury<sup>18,19</sup>. High mobility group box-1 (HMGB1), which is involved in the structural organization of DNA in eukaryotic cells, serves as a primary mediator of the innate immune response after release by necrotic cells or active release during sterile injury. HMGB1 is rapidly released upon I/R injury and is elevated after 30 min of ischemia<sup>20</sup>. Extracellular HMGB1 binding to Toll-like receptor 4 enhances the inflammatory

response to myocardial damage after I/R and induces cardiomyocyte apoptosis<sup>19,21</sup>. Therefore, synergistic interactions between HMGB1 and inflammatory factors amplify inflammatory responses and increase damage after I/R injury<sup>19</sup>. Plasma levels of HMGB1 are independently associated with increased mortality of STEMI patients treated with PCI<sup>22</sup>. Intravenous administration of glycyrrhizin, which attenuates extracellular release of HMGB1, significantly reduces the infarct size and decreased the levels of serum HMGB1, tumour necrosis factor (TNF)- $\alpha$ , and interleukin (IL)-6<sup>23</sup>. In a previous study using a middle cerebral artery occlusion rat model, both glycyrrhizin-mediated inhibition of HMGB1 and intracerebroventricular neutralizing antibody treatment significantly reduced the infarct volume<sup>24</sup>. Thus, HMGB1 is a valuable molecular target for new adjunctive therapies that extend the critical therapeutic window by blocking sterile inflammation during early myocardial damage after I/R injury. The exact mechanism by which hypothermia attenuates myocardial damage due to ischemia and reperfusion remains unknown<sup>25</sup>. It is critical to understand the direct functional and mechanistic relationships between TTM and HMGB1 in a clinically relevant model of AMI.

We previously demonstrated that TTM at both 33°C and 36°C equivalently helped rescue ischemic penumbra from exacerbated ischemic injury by attenuating pro-inflammatory cytokine production via HMGB1 blockade in a clinically relevant middle cerebral artery occlusion rat model<sup>24,26</sup>. Although TTM at 36°C is advantageous for ameliorating hypothermia-induced cardiac arrhythmia, shivering, and rewarming damage, whether core temperatures of 36°C and 33°C are equally effective in our

preclinical model of left anterior descending (LAD) coronary artery ligation remains unclear.

We hypothesized that TTM could attenuate the inflammatory response in peri-infarct regions by inhibiting the extracellular release of HMGB1 using a rat LAD coronary artery ligation model and subsequently reduce the myocardial infarcted area, resulting in increased myocardial protection after I/R injury. We investigated whether TTM at 36°C has a myocardial protective effect via the same mechanism.

## II. Materials and Methods

### 1. Preparation of experimental animals

Healthy, age-matched, adult male Wistar rats weighing 400–430 g were acquired from a single source breeder at Orientbio (Seongnam, Republic of Korea). All experiments and animal care were conducted in strict accordance with guidelines and protocols approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System (2016-0043) and National Institutes of Health.

### 2. Experimental rat model of myocardial I/R injury

Before surgery of the experimental rat model, anaesthesia was induced with 5% isoflurane in a mixture of 0.7 L/min nitrous oxide and 0.3 L/min oxygen and maintained using 2% isoflurane in the same gas mixture. After anaesthesia, tracheostomy was conducted using a midline neck incision and intravenous catheter

(4712-020-116. I.V Catheter 16G, Sewoon Medical Co., Cheon-An, Korea). Mechanical ventilation (tidal volume, 3.0 mL; respiratory rate, 50/min) was supported by a rodent ventilator (SAP-830/AP, CWE, Inc., Ardmore, PA). The heart was exposed by left vertical thoracotomy and pericardiectomy. Ligation of the LAD coronary artery was performed on rats as described previously<sup>27</sup>. An LAD coronary artery was ligated at the mid portion between the pulmonary artery and apex through a 6-0 ethilon suture. Immediately before ligation, the PE-10 tube (polyethylene tube, OD 0.61 mm) was placed between the LAD and suture. The suture was ligated with the PE-10 tube. Ischemia was confirmed, with cyanosis and dyskinesia of the myocardium supplied by LAD observed to be developed after ligation. Reperfusion was induced by removing the PE-10 tube after 30 min of LAD ligation and was sustained for 3 h 30 min. The skin was closed with 4-0 nylon sutures after reperfusion. The same surgical procedures were performed in sham animals except for ligation<sup>27</sup>.

### 3. Experiment protocol

We divided the present study into two main experiments. To assess the effects of myocardial protection through TTM at 33°C and 36°C, the rats were randomly divided into four experimental groups: sham (n = 5), LAD I/R + 37°C normothermia (n = 5), LAD I/R + 33°C TTM (n = 5), and LAD I/R + 36°C TTM (n = 5). The target core temperature was monitored in the rectum of rats and maintained during all experiments using a feedback-controlled heating pad (HB 101, Harvard Apparatus, Holliston, MA, USA). In the sham and normothermic groups, the target core temperature temperatures

were maintained at  $37.0 \pm 0.5^{\circ}\text{C}$ . In the TTM groups with target temperatures of  $33^{\circ}\text{C}$  and  $36^{\circ}\text{C}$ , external surface cooling was started at 15 min after LAD coronary ligation by placing ice packs on the animal's torso. The TTM target temperatures of  $33^{\circ}\text{C}$  and  $36^{\circ}\text{C}$  were maintained at  $33.0 \pm 0.5^{\circ}\text{C}$  and  $36.0 \pm 0.5^{\circ}\text{C}$ , respectively. To prevent shivering caused by TTM, vecuronium (0.9 mg/kg) was injected intramuscularly into all animals. Glycyrrhizin is a pharmacological inhibitor of HMGB1 and has been suggested to prevent HMGB1 release from cells by directly binding to HMGB1 (27-29). To verify the cardioprotective effect of TTM by specifically inhibiting HMGB1 in our animal model, rats were randomly assigned to three different experimental groups: sham (n = 5), LAD I/R (n = 5), and LAD I/R after pre-treatment with glycyrrhizin (n = 5). Glycyrrhizin (100 mg/kg) was injected intraperitoneally into the rats at 30 min before the ligation of the LAD coronary artery.

