Recombinant klotho protein ameliorates myocardial ischemia/reperfusion injury by attenuating sterile inflammation

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Directed by Professor Incheol Park, M.D., Ph.D.

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Jinwoo Myung

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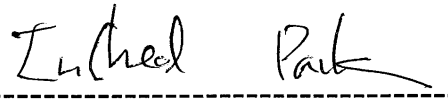
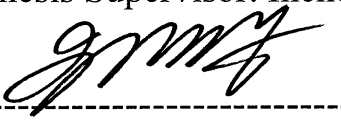
This certifies that the Doctoral Dissertation
of Jinwoo Myung is approved.



Thesis Supervisor: Incheol Park



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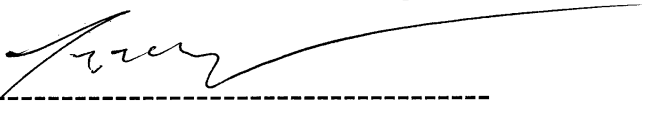

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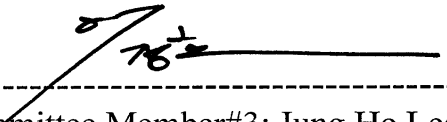

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First of all, praise the Lord for guiding me through this journey. Yes, there is no end to learning, but I am so thrilled and proud that I have completed my Ph.D. I am especially more elated because I have completed this program while working.

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My beloved family has been my greatest source of strength. I dedicate this dissertation to my dear family.

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My father- and mother-in-law, thank you for your unwavering support. Thanks to you, Younjin and I continue to grow personally and as a couple.

My dear maternal grandmother, your unconditional love raised me. I will always remember your love and kindness and live my life to the fullest as you have always wished. I also thank my adored little sister. I wish you a bright future.

Finally, my beloved father and mother, I thank you for raising me to be who I am today. I will be forever indebted to you, and I hope the fruits of my endeavors serve as great joy for you. Please stay healthy; I love you.

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Jinwoo Myung

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ABSTRACT

Recombinant klotho protein ameliorates myocardial ischemia/reperfusion injury by attenuating sterile inflammation

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(Directed by Professor Incheol Park, M.D., Ph.D)

Aims: Currently, no effective therapy and potential target have been elucidated for preventing myocardial ischemia and reperfusion injury (I/R). We hypothesized that the administration of recombinant klotho (rKL) protein could attenuate the sterile inflammation in peri-infarct regions by inhibiting the extracellular release of high mobility group box-1 (HMGB1).

Methods and Results: This hypothesis was examined using a rat coronary artery ligation model. Rats were divided into sham, sham+ rKL, I/R, and I/R+ rKL groups (n = 5/group). Furthermore, to verify the cardioprotective effect of rKL by specifically inhibiting HMGB1, rats were assigned to the sham, left anterior descending coronary artery (LAD) I/R, and LAD I/R after pre-treatment with glycyrrhizin groups (n = 5 per group). Administration of rKL protein reduced infarct volume and attenuated extracellular release of HMGB1 from peri-infarct tissue after myocardial I/R injury. The administration of rKL protein inhibited the expression of pro-inflammatory cytokines in the peri-infarct regions and significantly attenuated apoptosis and production of

intracellular reactive oxygen species by myocardial I/R injury. Klotho treatment significantly reduced the increase in the levels of circulating HMGB1 in blood at 4 h after myocardial ischemia. rKL regulated the levels of inflammation-related proteins.

Conclusions: This is the first study to suggest that exogenous administration of rKL exerts myocardial protection effects after I/R injury and provides new mechanistic insights into rKL that can provide the theoretical basis for clinical application of new adjunctive modality for critical care of acute myocardial infarction.

Key words: acute myocardial infarction, klotho, myocardial ischemia/reperfusion injury, high mobility group box-1, sterile inflammation

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I. INTRODUCTION

Acute myocardial infarction (AMI) is a common cardiac emergency associated with high rates of morbidity and mortality worldwide [1]. AMI is characterized by myocardial cell death via time-dependent ischemia due to prolonged occlusion of the infarct-related artery [2,3]. To minimize necrosis of the myocardium mediated by the infarct-related artery (IRA), the area at risk (AAR) and blood flow to the IRA should be restored as soon as possible by mechanical reperfusion using a coronary artery stent and thrombolytic therapy [2-4]. For safe and effective treatment, current guidelines recommend primary PCI as the preferred reperfusion strategy that should be performed for patients with ST elevation myocardial infarction (STEMI) within 12 h of symptom onset, which is considered as the therapeutic window [3,5]. The door to balloon time has been remarkably improved with advances in medical science. Nevertheless, in-hospital mortality has not been reduced significantly in patients with STEMI undergoing primary percutaneous coronary intervention (PCI) [1,6]. New treatments are needed to reduce myocardial infarct size and to preserve left

ventricular function. Studies examining treatment for myocardial reperfusion injury have not focused on finding a subsidiary therapeutic target. Myocardial reperfusion injury refers to cardiomyocyte death that paradoxically results from reperfusion of ischemic myocardium [7]. Currently, no effective therapy is available, and a potential target for preventing myocardial ischemia and reperfusion injury in patients with STEMI has not been identified [8,9].

Sterile inflammation has recently emerged as an important aggravator in the early stage of ischemic/reperfusion (I/R) injury. Therefore, new adjunctive therapies are required against AMI to widen the therapeutic window and ameliorate early myocardial damage induced by I/R injury. Myocardial I/R injury is very complex, and the pathophysiology is also poorly understood. In myocardial damage induced by I/R injury, induction of inflammatory response and apoptotic cell death are known to play critical roles in the propagation of ischemic injury [10-13]. High mobility group box-1 (HMGB1) serves as the structural organizer of DNA in eukaryotic cells. Upon I/R injury, this protein is rapidly released, and its levels are increased after 30 min of ischemia [14]. HMGB1, which is one of the primary mediators of the innate immune response, is passively released by necrotic cells or actively released during sterile injury [15]. Binding of extracellular HMGB1 to Toll-like receptor 4 (TLR-4) activates several inflammatory mediators that amplify and expand the extent of damage after I/R and induce cardiomyocyte apoptosis after I/R [15]. Administration of glycyrrhizin, a known pharmacological inhibitor, which binds directly to HMGB1 and blocks extracellular release of HMGB1, significantly decreases the infarct size and the levels of serum HMGB1 and inflammatory cytokines

including interleukin (IL)-1β, IL6, and tumor necrosis factor (TNF)-α [10,16]. Clinically, increased levels of HMGB1 in plasma were significantly associated with a higher risk of mortality in patients with STEMI receiving PCI [17]. This insight into the pathophysiological features of acute STEMI expands the therapeutic scope of this disease beyond the traditional strategies that focus on reducing stenosis.

