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Research Article

The Effects of Targeted Temperature Management on Oxygen-Glucose Deprivation/Reperfusion-Induced Injury and DAMP Release in Murine Primary Cardiomyocytes

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Introduction. Ischemia/Reperfusion (I/R) is a primary cause of myocardial injury after acute myocardial infarction resulting in the release of damage-associated molecular patterns (DAMPs), which can induce a sterile inflammatory response in the myocardial penumbra. Targeted temperature management (TTM) after I/R has been established for neuroprotection, but the cardioprotective effect remains to be elucidated. Therefore, we investigated the effect of TTM on cell viability, immune response, and DAMP release during oxygen-glucose deprivation/reperfusion (OGD/R) in murine primary cardiomyocytes. *Methods*. Primary cardiomyocytes from P1-3 mice were exposed to 2, 4, or 6 hours OGD (0.2% oxygen in medium without glucose and serum) followed by 6, 12, or 24 hours simulated reperfusion (21% oxygen in complete medium). TTM at 33.5°C was initiated intra-OGD, and a control group was maintained at 37°C normoxia. Necrosis was assessed by lactate dehydrogenase (LDH) release and apoptosis by caspase-3 activation. OGD-induced DAMP secretions were assessed by Western blotting. Inducible nitric oxide synthase (iNOS), cytokines, and antiapoptotic RBM3 and CIRBP gene expressions were measured by quantitative polymerase chain reaction. *Results*. Increasing duration of OGD resulted in a transition from apoptotic programmed cell death to necrosis, as observed by decreasing caspase-3 cleavage and increasing LDH release. DAMP release and iNOS expression correlated with increasing necrosis and were effectively attenuated by TTM initiated during OGD. Moreover, TTM induced expression of antiapoptotic RBM3 and CIRBP. *Conclusion*. TTM protects the myocardium by attenuating cardiomyocyte necrosis induced by OGD and caspase-3 activation, possibly via induction of antiapoptotic RBM3 and CIRBP expressions, during reperfusion. OGD induces increased Hsp70 and CIRBP releases, but HMGB-1 is the dominant mediator of inflammation secreted by cardiomyocytes after prolonged exposure. TTM has the potential to attenuate DAMP release.

1. Introduction

Acute myocardial infarction (AMI) is a leading cause of death worldwide. Myocardial ischemia followed by therapeutic reperfusion can induce a sterile inflammatory response by enhancing the production of cytokines and reactive oxygen species (ROS), which usually occurs in the early reperfusion phase. ROS can cause direct damage to cell structures and genomic DNA, as well as recruit neutrophil granulocytes, monocytes, and macrophages to the infarct area by chemotaxis and initiate an inflammatory response [1]. Typical promoters of inflammation are the cytokines interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), which are secreted by the damaged cells themselves, as well as by the recruited immunocompetent cells. This inflammatory response can also be referred to as sterile inflammation, since it is triggered in the absence of pathogens.

Other contributing factors to inflammation are damage-associated molecular patterns (DAMPs), which are released or secreted into the extracellular matrix by ischemia/reperfusion- (I/R-) induced injured cells. These DAMPs bind to pattern recognition receptors (PRR), including toll-like receptors (TLR), of neighboring or recruited immunocompetent cells. The subsequent induction of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), further stimulates the synthesis of cytokines, thereby maintaining or even exacerbating the inflammatory response [2]. Molecules that are referred to as DAMPs can be proteins or ADP/ATP.

Cold-inducible RNA-binding protein (CIRBP) is a nuclear protein, which when localized intracellularly provides cytoprotection by suppressing apoptosis and preserving RNA and DNA stability [3, 4]. Stimuli that lead to the upregulation of its expression include hypoxia and hypothermia, thus categorizing CIRBP as a so-called cold-shock protein [3, 5]. During hypothermia, CIRBP binds to different transcripts to stabilise and accelerate their translation [6]. These cytoprotective effects have already been confirmed *in vivo* by Wang et al., who showed that cooling rats to a temperature of $31 \pm 0.5^{\circ}$C after a traumatic brain injury leads to the suppression of apoptosis in certain brain regions, which correlated with increased CIRBP mRNA and protein expressions in the same regions and was abolished by silencing CIRBP [7]. However, in the case of cellular stress, CIRBP is methylated and translocates from the nucleus to the cytosol where it either binds to the mRNA of stress-induced molecules or is preserved in stress granules [6]. Furthermore, cellular damage can lead to the release of CIRBP to the extracellular matrix where it acts as a DAMP and contributes to the inflammatory response by stimulating the secretion of TNF-α and high-mobility group box-1 (HMGB-1) [6]. A clinical study by Zhou et al. demonstrated that plasma levels of CIRBP in septic patients were significantly lower in surviving patients than in those who died of sepsis, and high plasma levels of CIRBP correlated with a poorer outcome as assessed by Acute Physiology And Chronic Health Evaluation II (APACHE II) and sequential organ failure assessment (SOFA) scores [8]. These findings further emphasize the increasingly important role of extracellular CIRBP in the clinical setting.