#### 4. Assessment of infarct volume

To assess myocardial infarction, 2,3,5-triphenyltetrazolium chloride (TTC) (T8877, Sigma-Aldrich, St. Louis, MO, USA) staining was performed. The chest of anesthetized rats was re-opened at 4 h after sham treatment or LAD I/R surgery. The heart was quickly removed and sectioned into 2-mm-thick slices in a pre-chilled coronal matrix device (HSRA001-1, Zivic Instruments, Pittsburgh, PA, USA). Coronal sections were immersed for 30 min in a 1% TTC solution in sterile distilled water at  $37^{\circ}\text{C}$  and then fixed in 4% paraformaldehyde in phosphate-buffered saline for 48 h. Each stained section was scanned with a flatbed scanner (PERFECTION V800 PHOTO,

EPSN, Nagano, Japan). To measure the infarct volume, heart tissue between 0 and 8 mm from the apex of the heart was used. The infarct volume in each stained section was analysed with the Image J 1.48v software (National Institutes of Health, Bethesda, MD, USA) and the infarct area (%) was measured by multiplying the area of the infarcted area relative to the total area for each slice by the thickness of the section.

## 5. Immunohistochemistry analysis

For immunohistochemistry analysis, 2,3,5-TTC staining was performed to confirm the peri-infarct area in the left ventricle <sup>28</sup>.

Next, 2-mm-thick slices between 4 and 6 mm from the apex of the rat heart were selected, fixed with a 4% paraformaldehyde solution and embedded in paraffin. Using a microtome (LEICA RM 2335, Wetzlar, Germany), the heart sections were cut at 4  $\mu$ m thickness on New Silane III-coated microslides (Muto Pure Chemical, Tokyo, Japan) from a region including the infarct area. The sections were permeabilized and blocked with citrate buffer, 3% H<sub>2</sub>O<sub>2</sub>, and 5% bovine serum albumin in Tris-buffered saline (TBS) for 1 h at room temperature (RT). The sections were incubated in TBS containing Tween 20 and anti-HMGB1 polyclonal primary antibody overnight at 4 °C (1:100, ab18256; Abcam, Cambridge, UK). The sections were washed three times with TBS for 5 min and incubated for 1 h at RT with fluorescent secondary antibodies conjugated to Alexa-fluor 594 (1:100, A11032; Invitrogen, Carlsbad, CA, USA). The sections were washed three times with TBS and mounted with ProLong<sup>TM</sup>Diamond Antifade

Mountant containing DAPI (P36962, Invitrogen). The peri-ischemic areas of stained sections were observed with a confocal microscope (LSM 700; Carl Zeiss GmbH, Jena, Germany).

#### 6. Enzyme-linked immunosorbent assay (ELISA) for cardiac troponin T (cTnT)

To obtain serum samples from rats, blood was drawn from the right atrium at 4 h after ligation of the LAD coronary artery with a 22-gauge needle. One millilitre of collected blood was transferred into a Z Serum Sep Clot Activator (Greiner Bioone, Kremsmunster, Austria), followed by centrifugation for 15 min at 3,000 rpm. The cTnT concentrations were determined using an cTnT ELISA kit (MBS2024997, MyBioSource, San Diego, CA, USA).

#### 7. Real-time polymerase chain reaction (RT-PCR)

To prepare peri-infarcted myocardium tissue, 2,3,5-TTC staining was conducted to confirm the peri-infarct area in the left ventricle <sup>29</sup>. Tissue RNA was isolated using a Hybrid-R kit (305-010, GeneAll Biotechnology, Seoul, Korea). PrimerQuest (IDT, Skokie, IL, USA) was used to design primers for glyceraldehyde-3-phosphate dehydrogenase, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Single-stranded cDNA was synthesized from

500 ng of total RNA using the PrimeScript 1<sup>st</sup> strand cDNA Synthesis Kit (6110A, Takara Bio, Shiga, Japan). Quantitative PCR was performed using a 7500 ABI system (Applied Biosystems, Foster City, CA, USA) utilizing the SYBR-Green reagent (Q5602, Gendepot, Katy, TX, USA).

## 8. Statistical analysis

All experimental results are expressed as the mean  $\pm$  standard error of the mean. Statistical analyses were performed using unpaired *t*-test or by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests for multiple comparisons between groups. Differences with  $P < 0.05$  were considered as significant.