Klotho, which is a membrane-bound, soluble, and anti-aging protein, demonstrates protective activities in multiple organs [18]. In klotho-deficient mice, inherited phenotypes resemble aging in humans, whereas the lifespan is extended in animals with overexpression of klotho [19,20]. Klotho is a key factor in fibrous growth factors (FGF) signaling and is associated with the modulation of mineral metabolism and energy [21,22]. Exogenous kl gene insertion into cells leads to significant attenuation of apoptosis [23]. Moreover, this protein acts as a hormone exerting anti-inflammation and antioxidative effects, thereby regulating inflammation, oxidative stress, and fibrosis via attenuation of transforming growth factor-β1 (TGF-β1) and insulin/insulin-like growth factor-1 (IGF-1) signaling pathways [22,24,25]. In muscle progenitor cells, decreased expression of α-Klotho is associated with increased cytoplasmic expression of HMGB1 [26]. It is critical to understand the direct functional and mechanistic links between Klotho and HMGB1 in association with sterile inflammation in a clinically relevant model of AMI.

Several recent studies have demonstrated that klotho deficiency is related to the development of cardiomyopathy and cardiovascular diseases, and a higher concentration of plasma klotho was an independent predictor of a lower

likelihood of cardiovascular disease [27-29]. Until now, although some scientists have focused on the role of klotho in the development of cardiovascular diseases, their studies were limited to the pathophysiology of chronic cardiovascular diseases. No effective study has been published examining the therapeutic effectiveness of direct administration of recombinant klotho (rKL) in early myocardial damages after I/R injury. We hypothesized that the administration of rKL protein could attenuate the sterile inflammation in peri-infarct regions by inhibiting the extracellular release of HMGB1. We aimed to examine this hypothesis using the left anterior descending coronary artery (LAD) ligation rat model. We aimed to determine the subsequent reduction in the myocardial infarct area after I/R injury. We aimed to investigate whether the administration of rKL protein exerts a myocardial protective effect after I/R injury with the same underlying mechanism.

II. MATERIALS AND METHODS

1. Preparation of experimental animals

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 (2018-0262) and the National Institutes of Health. A total of 50 healthy, age-matched, adult male Wistar rats (Orientbio, Seongnam, Republic of Korea) weighing 400–430 g were used in this study. All the animals were acclimatized in plastic cages under sterile conditions, with a 12/12-h light/dark cycle, 50 ± 10% humidity, and a temperature of 22 ± 2 °C for 1 week. During this period, the rats were fed a standard diet, with fresh water supplied ad libitum [10].

2. Experimental rat model of myocardial I/R injury

Rats were anesthetized with 5% isoflurane administered in a mixture of nitrous oxide (0.7 L/min) and oxygen (0.3 L/min). The anesthetic conditions were maintained using 2% isoflurane in the same gas mixture during the surgery. After a midline neck incision, tracheostomy was conducted using an intravenous catheter (4712-020-116. I.V Catheter 16G, Sewoon Medical Co., Cheon-An, Republic of 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) during the surgery. The heart was exposed by left vertical thoracotomy and pericardiectomy. The ligation of the LAD coronary artery was performed as described in the previous study [30]. In summary, the

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 as cyanosis and dyskinesia of the myocardium induced by LAD was developed after ligation. Reperfusion was induced by removing the PE-10 tube after 30 min of LAD ligation, and the skin was closed with 4-0 nylon sutures after reperfusion. The same surgical procedures, except ligation, were performed in sham animals [30]. After 4 or 24 h of LAD ligation, anesthesia was administered via 5% isoflurane in a mixture of 0.7 L / min nitrous oxide and 0.3 L / min oxygen by inhalation, and the animals were euthanized [10].

3. Experiment protocol

To assess the optimal dose of rKL for myocardial protection, first, the rats were randomly divided into four experimental groups; sham, LAD I/R+ 0.025 μg of rKL /g of body mass of, LAD I/R+ 0.05 μg of rKL /g of body mass, LAD I/R+ 0.075 μg of rKL/g of body mass. We determined 0.05 μg/g as the optimal dose of rKL for myocardial protection. We assessed the optimal dose of rKL for myocardial protection. To identify the protective effects of klotho administration against myocardium after myocardial I/R injury, the rats were randomly divided into four experimental groups: sham, sham + klotho, LAD I/R, and LAD I/R + klotho. A total of 0.05 μg/g of rKL in 0.5ml of saline was also administered via intraperitoneal injection 15 min after LAD ligation. The same volume of saline was administered via intraperitoneal injection to control

groups. Reperfusion was induced after 15 min of rKL administration. The core temperature was monitored in the rectum of rats and maintained at 37.0 ± 0.5°C during all experiments using a feedback-controlled heating pad (HB 101, Harvard Apparatus, Holliston, MA, USA). It has been suggested that as a pharmacological inhibitor of HMGB1, glycyrrhizin can prevent the extracellular HMGB1 release from damaged cells by directly binding to HMGB1 [30-32]. To verify the cardioprotective effect of klotho administration by specifically inhibiting HMGB1 in our animal model and also mechanistically identify the link between klotho administration and extracellular HMGB1-release inhibition, the rats were randomly assigned to three different experimental groups—the sham, LAD I/R, and LAD I/R after pre-treatment with glycyrrhizin groups (n = 5 per group). The rats were administered 100 mg/kg glycyrrhizin intraperitoneally 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 at 4 h or 24 h after LAD I/R. The chest of anesthetized rats was re-opened at 4 h or 24 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°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, the heart tissue between 0 and 8 mm from the apex of the heart was used. We measured the infarct area in the anterior and posterior sides of each 2 mm thick slice using ImageJ 1.48v software. To determine the infarct volume in each slice, the average value of the infarct area on the anterior and posterior sides was multiplied by the thickness (2 mm) [thickness × (top area + bottom area)/2]. In addition, the total infarct volume was calculated as the sum of the infarct volume per slice [10].

5. Immunohistochemistry analysis

For immunohistochemistry analysis, 2,3,5-TTC staining was performed to confirm the peri-infarct area in the left ventricle [31]. Next, 2-mm-thick slices were selected between the section that was 4 and 6 mm from the apex of the rat heart, fixed with a 4% paraformaldehyde solution and embedded in paraffin. The section that was between 4 and 6 mm from the apex of the rat heart was selected because the peri-infarcted region was easily observed given that it was properly mixed with normal and infarct tissue after TTC staining. Using a microtome (LEICA RM 2335, Wetzlar, Germany), the heart sections were cut to obtain segments with 4 μm 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% H2O2, 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℃ (1:100,

ab18256; Abcam, Cambridge, UK). The sections were washed thrice 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 thrice with TBS and mounted with ProLongTMDiamond 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) [10].

6. Detection of intracellular levels of reactive oxygen species (ROS)

We used cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) stain to detect intracellular ROS levels (Thermofisher, MA, USA). We deparaffinized the sectioned slide and added 10 μM of H2DCFDA on the slide and incubated it for 15 minutes at 37°C in the dark. After H2DCFDA staining, we also observed ROS generation in the cells using a confocal microscope (LSM 700; Carl Zeiss GmbH, Jena, Germany). In the presence of ROS, H2DCFDA is oxidized to 2',7'-dichlorofluorescein (DCF) detected by green fluorescence that cannot penetrate the cell membrane, and its fluorescence intensity is proportional to the level of intracellular ROS. We measured fluorescence intensity using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices, CA, USA).