RNA-binding motif protein 3 (RBM3) is another cold-shock protein with antiapoptotic and cytoprotective properties that is closely related to CIRBP. In contrast to CIRBP, RBM3 has so far not been described as a DAMP [3]. To date, both cold-shock proteins have mostly been observed in neuronal cells and are associated with hypothermia-induced neuroprotection.

Therapeutic hypothermia (TH), also referred to as targeted temperature management (TTM), has been established for neuroprotection and is a promising cardioprotective therapeutic strategy, as suggested by many *in vitro* and *in vivo* studies. However, the effect of TTM on the mechanisms and kinetics of the sterile inflammatory response after AMI remains to be elucidated. Therefore, we investigated the effect of intraischemic moderate TH (33.5°C) on murine primary cardiomyocytes viability after exposure to oxygen-glucose deprivation (OGD) and reperfusion (OGD/R). We also investigated the expression and release of DAMPs induced by exposure to OGD, as well as the sterile inflammatory response. Moreover, we also investigated the effect of TTM on RBM3 and CIRBP expressions in OGD/R-injured cardiomyocytes.

2. Materials and Methods

2.1. Animals and Preparation of Primary Cultures. All animal experiments were approved and performed in accordance with the guidelines of the Charité – Universitätsmedizin Berlin, Germany. Animals were housed in a conventional animal facility at the Center for Anatomy, Charité – Universitätsmedizin Berlin, Germany.

2.2. Preparation of Murine Primary Cardiomyocytes. Murine primary cardiomyocytes were isolated from P1-3 C57/BL6N mice using the Pierce™ Primary Cardiomyocyte Isolation Kit (Thermo Fisher) according to the manufacturer's recommended protocol. Briefly, dissected hearts were cut into 1-3 mm^2 pieces in Hanks' Balanced Salt Solution (HBSS), washed, and digested with two tissue-specific dissociation enzymes (papain and thermolysin) for 30 minutes at 37°C. Isolated cells were then resuspended in Dulbecco's modified eagle medium (DMEM) formulated for primary cell culture (Pierce Primary Cardiomyocyte Isolation Kit) supplemented with 10% fetal bovine serum (FBS, Merck Millipore) and 0.2% primocin (Invivogen). For each experimental group, 3×10^6 cells were plated out in 40 mm diameter Petri dishes (TPP) and cultivated for 24 hours at 37°C in a humidified incubator with 21% O_2 and 5% CO_2. Afterward, the medium was changed to a complete medium containing a growth supplement (1 : 1000, Pierce Primary Cardiomyocyte Isolation Kit) to promote the isolation, enrichment, and growth of functional cardiomyocytes. Cells were further cultivated for an additional 6 days with a medium change every subsequent two days.

2.3. Oxygen-Glucose Deprivation/Reperfusion (OGD/R). Ischemia-reperfusion injury was simulated *in vitro* by exposure to OGD/R, as previously established in our laboratory [9]. Briefly, primary cardiomyocytes were deprived of oxygen and glucose for up to 6 hours in glucose/serum free DMEM (Thermo Fisher) at 0.2% O_2 and 5% CO_2 in a humidified CO_2 incubator (Binder) [10]. Control groups were kept at normoxia (21% O_2) in complete medium (DMEM containing glucose and 10% FBS (Merck Millipore)). After 2-6 hours of OGD, reperfusion was simulated by restoration of nutrients and oxygen in complete medium at 21% O_2. All experimental media were supplemented with 0.2% Primocin (InvivoGen) and 2 mM L-glutamine (Merck Millipore).

2.4. Targeted Temperature Management (TTM). We previously established a time-temperature protocol for intraischemic cooling (33.5°C) of the cardiomyocytes (see Figure 1) [10, 11]. Briefly, normothermic OGD/R-injured groups were maintained at 37°C for the duration of the experiment, while TTM groups were cooled to 33.5°C after 1-hour exposure to OGD and maintained during simulated reperfusion for 24 hours. Samples for determining the