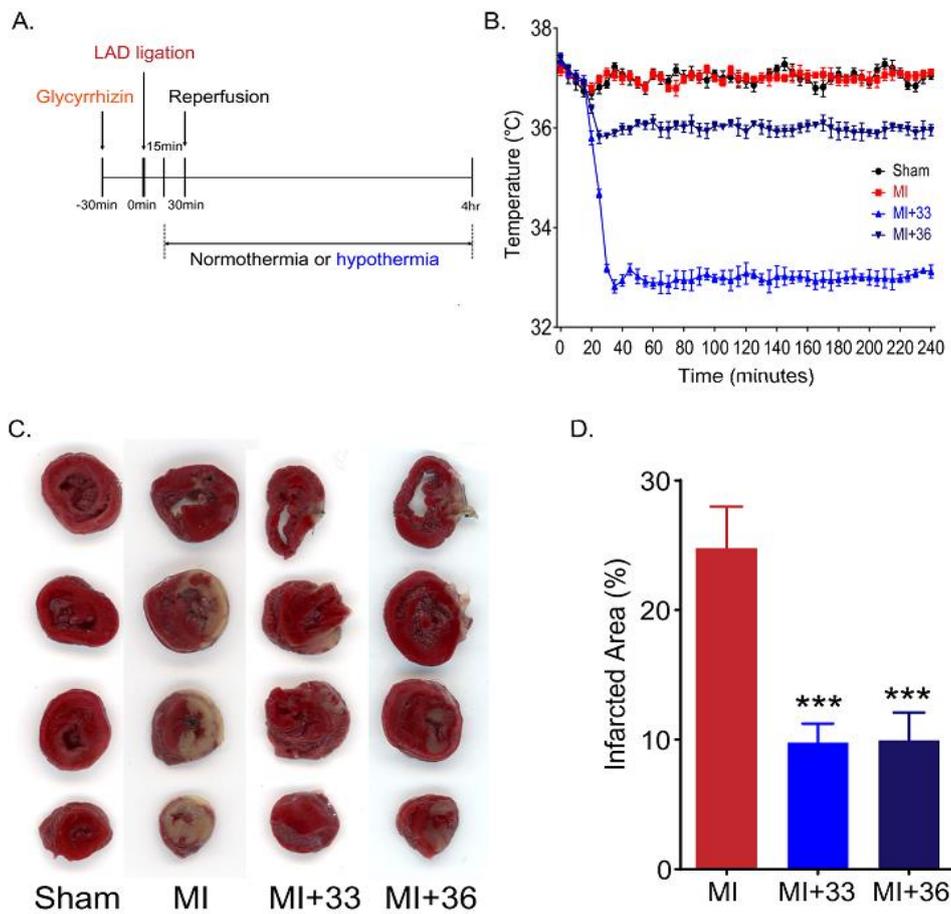
## III. Results

### 1. Target temperatures of 33°C and 36°C equivalently reduce infarct volume in myocardial I/R injury

The core target temperatures of  $33 \pm 0.5^\circ\text{C}$  and  $36 \pm 0.5^\circ\text{C}$  were reached within 18 and 8 min after the onset of TTM, respectively, and then maintained for 4 h (Fig. 1A and B). Infarct volumes were assessed by TTC staining after 4 h of ischemic injury (Fig. 1C and D). In the normothermic group, the mean ratio of the infarcted area after

myocardial I/R was  $24.78 \pm 3.20\%$  compared to the total area between 0 and 8 mm from the apex, whereas the mean ratio of the infarcted area at  $33^{\circ}\text{C}$  and  $36^{\circ}\text{C}$  of TTM was  $9.78 \pm 1.45\%$  and  $9.94 \pm 2.15\%$ , respectively. There was a significant difference between the normothermic group and TTM groups ( $P = 0.001$ ).

To detect differences in myocardial protective effects at  $33^{\circ}\text{C}$  and  $36^{\circ}\text{C}$  in TTM after myocardial I/R injury, we compared the degree of reduction of the infarct volume in the  $33^{\circ}\text{C}$  and  $36^{\circ}\text{C}$  TTM groups. There was no significant difference between the infarct volumes of the  $33^{\circ}\text{C}$  TTM group and the  $36^{\circ}\text{C}$  TTM group ( $P = 0.999$ ). These results suggest that application of TTM at both  $33^{\circ}\text{C}$  and  $36^{\circ}\text{C}$  has significant myocardial protective effects and that these temperatures lead to equivalent protection against myocardial I/R injury.

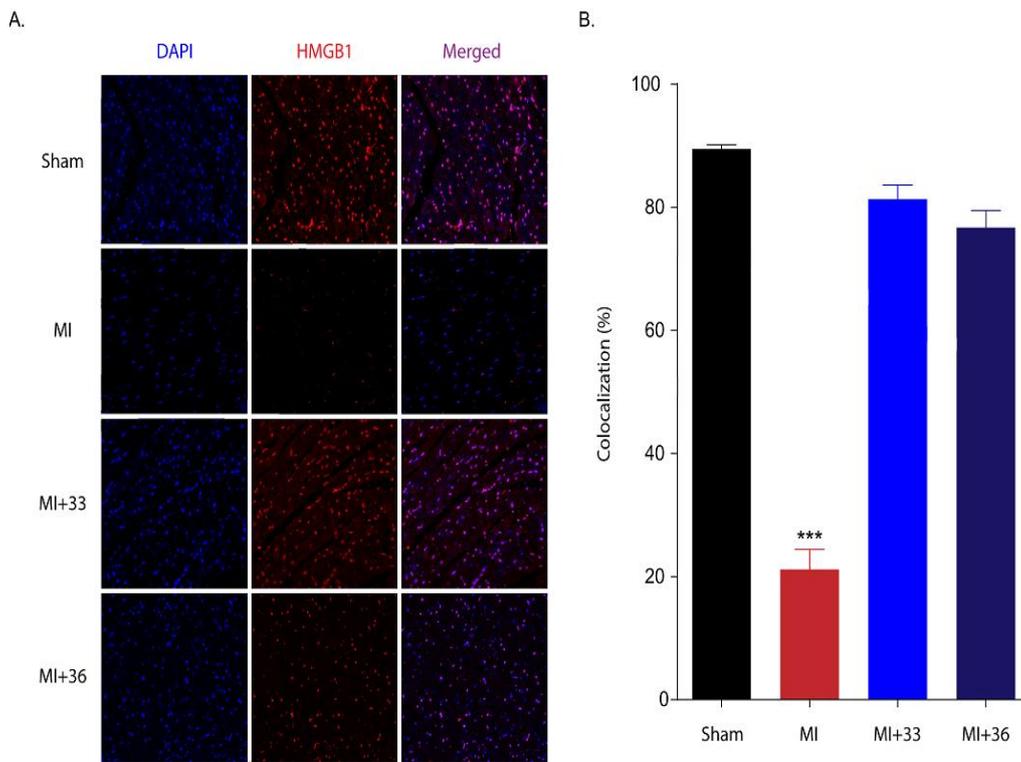


**Figure 1.** Targeted temperature management at 33°C and 36°C similarly reduces infarct volume in myocardial I/R injury. A. Experimental schedule; B. Traces of rat body temperature after LAD ligation; C. Representative image of 2,3,5-triphenyltetrazolium chloride (TTC) staining; D. Volume of myocardial infarction stained with TTC, \*\*\* $P < 0.001$ , comparison of myocardial I/R with normothermia and hypothermia (33°C and 36°C), one-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* test.