7. Enzyme-linked immunosorbent assay (ELISA) for detection of cardiac troponin T (cTnT) and HMGB1

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. A total of 1 ml 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 a cTnT ELISA kit (MBS2024997, MyBioSource, San Diego, CA, USA), and HMGB1 concentrations were determined using the Rat HMGB1 ELISA kit (Solarbio, Beijing, China).

8. 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 [32]. 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, tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, and IL-6. Single-stranded cDNA was synthesized from 500 ng of total RNA using the PrimeScript 1st strand cDNA Synthesis Kit (6110A, Takara Bio, Shiga, Japan) (Supplement 1). 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) [10].

9. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

Apoptotic cells were detected by TUNEL assay using the DeadEndTM Fluorometric TUNEL system (Promega, WI, USA) according to the manufacturer's instructions. A confocal microscope (LSM700, Carl Zeiss GmbG, Jena, Germany) was used to identify the stained sections. One slide from each animal was selected and stained. The two peri-ischemic areas of the stained sections were observed with a confocal microscope (LSM 700; Carl Zeiss GmbH, Jena, Germany). The average values of TUNEL-positive cells in the peri-infarct area were derived from two areas on the stained sections. Numbers of TUNEL-positive cells in the infarct area were normalized using values associated with the hearts of sham animals [10].

10. Cytokine array

The peri-infarct tissue of the rat heart was homogenized with a homogenizer (Bertin technologies Montigny) in PBS with protease inhibitor. Protein quantitation was performed with a BCA kit according to the manufacturer's protocol. Proteome Profiler array was performed using the Mouse Cytokine Array Panel A (R&D system) according to the manufacturer's instructions. Blots were visualized using the ECLTM Western Blotting Analysis System (GE Healthcare) and imaged using LAS 4000 mini (Fujifilm). The blots were quantized by HLImage++ (Western Vision software) for array analysis [10].

11. Statistical analysis

All experimental results are expressed as the mean ± 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 significant.

III. RESULTS

1. Administration of rKL protein reduces infarct volume in myocardial I/R injury

In the present study, we assessed infarct volumes by TTC staining after 4 h or 24 h of ischemic reperfusion injury (Figure. 1A-E). In the myocardial I/R group without rKL treatment, the mean ratios of the infarct area at 4 h and 24 h after myocardial I/R were 15.72 ± 0.82 % and 16.38 ± 3.46 % compared to those of the total area between 0 and 8 mm from the apex, whereas the mean ratio of the infarcted area in klotho-treated group after myocardial I/R was 5.18 ± 0.70 % and 5.22 ± 0.77 % after 4 h and 24 h. There was a significant difference between the myocardial I/R group and the klotho-treated group at both 4 h and 24 after myocardial I/R ($P< 0.0001$ and $P= 0.014$).

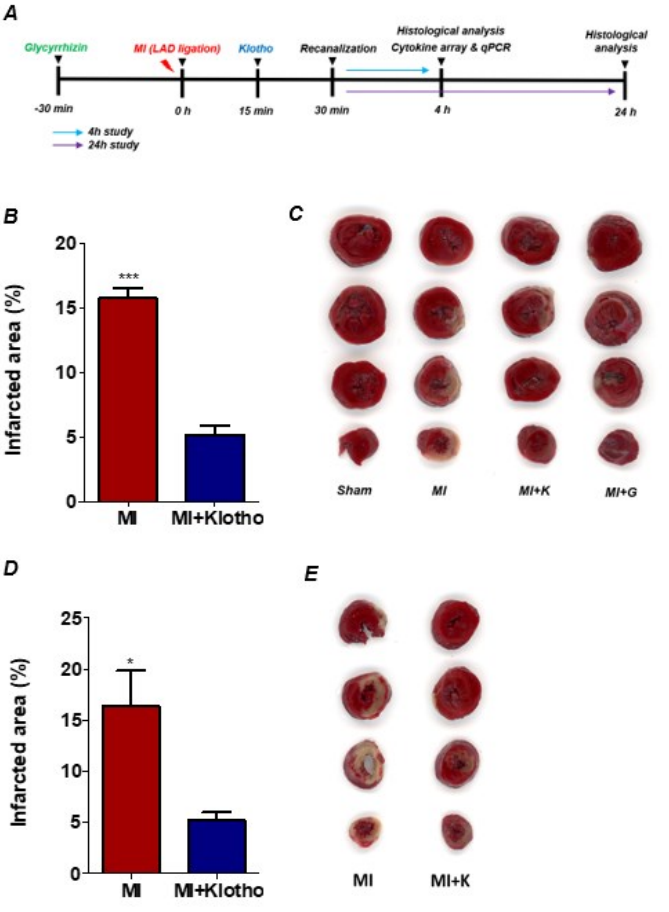

Figure 1. Recombinant klotho (rKL) reduces infarct volume in myocardial I/R injury. A. Experimental schedule; B. Volume of myocardial infarct area stained with of 2,3,5-triphenyltetrazolium chloride (TTC) at 4 h, ***P < 0.001, comparison of myocardial I/R with rKL, one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test. C. Representative image of 2,3,5-triphenyltetrazolium chloride (TTC) staining at 4 h; D. Volume of myocardial infarct area stained with TTC observed at 24 h, *P < 0.05, comparison of myocardial I/R with rKL, unpaired t-test. E. Representative image of 2,3,5-triphenyltetrazolium chloride (TTC) staining at 24 h (the number of animals: n = 5, respectively).

2. Administration of rKL protein reduces intracellular levels of reactive oxygen species (ROS)

We assessed intracellular ROS levels using H2DCFDA staining after 4 h. After myocardial I/R, intracellular ROS levels, which were represented by fluorescence intensities, were significantly reduced in the klotho-treated myocardial I/R injured group (8.98 ± 3.42) compared to the myocardial I/R injured group (40.70 ± 4.29) ($P < 0.001$) (Figure. 2A and B).