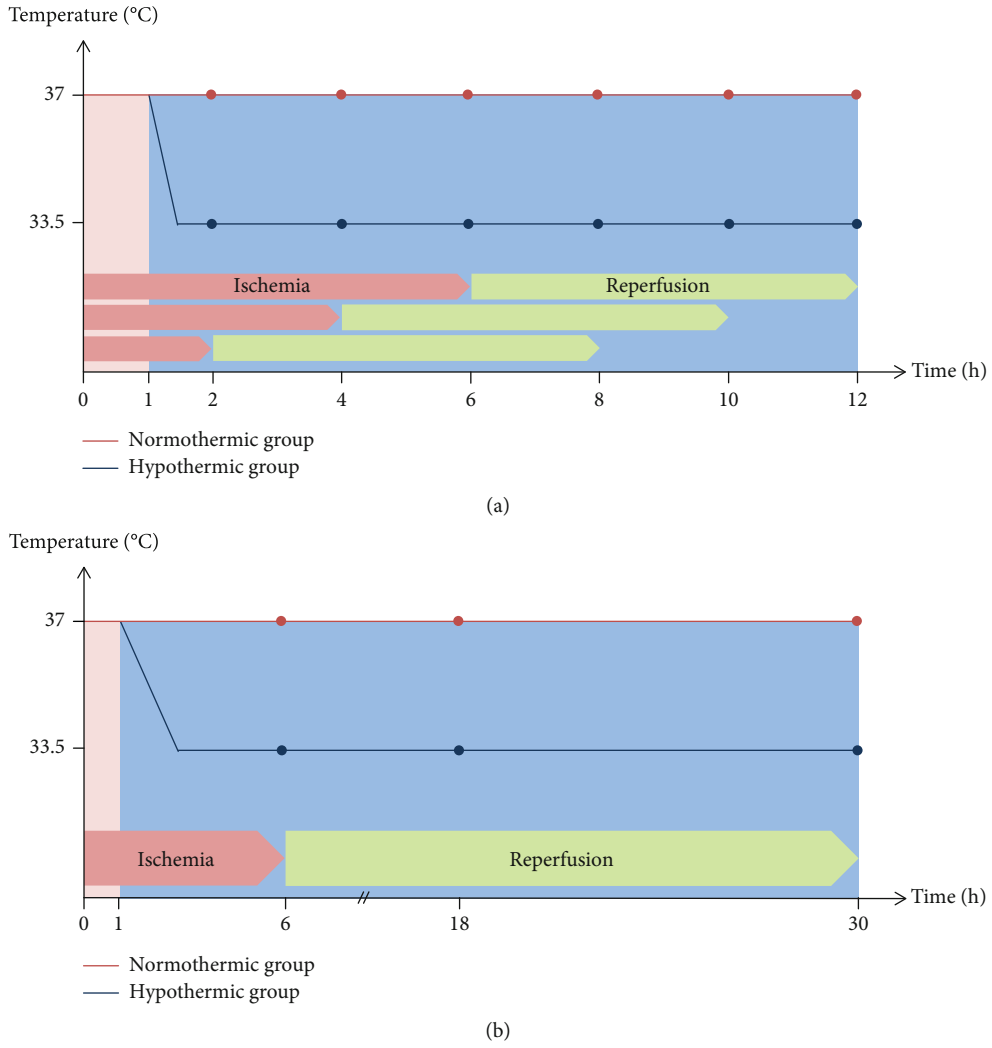

Figure 1: Experimental time-temperature protocol. Normothermic OGD/R-induced injury groups were maintained at 37°C for the duration of the experiment, while TTM-treated groups were cooled to 33.5°C after 1 hour exposure to OGD and maintained during the simulated reperfusion phase. Experimental samples were analyzed (a) directly after exposure to increasing duration of OGD (2-6 h) and 6 hours of simulated reperfusion (8-12 h after experimental start, respectively) or (b) directly after exposure to 6 hours OGD and 12 or 24 hours of simulated reperfusion (18 and 30 h after experimental start, respectively).

underlying cell death mechanisms were analyzed directly after OGD (2-6 h) and 6 hours reperfusion (8-12 h), respectively. In order to thoroughly investigate the effect of OGD/R and TTM on the cardiomyocytes, samples were also analyzed after 6 hours OGD followed by 12 and 24 hours reperfusion.

2.5. Assessment of Lactate Dehydrogenase (LDH) Release. Necrotic cell death was quantified by LDH release into the cultured supernatant using a colorimetric Cytotoxicity Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. After each experimental time point, cultured supernatants were collected, centrifuged, and mixed with reagents (1 : 1) in a 96-well plate. Extinction was measured at 570 nm minus 630 nm using a microtiter plate reader (Multiskan Reader Ascent, Thermo Fisher). LDH release is expressed as percent cytotoxicity relative to total LDH content, as determined from lysed normoxic control cells using a freeze/thaw method at -80°C, and normothermic normoxia untreated group as baseline control for spontaneous LDH release.