## 2. Different target temperatures of 33°C and 36°C TTM similarly suppress extracellular release of HMGB1 from peri-infarct tissue after myocardial I/R injury

When ischemic damage to the myocardium is induced by LAD ligation of the heart, HMGB1 is released from the nucleus of myocardial cells<sup>30,31</sup>.

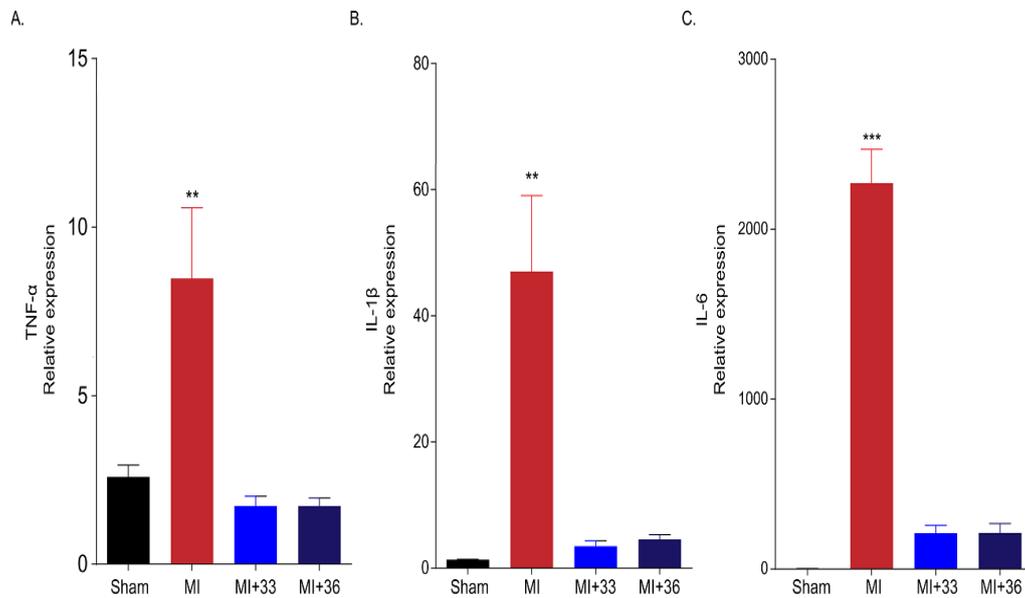
We found that the HMGB1 immunoreactivity was significantly decreased in the myocardium after ligation of LAD in rats. To investigate whether TTM at 33°C and 36°C significantly reduced the release of extracellular HMGB1 following I/R injury, we compared HMGB1 immunoreactivity between the normothermia and TTM groups after LAD ligation. We found that  $21.15 \pm 3.26\%$  of 4,6-diamidino-2-phenylindole (DAPI)-positive cells in the peri-ischemic myocardium of LAD ligation rats were HMGB1-positive. However, we also found that target temperatures of 33°C and 36°C similarly restored the number of HMGB1-positive cells in post-infarct tissues. The percentages of HMGB1-positive cells were  $81.28 \pm 2.33\%$  and  $76.68 \pm 2.80\%$  for TTM at 33°C and 36°C, respectively. While significant increases for the proportion of HMGB1-positive cells were observed for TTM at 33°C and 36°C compared to the normothermic group ( $P < 0.001$ ), there was no significant difference between the 33°C and 36°C groups ( $P = 0.999$ ). This suggests that both 33°C and 36°C TTM cause similarly significant reductions in the extracellular release of HMGB1 after ischemic myocardial damage (Fig 2).



**Figure 2.** Hypothermia suppresses extracellular release of HMGB1 after myocardial I/R injury. A. Representative immunohistochemistry results for 33°C and 36°C targeted temperature management after myocardial I/R injury; B. Immunohistochemistry results, \*\*\* $P < 0.001$ , comparing myocardial I/R with normothermia and hypothermia (33°C and 36°C), one-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* test.

### 3. TTM at 33°C and 36°C similarly inhibited inflammatory cytokine expression from peri-infarct regions

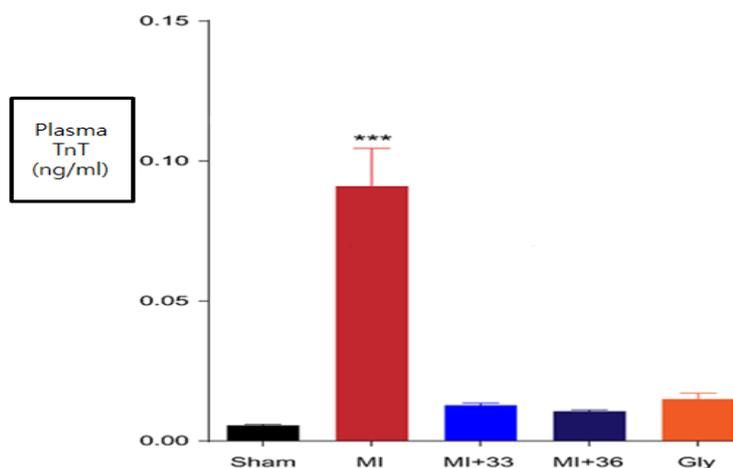
Cardiac mRNA expression of three major inflammatory cytokines (i.e., TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) was assessed by quantitative RT-PCR in the peri-infarcted myocardium 4 h after LAD ligation. In normothermic rats maintained at 37°C after myocardial I/R, the expression levels of TNF- $\alpha$  ( $8.48 \pm 2.09$ ,  $P = 0.001$ ), IL-1 $\beta$  ( $47.03 \pm 12.03$ ,  $P < 0.001$ ), and IL-6 ( $2270.84 \pm 199.60$ ,  $P < 0.001$ ) were significantly increased. Compared to the normothermic I/R group, TTM treatment at 33°C was closely associated with lower expression of inflammatory cytokines in the peri-infarcted myocardium (TNF- $\alpha$  ( $1.72 \pm 0.29$ ,  $P = 0.001$ ), IL-1 $\beta$  ( $3.46 \pm 0.86$ ,  $P < 0.001$ ), IL-6 ( $211.69 \pm 45.29$ ,  $P < 0.001$ ) (Fig. 3)). TTM at 36°C also decreased the expression of these cytokines in the peri-infarcted myocardium (TNF- $\alpha$  ( $1.72 \pm 0.24$ ,  $P = 0.001$ ), IL-1 $\beta$  ( $4.54 \pm 0.76$ ,  $P < 0.001$ ), IL-6 ( $212.22 \pm 54.73$ ,  $P < 0.001$ )). First, there were no significant differences in the mRNA expression of three inflammatory cytokines between the 33°C and 36°C TTM groups ( $P < 0.999$ ,  $P = 0.999$ ,  $P < 0.999$ , respectively). Thus, the application of TTM prevents the aggravation of damage by suppressing the production of inflammatory cytokines in the peri-infarct area after myocardial I/R injury. This indicates that the different target temperatures of 33°C and 36°C TTM similarly attenuate inflammatory cytokine expression after cardiac I/R injury.