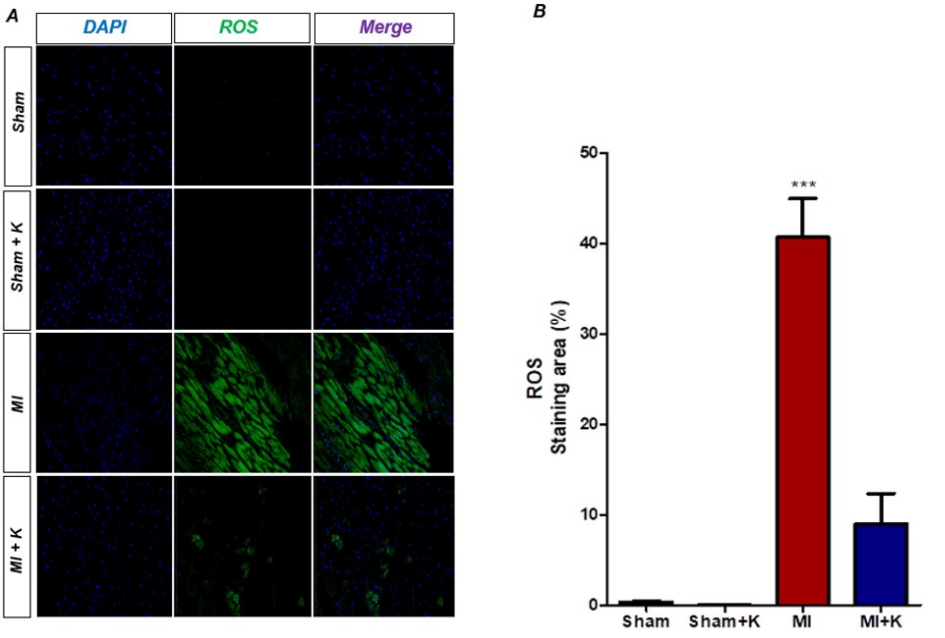

Figure 2. Intracellular reactive oxygen species (ROS) level. A. Representative intracellular reactive oxygen species (ROS) results observed following administration of klotho after myocardial ischemic/reperfusion (I/R) injury; B. ROS staining area, ***$P < 0.001$, comparison of myocardial I/R with and without administration of recombinant klotho (rKL) by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (number of animals: n = 5).

3. Administration of rKL protein attenuates the extracellular release of HMGB1 from peri-infarct tissue after myocardial I/R injury

When ischemic damage to the myocardium is induced by LAD ligation in the heart, HMGB1 is released from the nucleus of myocardial cells [33,34]. We found that the HMGB1 immunoreactivity was significantly decreased in the myocardium after ligation of LAD in rats. To investigate whether rKL protein significantly reduced the release of extracellular HMGB1 following I/R injury, we compared HMGB1 immunoreactivity between the myocardial I/R injury and Klotho-treated groups after myocardial I/R injury. We found that 18.25 ± 7.52% of 4,6-diamidino-2-phenylindole (DAPI)-positive cells in the peri-ischemic myocardium of rats with LAD ligation were also positive for the expression of HMGB1. However, we also found that klotho-treated rats demonstrated restoration of the number of HMGB1-positive cells in post-infarct tissues. The percentages of HMGB1-positive cells were 79.45 ± 2.84% after administration of rKL. Significant increases were observed in the proportion of HMGB1-positive cells in the klotho-treated myocardial I/R group compared to that in the myocardial I/R group ($P < 0.001$). This suggests that the administration of rKL protein significantly reduced the extracellular release of HMGB1 after ischemic myocardial damage (Figure. 3A and B).

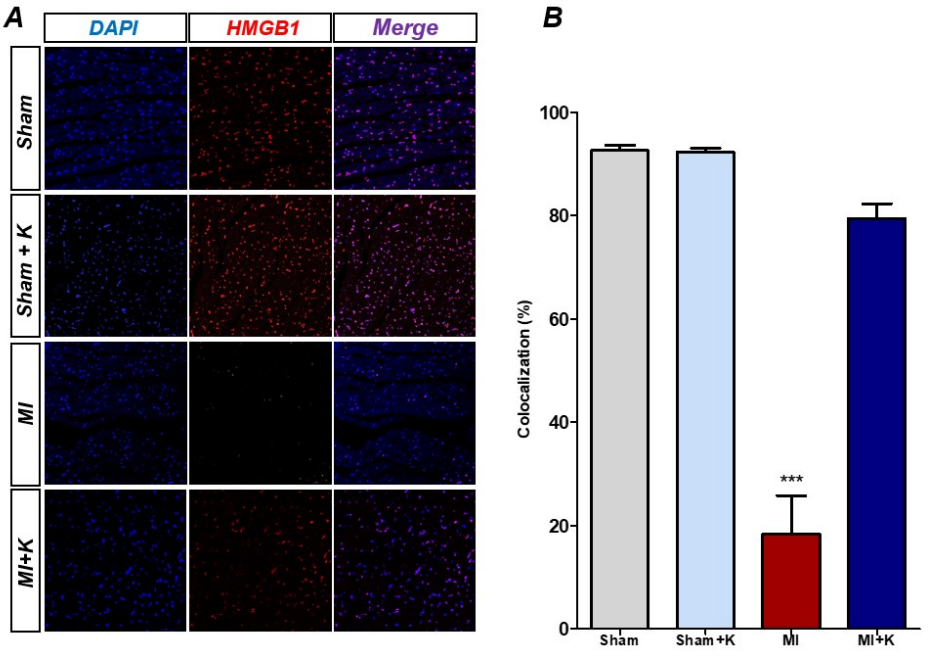

Figure 3. Recombinant klotho (rKL) suppresses the extracellular release of high mobility group box-1 (HMGB1) after myocardial ischemic/reperfusion (I/R) injury. A. Representative immunohistochemistry results for rKL management after myocardial I/R injury; B. Immunohistochemistry results, ***P < 0.001, comparing myocardial I/R with rKL using one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test (the number of animals: n = 5, respectively).

4. Administration of rKL protein inhibited expression of pro-inflammatory cytokines from peri-infarct regions

Cardiac mRNA expression of three major inflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) was assessed by quantitative RT-PCR in the peri-infarcted myocardium 4 h after LAD ligation. The expression levels of TNF-α (18.71 ± 6.141, P =0.031), IL-1β (73.32 ± 12.60, P < 0.001), and IL-6 (3986.57 ± 1588.10, P =0.045) were significantly increased in rats with myocardial I/R injury. Klotho-treated rats with myocardial I/R injury demonstrated a decrease in the expression of these cytokines in the peri-infarcted myocardium (TNF-α (4.60 ± 0.25, P =0.031), IL-1β (23.39 ± 1.72, P < 0.001), IL-6 (550.19 ± 96.99, P =0.045) in comparison with rats with myocardial I/R injury (Figure. 4A-C). Thus, the administration of recombinant klotho protein attenuated the aggravation of myocardial damage via suppression of the production of inflammatory cytokines in the peri-infarct area after myocardial I/R injury.

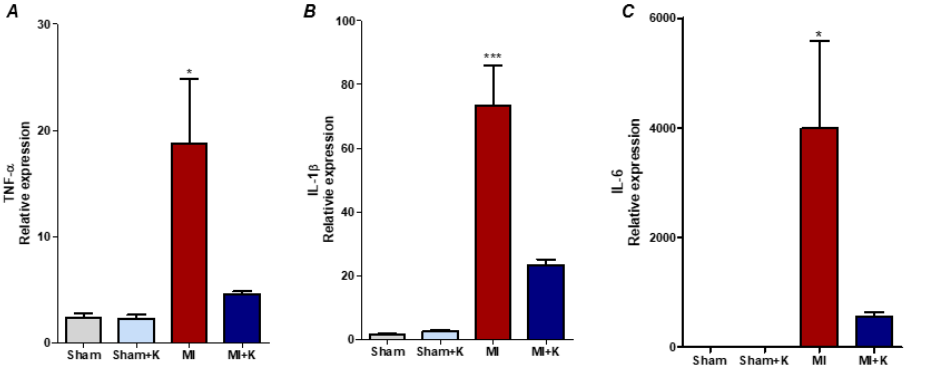

Figure 4. Inflammatory cytokine expression in peri-infarct regions. A. Quantification of the expression of tumor necrosis factor-α (TNF-α) by RT-PCR, *P < 0.05, comparison of myocardial ischemic/reperfusion (I/R) injury treated with klotho by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. B. Quantification of interleukin-1β (IL-1β) expression by RT-PCR, ***P < 0.001. Comparison of myocardial I/R injury treated with recombinant klotho (rKL) by ANOVA followed by Bonferroni post hoc test. C. Quantification of IL-6 expression by RT-PCR, *P < 0.05. Comparison of myocardial I/R treated with klotho by ANOVA followed by Bonferroni post hoc test (the number of animals: n = 5, respectively).

5. rKL protein attenuates apoptosis in the myocardium after myocardial I/R injury (TUNEL assay)

After myocardial I/R, the number of TUNEL-positive apoptotic cells, which appear as light green dots under the confocal microscope, was significantly increased in the myocardial I/R injury group (93.53 ± 10.93) compared to the klotho-treated myocardial I/R injury group (34.13 ± 4.57 $P < 0.001$). These results also imply that the application of recombinant klotho protein demonstrated significant myocardial protection effects by attenuating apoptosis induced by myocardial I/R injury (Figure 5A and B).

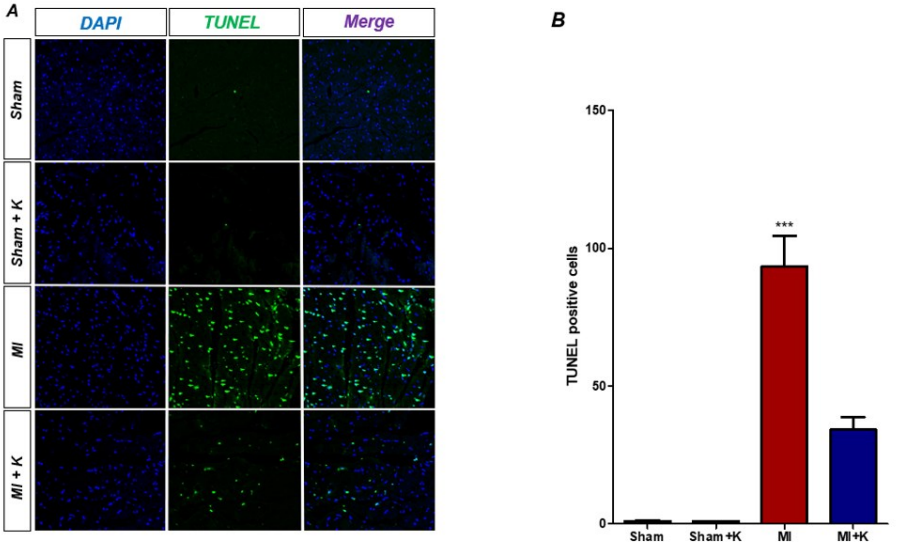

Figure 5. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. A. Representative TUNEL assay results for recombinant klotho (rKL) management after myocardial ischemic/reperfusion (I/R) injury; B. TUNEL assay results, ***$P < 0.001$, comparison of myocardial I/R treated with rKL by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test.

6. Effects of the administration of rKL protein on cTnT and HMGB1 levels in the plasma

To measure HMGB1 levels in serum samples, we conducted ELISA with blood samples obtained at 4 h after the onset of ischemia. As expected, myocardial I/R injury increased the level of circulating HMGB1 (104.06 ± 16.09 pg/mL). However, klotho treatment significantly reduced the increase in the levels of circulating HMGB1 (34.34 ± 5.49 pg/mL), $P < 0.001$) (Figure. 6).

In addition, to identify the myocardial protective effects of klotho protein on cTnT levels reflecting myocardial damage, we measured cTnT levels in the plasma. The levels of cTnT were higher in the myocardial I/R injury group in comparison with those in the sham-operated group (3.78 ± 0.50 and 0.37 ± 0.09 ng/mL, respectively; $P < 0.001$). However, rats treated with rKL protein showed lower cTnT levels than those in the myocardial I/R injured group (1.328 ± 0.05 and 3.78 ± 0.50 ng/mL, respectively; $P < 0.001$). No significant difference was observed in plasma cTnT between sham-operated and klotho-treated groups ($P > 0.05$), indicating that klotho treatment reduced myocardial damage (Figure. 7)

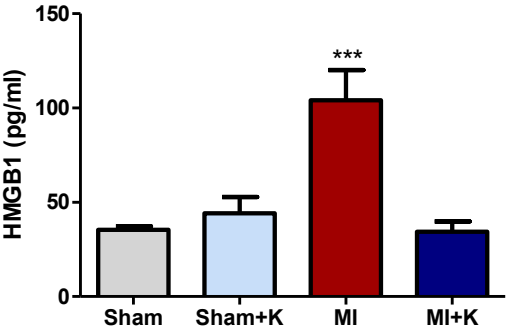

Figure 6. Levels of circulating of HMGB1 in plasma, ***P < 0.001, comparison of myocardial ischemic/reperfusion (I/R) injury with and without recombinant klotho (rKL) administration by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test (the number of animals: n = 5, respectively).

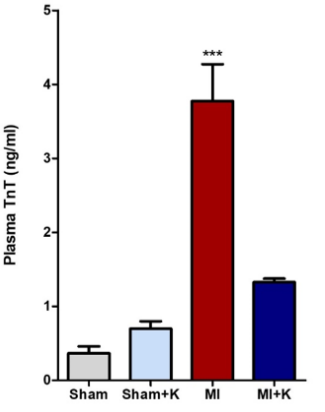

Figure 7. Levels of cardiac troponin T (cTnT) in plasma, ***P < 0.001, comparison of myocardial ischemic/reperfusion (I/R) injury with and without recombinant klotho (rKL) administration by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (the number of animals: n = 5, respectively).

7. Administration of rKL protein modulated cytokine production in the myocardial I/R injury

To identify specific cytokines and compare the expressions of cytokines according to the administration of rKL protein, we performed cytokine array analysis with the whole heart tissue at 24 h after myocardial I/R injury (Figure. 8A). We found that the expressions of several cytokines increased in the heart following myocardial I/R injury. In comparison with the myocardial I/R injury group not treated with rKL, administration of rKL protein significantly reduced the expression levels of several cytokines, such as CINC-1, intracellular adhesion molecule-1 (sICAM-1/CD54), LIX, and L-selectin (Figure. 8B). Thus, we found that rKL protein regulated the levels of inflammation-related proteins.