**Figure 3.** Inflammatory cytokine expression in peri-infarct regions. A. Quantification of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression by RT-PCR,  $**P < 0.01$ , comparison of myocardial I/R with normothermia and hypothermia (33°C and 36°C) by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. B. Quantification of interleukin-1 $\beta$  (IL-1 $\beta$ ) expression by RT-PCR,  $**P < 0.01$ . Comparison of myocardial I/R with normothermia and hypothermia (33°C and 36°C) by ANOVA followed by Bonferroni *post hoc* test. C. Quantification of IL-6 expression by RT-PCR,  $***P < 0.01$ . Comparison of myocardial I/R with normothermia and hypothermia (33°C and 36°C) by ANOVA followed by Bonferroni *post hoc* test.

#### 4. Effects of 33°C and 36°C TTM on cTnT levels in the plasma

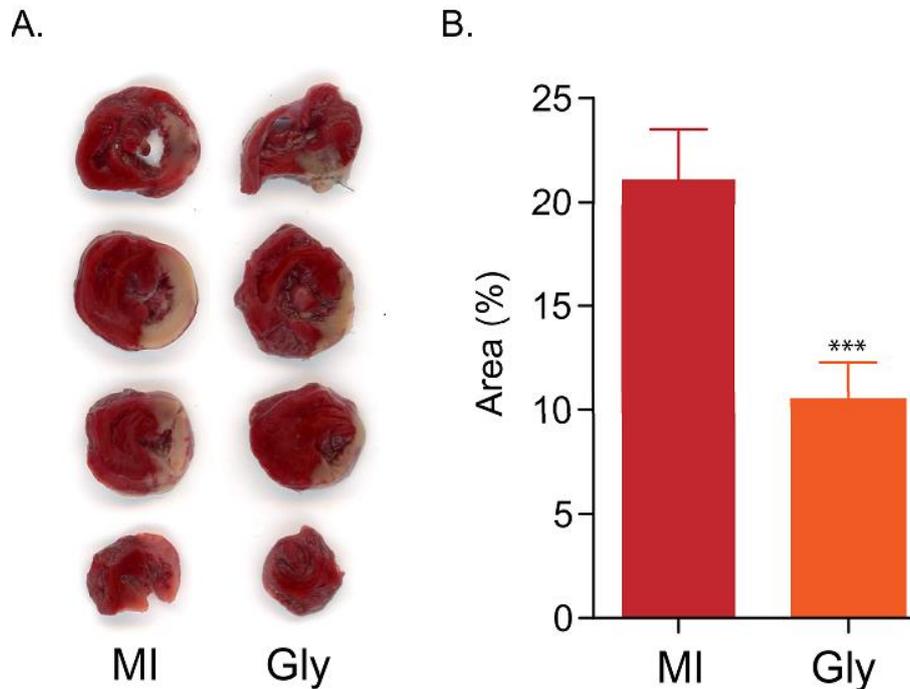
To examine the myocardial protective effects of 33°C and 36°C TTM on cTnT levels reflecting myocardial damage, we measured cTnT levels in the plasma. The levels of cTnT were higher in the normothermia group after LAD ligation compared to those in the sham-operated group ( $0.091 \pm 0.013$  and  $0.0056 \pm 0.001$  ng/mL, respectively;  $P < 0.001$ ). However, rats subjected to either 33°C or 36°C TTM showed lower cTnT levels than those in the normothermia group ( $0.013 \pm 0.001$  in 33°C and  $0.011 \pm 0.001$  ng/mL in 36°C TTM group). There was no significant difference in plasma cTnT at 33°C and 36°C TTM ( $P = 0.999$ ), indicating that both target core temperatures for TTM equivalently reduced myocardial damage (Fig 4).



**Figure 4.** Levels of cardiac troponin T (cTnT) in plasma, \*\*\* $P < 0.001$ , comparison of myocardial I/R with normothermia and hypothermia (33°C and 36°C) and glycyrrhizin pre-treatment by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test.