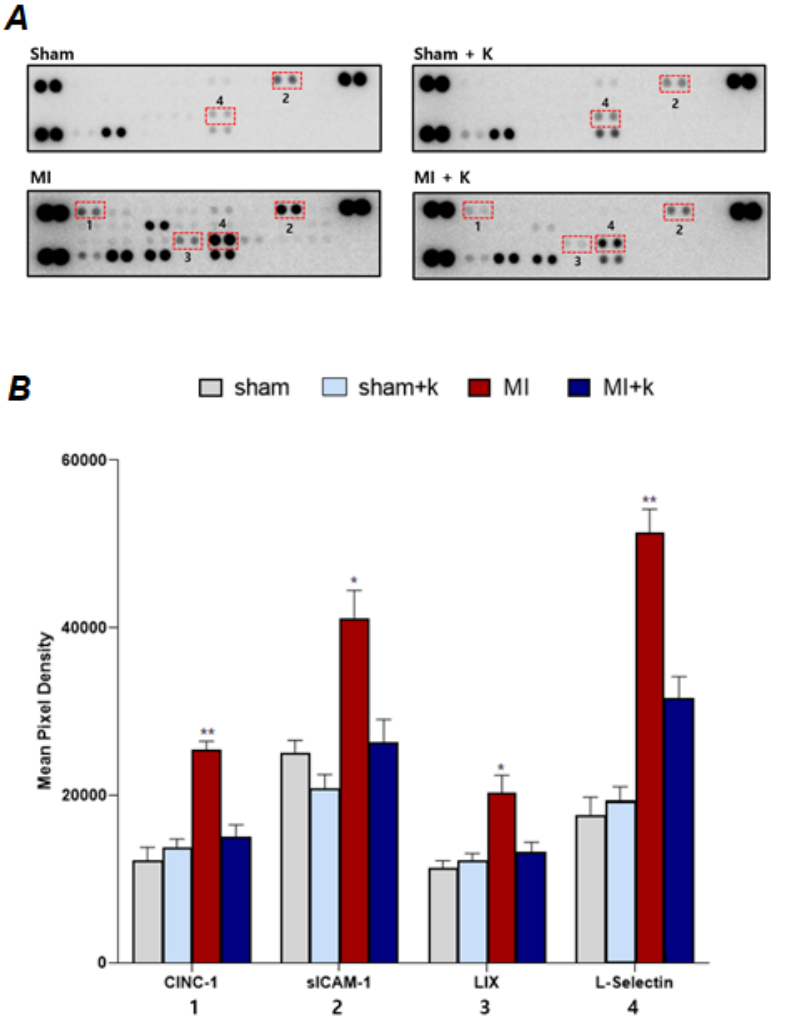

Figure 8. Expression levels of several cytokines A. Representative chemiluminescence images of the proteome profiler array after administration of Klotho following myocardial I/R injury. The dotted line box indicates the selected candidate cytokines. B. Expression levels of several cytokines, *P < 0.05, **P < 0.01, comparison of myocardial ischemic/reperfusion (I/R) injury with and without recombinant klotho (rKL) administration by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test (the number of animals: n = 5, respectively).

8. Glycyrrhizin alleviates myocardial damage by suppressing extracellular HMGB1 release in myocardial I/R injury

Glycyrrhizin is a pharmacological HMGB1 inhibitor; it binds directly to HMGB1 and prevents its extracellular release, thereby blocking its cytokine function [35-37]. We compared the effects of glycyrrhizin treatment on infarct volume, extracellular HMGB1 release, inflammatory cytokines expression, and plasma cTnT level in our animal model. The infarct volume decreased significantly in the myocardial I/R group treated with intraperitoneal glycyrrhizin (6.17 ± 1.02%) than in the non-treated myocardial I/R group (15.49 ± 1.91%, P = 0.003; Figure. 9A and B). Furthermore, glycyrrhizin treatment significantly increased the proportion of HMGB1-positive cells in the I/R-injured myocardium (18.20 ± 7.52% in rats after myocardial I/R vs. 78.80 ± 2.84% in glycyrrhizin-treated myocardial I/R rats, P < 0.001; Figure. 9C and D). In glycyrrhizin-treated AMI rats, the levels of TNF-α (10.15 ± 0.87, P = 0.048), IL-1β (17.16 ± 3.02, P = 0.035), and IL-6 (416.87 ± 103.52, P = 0.034) decreased compared with those in the myocardial I/R group without glycyrrhizin treatment (TNF-α, 18.30 ± 3.40; IL-1β, 77.41 ± 24.61; and IL-6, 3656.64 ± 1322.77; respectively; Figure. 10A, B, and C). Additionally, cTnT levels were significantly lower in the glycyrrhizin-treated myocardial I/R group (1.21 ± 0.17 ng/mL) than in the normothermic group after myocardial I/R injury (3.78 ± 0.91 ng/mL, P = 0.016; Figure. 10D), and the counts of TUNEL-positive apoptotic cells significantly increased in the myocardial I/R injured group (128.20 ± 25.28) compared with that in the klotho-treated myocardial I/R injured group (28.60 ± 4.35 P = 0.001; Figure. 11A and B).

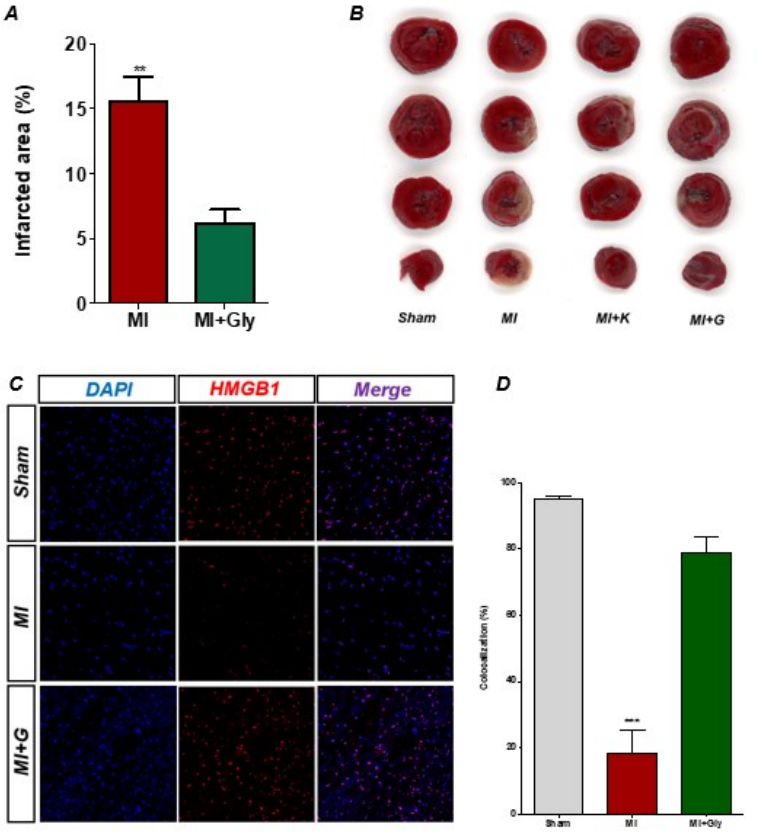

Figure 9. Effects of glycyrrhizin on infarct volume and the extracellular release of high mobility group box-1 (HMGB1) in myocardial I/R injured rats. A. Comparison of TTC staining results corresponding to myocardial I/R injury with and without glycyrrhizin treatment via unpaired t-test (**P < 0.01). B. Representative image for 2,3,5-triphenyltetrazolium chloride (TTC) staining comparing myocardial I/R injury with and without glycyrrhizin pre-treatment. C. Representative immunohistochemistry results for glycyrrhizin management before myocardial I/R injury. D. Immunohistochemistry results comparing myocardial I/R with and without glycyrrhizin treatment, one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test (***P < 0.001).

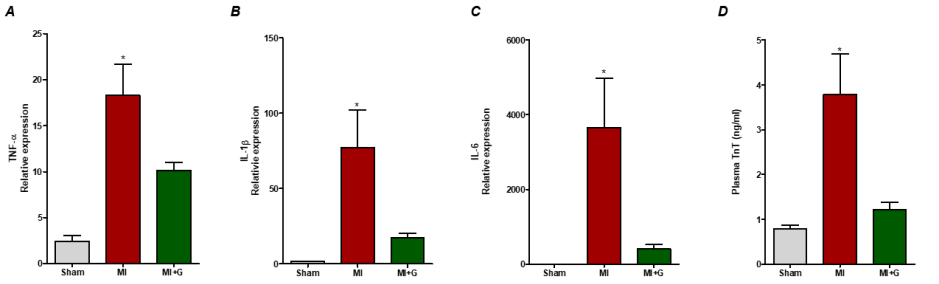

Figure 10. Inflammatory cytokine expression in peri-infarct regions in myocardial I/R injury with and without glycyrrhizin treatment. A. Comparison of tumour necrosis factor-α (TNF-α) expression in myocardial I/R injury with and without glycyrrhizin treatment (*P < 0.05 based on one-way ANOVA followed by Bonferroni post hoc test). B. Comparison of interleukin-1β (IL-1β) expression in in myocardial I/R injury with and without glycyrrhizin treatment (*P < 0.05 based on one-way ANOVA followed by Bonferroni post hoc test). C. Comparison of IL-6 expression levels in myocardial I/R injury with and without glycyrrhizin treatment (*P < 0.05 based on one-way ANOVA followed by Bonferroni post hoc test). D. Plasma cardiac troponin T (cTnT) levels corresponding to glycyrrhizin-treated and non-treated rats with acute myocardial infarction (***P < 0.001 based on one-way ANOVA followed by Bonferroni post hoc test).

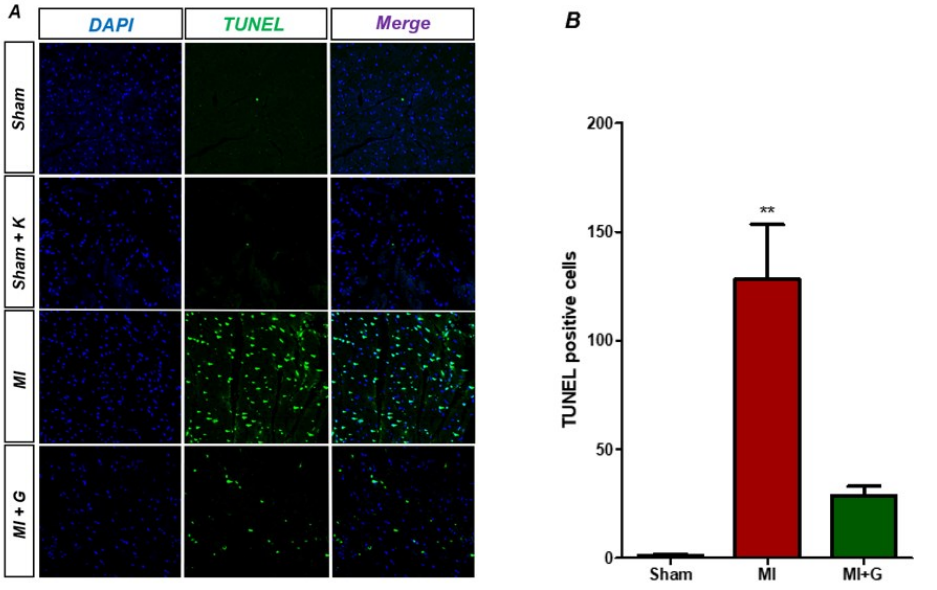

Figure 11. TUNEL assay results corresponding to glycyrrhizin management. A. Representative TUNEL assay results for glycyrrhizin management before myocardial I/R injury. B. Comparison of TUNEL assay results corresponding to myocardial I/R injury with and without glycyrrhizin treatment (**P < 0.01, one-way ANOVA, followed by Bonferroni post hoc test).

IV. DISCUSSION

In our study, we established a rat model to evaluate the therapeutic effects of rKL after myocardial I/R injury, similar to those observed with recanalization after AMI. First, administration of rKL protein significantly reduced infarct volume in rats with myocardial I/R injury. After myocardial I/R injury, the increase in the levels of cardiac troponin, which is a marker of heart muscle damage, was significantly inhibited by rKL protein.

Although inflammation is fundamental in wound healing after AMI, cardiomyocytes can be damaged significantly with a potent inflammatory response induced against I/R injury [8,10]. Cell death through a combination of necrosis and apoptosis begins early after the cessation of blood flow (within 30 min to 1 h) [8,10]. The onset of ischemia initiates inflammation, which leads to devastating myocardial damage. Reperfusion also sustains inflammation over several hours [8,10]. During ischemia and other forms of sterile cell injury, HMGB1 is released as an early mediator that mediates the release of several cytokines, such as TNF, and tissue damage via molecular mechanisms involving signaling via binding of HMGB1 and Toll-like receptor 4 (TLR4) [15]. Therefore, it is important to mitigate the early effects of HMGB1 to attenuate I/R injury of the myocardium [10]. This study is the first to establish a direct functional and mechanistic link between the administration of rKL and HMGB1 in a clinically relevant acute myocardial I/R animal model. After acute myocardial I/R injury, administration of rKL protein could attenuate sterile inflammation in peri-infarct regions by inhibiting the extracellular release of HMGB1 and

subsequently reducing myocardial damage, resulting in effective protection of myocardium after I/R injury. In addition, rKL protein suppressed the production of inflammatory cytokines associated with signaling involving the binding of HMGB1 and TLR4 in the peri-infarct area after myocardial I/R injury. The rKL attenuated the extracellular release of HMGB1 and reduced the mRNA expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in I/R injured rat hearts, similar to the effects of HMGB1 inhibitor glycyrrhizin [10]. Interestingly, rats treated with rKL after myocardial I/R injury demonstrated protective effects on myocardium similar to those observed in the rats that were treated glycyrrhizin before I/R injury. Our finding suggests that rKL helps to prevent this devastating propagation of damage by inhibiting the extracellular release of HMGB1 after myocardial I/R injury, and inhibition of HMGB1 by rKL accounts for its suppression of peri-infarct inflammation. Further, glycyrrhizin showed a myocardial protective effect in pretreatment; however, it did not show the same effect after I/R injury. Unlike glycyrrhizin, rKL administration demonstrated effective myocardial protection after I/R injury. This finding showed the potential of klotho as a therapeutic agent. The findings of this study support the new hypothesis that administration of rKL acts to inhibit HMGB1 in acute myocardial I/R injury. rKL subsequently attenuates sterile inflammation after I/R injury. This is the first study to suggest that exogenous administration of rKL exerts myocardial protection effects after I/R injury and provides novel mechanistic insights into rKL that can provide the theoretical basis for clinical application of new adjunctive modality to the critical care of AMI.