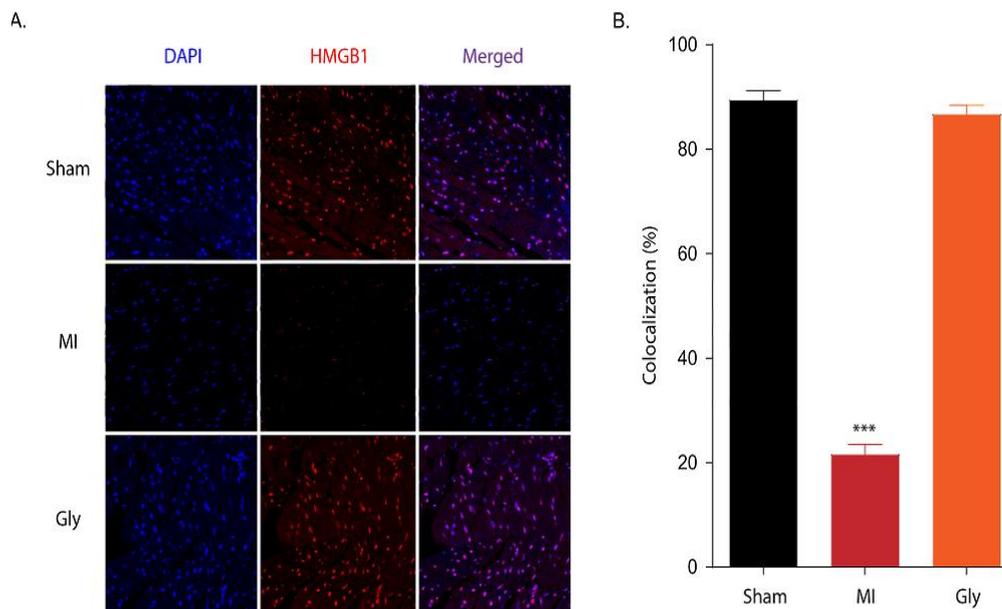
5. Glycyrrhizin alleviates myocardial damage by suppressing the extracellular release of HMGB1 in myocardial I/R injury

Glycyrrhizin is a pharmacological HMGB1 inhibitor that binds directly to HMGB1 and prevents the extracellular release of HMGB1 to block its cytokine function<sup>32-34</sup>. We compared the effects of glycyrrhizin treatment on infarct volume, extracellular release of HMGB1, expression of inflammatory cytokines, and plasma level of cTnT in our animal model (Fig 4). In the myocardial I/R group treated with an intra-peritoneal injection of glycyrrhizin, the infarct volume was significantly decreased ( $10.48 \pm 1.79\%$ ) compared to that in the normothermic myocardial I/R group ( $21.10 \pm 2.40\%$ ,  $P = 0.008$ ) (Fig 5).



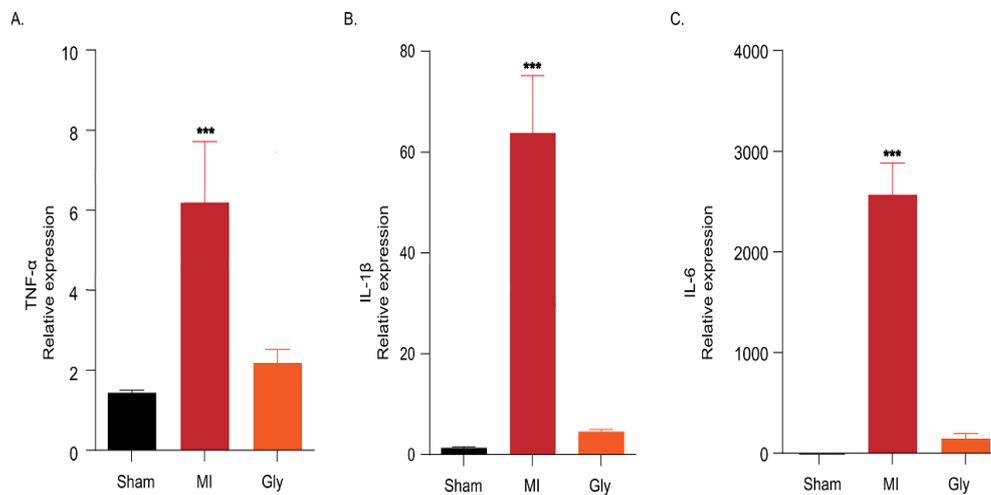
**Figure 5.** Glycyrrhizin reduces infarct volume in myocardial I/R injury. A. Representative image for 2,3,5-triphenyltetrazolium chloride (TTC) staining comparing myocardial I/R injury with normothermia and glycyrrhizin pre-treatment; B. Quantification of TTC staining results in A, \*\*\* $P < 0.001$  comparing myocardial I/R injury with and without glycyrrhizin, unpaired  $t$ -test.

Glycyrrhizin also significantly increased the proportion of HMGB1-positive cells in the I/R injured myocardium ( $17.18 \pm 2.86\%$  in normothermic rats after myocardial I/R versus  $89.34 \pm 1.81\%$  in glycyrrhizin-treated myocardial I/R rats,  $P < 0.001$ ) (Fig 6).



**Figure 6.** Glycyrrhizin suppresses extracellular release of HMGB1 in myocardial I/R injury. A. Representative images showing HMGB1 immunoreactivity from myocardial I/R injury with and without glycyrrhizin treatment; B. Quantification of immunohistochemistry results in A, \*\*\* $P < 0.001$ , comparing myocardial I/R injury with and without glycyrrhizin by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test.

In glycyrrhizin-treated AMI rats, TNF- $\alpha$  ( $2.18 \pm 0.34$ ,  $P < 0.001$ ), IL-1 $\beta$  ( $4.59 \pm 0.42$ ,  $P < 0.001$ ), and IL-6 ( $145.78 \pm 47.97$ ,  $P < 0.001$ ) levels were decreased compared to those in the myocardial I/R group (TNF- $\alpha$ ;  $6.18 \pm 1.52$ , IL-1 $\beta$ ;  $63.78 \pm 11.32$ , and IL-6;  $2565.74 \pm 316.32$ , respectively) (Fig 7).