C-X-C motif chemokine 5 (CXCL5) is a powerful chemo-attractant of neutrophils and pro-angiogenic factors involved in the process of the innate immune response. CINC-1 plays a critical role by attracting neutrophils to the site of inflammation in neutrophil-mediated inflammatory diseases [38]. ROS are indispensable messengers, and NFκB is a ubiquitous and redox-sensitive transcription factor that plays a critical role in CINC-1 production [38]. Cytokine-induced neutrophil chemoatractant 1 (CINC-1) signaling pathway for CINC-1 production is significantly associated with NFκB-mediated transcription and ROS generation in the mitochondria [38]. HMGB1-TLR4 signaling upregulates the expression of cytokines and other inflammatory mediators by activating MyD88-dependent nuclear translocation of NF-κB. Our findings demonstrated that rKL suppressed the extracellular release of HMGB1 and ROS production after I/R injury. In consequence, this may lead to attenuation of the activation of CINC-1 signaling. L-selectin regulates leukocyte adhesion and recruitment to lymph nodes in the periphery and sites of acute and chronic inflammation [39]. IL-1 and TNF can induce the expression of ICAM-1, which is expressed by vascular endothelium, macrophages, and lymphocytes [40]. Leukocytes adhere to endothelial cells via ICAM-1/LFA-1, thereby penetrating the tissues [40]. CXCL5 is also a powerful chemo-attractant of neutrophils and pro-angiogenic factors involved in the process of the innate immune response [41]. The production of CXCL5 is similar to that of ICAM-1, which is mediated by the stimulation of cells with IL-1 or TNF that act as inflammatory cytokines [41]. Our study demonstrated that rKL protein significantly reduced the production of

the protein levels of several cytokines, such as CXCL5: LIX, CINC-1, intracellular adhesion molecule-1 (sICAM-1/CD54), and L-selectin.

As klotho prevents oxidative stress, senescence, and apoptosis, decreased klotho levels have been significantly associated with an increase in oxidative stress. Oh et al suggested that klotho plays a role in preventing progressive apoptosis via antioxidative effect in the contrast-associated acute kidney injury (CA-AKI) model [22]. Moreover, we also showed that exogenous supplementation of rKL may attenuate progressive apoptosis via the antioxidative effect by reducing intracellular ROS levels in the early stage of I/R injury. In this study, the activity of ROS was significantly attenuated after 4 h of rKL supplementation. Oh et al. demonstrated that rKL supplementation significantly decreased ROS activity after 16 h, but not at 24 h, in the CA-AKI model [22]. The antioxidative effect of rKL supplementation may be limited overtime after I/R injury. rKL may be supplemented in the early stages after I/R injury to maximize the antioxidative effect. We also found that the administration of rKL exerted significant myocardial protective effects against cellular apoptosis compared to those observed in rats with myocardial I/R injury not treated with rKL. We identified that rKL intuitively attenuated cell death in the myocardium with significant improvements in aggravation of the infarcted area that lasted up to 24 h.

This study has several limitations. Although protective effects similar to those observed with glycyrrhizin treatment were reported, explicit evidence demonstrating a direct association between rKL and HMGB1 inhibition is lacking. In future research, to explore the specific mechanism of rKL, it would

be necessary to elucidate a mechanism by comparing the effects of rKL with those of direct antibodies or substances with a known mechanism. The final goal of this study was to find a drug that is helpful in the treatment and prognosis of patients with myocardial infarction in actual clinical practice. Therefore, klotho injection was administered with a focus on reperfusion in this study. In future experiments, studies on the time and administration route that can optimize the effects for the treatment of reperfusion injury should be conducted.

Supplementation of rKL can be a viable and adjunctive therapeutic modality for patients with I/R injury including those with out-of-hospital cardiac arrest, hemorrhagic shock, AMI, and ischemic stroke. In addition, the positive effect of Klotho observed in various organs can be expected to play an important role in the treatment of AMI in the future. However, further studies are needed to verify the clinical benefits of supplementation of rKL and the mechanisms for myocardial protection of rKL against sterile inflammation in patients with AMI.

V. CONCLUSION

Our study suggested that the administration of rKL exerted myocardial protective effects by attenuation of the extracellular release of HMGB1, inflammation, apoptosis, production of intracellular ROS, and cell death after I/R injury. This study provides insights into the mechanism of rKL that can provide the theoretical basis for clinical applications of new adjunctive modalities for the critical care of AMI.

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ABSTRACT(IN KOREAN)

심근 허혈/재관류 손상에서 klotho의 무균염증반응 약화

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명 진 우

급성 심근경색은 높은 사망률을 초래하는 심장 응급 질환이다. 현재까지 심근경색 환자의 치료는 재관류 치료 외에 효과적인 방법이나 잠재적 표적이 없는 상태이다. 우리는 재조합 klotho 단백질을 이용하여 이 물질이 Rat의 LAD 관상 동맥 결찰 모델에서 HMGB1의 세포외 방출을 억제함으로써 경색 주변 영역에서 멸균 염증을 약화시키고 I/R 손상 후 심근 경색 영역을 감소시킬 수 있다고 가정하였다. 또한 우리는 재조합 klotho 단백질의 투여가 어떤 메커니즘을 심근 보호 효과를 제공하는지 여부를 조사했다.

심근 허혈/재관류 손상에서 재조합 klotho 단백질의 투여는 경색 부피를 감소시켰고 경색 주변 조직에서 HMGB1의 세포외 방출이 감소되었다. 또한 재조합 Klotho 단백질의 투여는 경색 주변 부위로부터 염증성 사이토카인의 발현을 억제하고 apoptosis를 감소시켰다. 허혈 후 시행한 ELISA에서 Klotho 투여는 HMGB1의 혈액내

농도를 유의하게 감소시켰고 여러 염증 관련 사이토카인의 발현 수준을 유의하게 감소시켰다. 따라서 우리는 재조합 Klotho 단백질이 염증 관련 단백질의 수준을 조절한다는 것을 예상할 수 있다.

우리 연구에서는 재조합 klotho의 투여가 심근 허혈/재관류 손상 후 효과적인 심근 보호 효과를 보인다는 것을 보여주었고 따라서 본 연구는 급성심근경색 환자 치료에 대한 새로운 이론적 기초를 제공한다.

핵심되는 말 : Klotho, HMGB1, 심근 허혈/재관류 손상