**Figure 7.** Inflammatory cytokine expression in myocardial I/R injury with and without glycyrrhizin treatment. A. Quantification of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression by RT-PCR comparing myocardial I/R injury with and without glycyrrhizin treatment, \*\*\* $P < 0.001$ , comparing myocardial I/R injury with and without glycyrrhizin by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test; B. Quantification of interleukin-1 $\beta$  (IL-1 $\beta$ ) expression by RT-PCR comparing myocardial I/R injury with and without glycyrrhizin, \*\*\* $P < 0.001$ , comparing myocardial I/R injury with and without glycyrrhizin treatment by one-way ANOVA followed by Bonferroni *post hoc* test; C. Quantification of interleukin-6 (IL-6) expression by RT-PCR comparing myocardial I/R injury with and without glycyrrhizin treatment, \*\*\* $P < 0.001$ , comparing myocardial I/R injury with and without glycyrrhizin treatment by one-way ANOVA followed by Bonferroni *post hoc* test.

Additionally, cTnT levels were significantly lower in the glycyrrhizin-treated myocardial I/R group ( $0.014 \pm 0.002$  ng/mL) than in the normothermic group after myocardial I/R ( $0.091 \pm 0.013$  ng/mL,  $P < 0.001$ ) (Fig 4).

#### IV. Discussion

Our results suggest that TTM at both 33°C and 36°C reduces myocardial injury following acute myocardial I/R injury by suppressing the extracellular release of HMGB1. We found that TTM attenuated the increase in infarct size, extracellular release of HMGB1, and pro-inflammatory effect against the propagation of injury in rats with AMI. We also showed that TTM at both 33°C and 36°C significantly attenuated the elevation of cardiac troponin, which is a sensitive and specific marker of heart muscle damage, after myocardial I/R injury. TTM at 36°C showed similar myocardial protective effects against myocardial I/R injury as TTM at 33°C in our rat AMI model.

The inflammatory cascade in myocardial injury and infarction is significantly associated with debris removal and scar formation. Despite the fundamental role of inflammation in wound healing after AMI, an overwhelming inflammatory response can lead to devastating effects on cardiomyocytes. The onset of cell death begins within 30 min to 1 h after the cessation of blood flow through a combination of necrosis and apoptosis. Inflammation, which contributes to lethal myocardial injury, is initiated during ischemia and is sustained over several hours after reperfusion<sup>35</sup>. Inhibiting the inflammatory process can provide a potential therapeutic window for cardioprotection<sup>35</sup>. HMGB1 is known to subsequently act as a pro-inflammatory cytokine that activates inflammatory cells by its extracellular release from necrotic cells in the pathophysiology of various diseases<sup>21,36,37</sup>. The serum concentration of HMGB1 is

significantly associated with infarct size and residual left ventricular function in patients with STEMI<sup>38</sup>. Furthermore, extracellular HMGB1 mediates inflammation and enhances the regeneration of damaged tissues. Takahashi et al. reported that HMGB1 has beneficial effects at low concentrations and deleterious effects at high concentrations<sup>36</sup>. HMGB1 levels are significantly increased within 1 h and are maintained for up to 24 h during I/R injury<sup>39</sup>. Therefore, it is important to prevent the action of HMGB1 to alleviate ischemic injury of the myocardium. The interaction between extracellular HMGB1 and Toll-like receptor 4 enhances the inflammatory response to myocardial damage after I/R by activating the release of pro-inflammatory cytokines, such as TNF- $\alpha$  from macrophage/monocytes<sup>21</sup>. TNF- $\alpha$  influences the production of other pro-inflammatory cytokines, such as IL-1  $\beta$  and IL-6, resulting in a negative cycle of pro-inflammatory cytokine production and aggravation of injury after myocardial infarction<sup>40,41</sup>.

Our previous study demonstrated that therapeutic application of TTM helps alleviate peri-infarct damage from the propagation of ischemic injury in an ischemic stroke model by reducing inflammatory cytokines through the blockage of HMGB1 release<sup>24,26</sup>. This is the first study to show a direct mechanistic and functional link between HMGB1 and TTM in a clinically relevant AMI animal model. Interestingly, myocardial I/R injured rats treated with glycyrrhizin showed equivalent myocardial protection as myocardial I/R rats that underwent TTM. This suggests that the extracellular release of HMGB1 is critical for the propagation of I/R injury by increasing the expression of proinflammatory cytokines in the peri-infarct myocardium

and that TTM helps attenuate this propagation of I/R injury by inhibiting HMGB1 after I/R injury. In addition, our previous study demonstrated that serum HMGB1 level was lower in the TTM group than the group without TTM, demonstrating the correlation between TTM and HMGB1 <sup>24,26</sup>. However, there is a lack of data regarding the link between HMGB1 and I/R injury and direct mechanisms in our study. In the case of the AMI animal model, we could not confirm the relationship between HMGB1 and I/R injury because HMGB1 neutralizing antibodies could not be injected locally. Instead, we indirectly confirmed the association of HMGB1 with I/R injury through our previously used middle cerebral artery occlusion model <sup>24,26</sup>. Therefore, further research is needed to directly confirm the association between HMGB1 and I/R injury in the AMI model.

Induced hypothermia may increase the rates of lethal arrhythmia, hypotension, shivering, infection, impaired coagulopathy, and rewarming injury and significantly alter the pharmacokinetics <sup>42</sup>. Its intrinsic adverse effects can significantly diminish the hypothermic benefits throughout the body <sup>42</sup>. TTM at 36 °C may be preferred to TTM at 33 °C in patients with cardiac arrest for several reasons <sup>26</sup>. In clinical practice, TTM consists of three phases: induction, maintenance, and rewarming <sup>43</sup>. TTM should be initiated as soon as possible according to international guidelines <sup>42</sup>. Moreover, rapidly induced hypothermia is important for modulating the efficacy of hypothermia in the clinical setting because minimizing the door to balloon time and reaching the target temperature within that at pre-reperfusion are critical for salvaging cardiac muscles <sup>44</sup>.

As TTM at 36°C is close to the lower margin of the normal body temperature, it has the advantage of quickly achieving the target core temperature<sup>42</sup>. Clinical management to control shivering and minimize the risk of the devastating complications of TTM should be considered to maximize the benefits<sup>43</sup>. Shivering, as a major adverse effect of TTM, leads to extremely uncomfortable and massive increases in the metabolic demand and systemic energy consumption<sup>43</sup>. TTM at 36°C may be preferred to minimize the risk of shivering in the induction and rewarming phases because peripheral vasoconstriction and shivering are triggered at 36.5°C and 35.5°C in healthy humans<sup>43</sup>. In the rewarming phase, a small temperature change between the maintenance and rewarming periods can be beneficial for reducing the increased risk of secondary damage due to the adverse consequences of rewarming on the whole body. TTM at 33°C is generally recommended as the safer margin for the target core temperature in critically ill patients because temperatures below 32°C can induce serious cardiac arrhythmia. Application of TTM at 36°C to AMI patients also helps ameliorate the risk of several adverse effects. Unlike patients who are resuscitated after cardiac arrest, most patients with AMI remain awake and breathe spontaneously during acute management<sup>43</sup>. Simple and well-tolerated TTM at 36°C is more feasible during the acute period of AMI. In this experiment, we did not study the rewarming phase when applying 33°C and 36°C TTM. Rewarming treatment of the 33°C TTM group is likely to be more damaging than 36°C TTM group. However, due to the technical problems of our study, it was difficult to implement rewarming experiments. In future studies, additional rewarming treatments will be necessary because they can provide

more meaningful interpretations compared to actual clinical practice. TTM has been shown to be safe and feasible in clinical practice, and there were no differences in mortality or neurological outcomes between patients who underwent TTM at 33 °C and those who underwent TTM 36 °C after out-of-hospital cardiac arrest in recent multi-centre clinical trials <sup>45</sup>. Recently, the target temperature of TTM tends to change accordingly from 33–34 °C to 36 °C during post-resuscitation care <sup>45</sup>. However, previous animal studies demonstrated that rapid application of therapeutic hypothermia at 32–34 °C prior to reperfusion significantly reduced the myocardial infarct size <sup>25,46,47</sup>. Although the infarct size in TTM at 35 °C is decreased, Dash et al. demonstrated that TTM at 32 °C is superior to TTM at 35 °C and normothermic porcine after AMI <sup>44</sup>. Several clinical trials of TTM after AMI have shown inconsistent results with the major findings of many experimental studies. This may be because of interspecies variability among animal models and differences in the immune response to I/R injury. Additionally, clinical outcomes may also be affected by disease- or organ-specific characteristics and molecular biological differences among organs <sup>16,17</sup>. To reduce the gap between the TTM beneficial effects for cardiac arrest and AMI, we investigated whether TTM at 36 °C has a potent myocardial protective effect. Our study showed that target temperatures of 33 °C or 36 °C in TTM similarly inhibited HMGB1 release and induced equivalent myocardial protection in terms of the infarct size in myocardial I/R injury in a rat model. This is the first study to suggest that a target core temperature of 36 °C is applicable for cardioprotection in myocardial I/R injury. However, we

compared the effects of two different temperatures in rats intubated with ventilation support, adequate sedation, and strict shivering control by vecuronium, mimicking the features of cardiac arrest. Therefore, further studies are needed to clarify the cardioprotective effects of both 33 °C and 36 °C TTM and the critical roles of HMGB1 in patients with AMI who are awake and exhibit spontaneous breathing. Finally, the importance of this study is that whilst there are many described pathways known to protect against I/R injury due to TTM, here we identified a novel pathway that inhibits the release of HMGB1.

## V. Conclusion

In summary, we describe a new mechanistic and clinical link showing that TTM at 36 °C is a therapeutic candidate that should be investigated in future clinical trials by reducing the propagation of myocardial damage to effectively inhibit the extracellular HMGB1 release after myocardial I/R injury.

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**Abstract (in Korean)**

심근 허혈 / 재관류 손상에서  
HMGB1 방출 억제를 통한 목표 체온 유지 치료

< 지도교수 정현수 >

연세대학교 대학원 의학과

범 진 호

급성 심근경색은 동맥의 지속적인 폐색으로 인한 시간 의존적인 허혈을 통해 심근의 괴사를 일으키는 치명적인 질환이다. 그러나 현재 심근 허혈 / 재관류 손상을 예방할 수 있는 효과적인 치료법이나 잠재적인 치료 목표가 없는 실정이다. 우리는 목표 체온 유지 치료를 통해 HMGB1의 세포외 방출을 억제하는 기전을 확인하고자 하였고, 33도와 36도에서의 목표 체온 유지 치료가 서로 유사한 심근 보호 효과가 있는지 알아보려고 하였다.

본 연구는 좌 전 하행 동맥을 결찰 유지 후 결찰을 제거함으로써 심근의 허혈 및 재관류손상을 유발하였고, 대조군, 37도 정상 체온 유지군, 33도와 36도 목표 체온 유지치료군, 글리세리진 전 처치군으로 나누어 실험을 진행하였다. 실험 결과 33도와 36도 목표 체온 유지 치료군 두 군 모두 비슷하게 심근경색의 범위 감소, 심근경색 주변부에서 HMGB1 의 세포 외 방출 억제, 염증성 사이토카인의 발현이 억제되었다. 또한 혈장 심장 트로포닌 T 에서도 두 군에서 유사하게 감소된 결과를 확인 할 수 있었다, 글리세리진 전 처치 군에서도 33도 및 36도 목표 체온 유지 치료군과 비슷한 결과를 보여주었다.

이를 통해 목표 체온 유지 치료는 심근의 허혈 / 재관류 손상에서 HMGB1 의 세포 외 방출을 억제함을 알 수 있었고, 33도와 36도 목표체온 유지 치료 모두 동등한 심근 보호 효과가 있음을 확인 할 수 있었다.

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**핵심되는 말** : 목표 체온 유지 치료, HMGB1, 심근 허혈 / 재관류 손상