2.6. Intracellular and Extracellular Proteins Isolation and Western Blot Analysis. After 2, 4, and 6 hours of OGD, intracellular and extracellular proteins were isolated for Western blot analysis. Briefly, intracellular proteins were isolated from attached cells that were washed with ice-cold PBS, dislodged using a cell scraper, and lysed using a modified RIPA buffer (50 mM Tris–HCl at pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecylsulfate, 0.5% Na-deoxycholate, 2 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, sodium fluoride, and protease inhibitor cocktail 3, all from Sigma-Aldrich) and quantified using a BCA Protein Assay Kit (Pierce Biotechnology). Extracellular proteins were isolated using a trichloroacetic acid precipitation method. Briefly, 1 mL cell cultured supernatants were incubated with 20% (*v*/*v*) trichloroacetic acid

(VWR) for 30 minutes on ice and centrifuged at 16,000xg for 20 minutes to precipitate proteins. The proteins were washed with ice-cold acetone, dried, and dissolved in 25 μL modified RIPA buffer. Subsequently, 30 μg intracellular and total extracellular isolated proteins were incubated with Pierce Lane Marker Reducing Sample Buffer (Thermo Scientific) at 95°C for 5 minutes, subjected to 15% SDS polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane (PALL Life Sciences) overnight at 30 V using a tank blot procedure (Bio-Rad). The membrane was blocked for 1 hour at room temperature using 5% bovine serum albumin (Carl Roth) for Hsp70 and HMGB-1 or 5% dry milk (AppliChem) for CIRBP in TBS + 0.1%Tween 20. Primary antibodies against Hsp70 (1 : 1000, Cell Signaling #4872), HMGB-1 (1 : 1000, Chondrex, #7028) and CIRBP (1 : 500, Proteintech (10209-2-AP)) were diluted in respective blocking solution and incubated overnight at 4°C, and HRP-conjugated secondary antibodies (anti-rabbit IgG (1 : 20,000), Dianova) were incubated for 1 hour at room temperature. Protein expression was visualized using Dura Super Signal West (Thermo Scientific) according to the manufacturer's instructions, and subsequent densitometry analysis was performed using the ChemiDoc™ Imaging Systems and Image Lab (Bio-Rad).

2.7. RNA Isolation and RT-qPCR Analysis. Total RNA from murine primary cardiomyocytes was isolated using the GenUp™ Total RNA Kit (Biotechrabbit) according to the manufacturer's recommended protocol. RNA concentration and purity were determined by spectrophotometric measurements at 260 and 280 nm using a Nanodrop 2000 (Nanodrop) and 1% agarose gel electrophoresis, respectively. CDNA was transcribed from 1000 ng total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and PTC200 Thermal Cycler (MJ Research). Expression of target genes and the endogenous control, gylcerinaldehyde-3-phosphate dehydrogenase (GAPDH), was assessed by real-time qPCR using the TaqMan® Gene Expression Assays (see Table 1) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to manufacturer's recommendations. Reactions with no reverse transcripts and templates were included as negative controls. Relative quantification of target gene expression was normalized to the housekeeping gene using the $2 - \Delta\Delta$ct method and illustrated as fold change [12].

2.8. Statistical Analysis. Data were analyzed and graphed using GraphPad Prism 5 (GraphPad Software). Results were expressed as means ± standard deviations (SD). Experiments were independently repeated at least three times. One-way ANOVA followed by Tukey's posttest was used for multiple group comparisons and $p < 0.05$ was considered statistically significant (*, group comparison; $^\#$, compared to normoxia control).

3. Results

3.1. The Effect of TTM on OGD/R-Induced Necrosis and Apoptosis in Murine Primary Cardiomyocytes. Increasing duration of exposure to OGD resulted in increased cytotoxicity in the murine primary cardiomyocytes, as assessed by the increased release of LDH into the cultured medium (see Figure 2(a)). Maximum cytotoxicity (29.6%) was observed in the uncooled OGD group after 6 hours exposure and was significantly higher relative to 4 hours (16.4%) and 2 hours (4.6%) exposure to OGD. Hypoxia has been shown to induce oxidative stress leading to apoptosis in the myocardium [13]. Apoptotic programmed cell death as assessed by cleavage of caspase-3 via Western blotting and was observed in the reperfusion phase after exposure to OGD (see Figure 2(b)). Maximum cleavage of caspase-3 (1.7-fold increase) was observed after exposure to OGD for 2 hours followed by reperfusion for 6 hours and significantly decreased with extended exposure to OGD (0.9-fold increase for 4 hours and undetectable for 6 hours OGD), relative to normoxic control. In general, we observed that increasing duration of exposure to OGD resulted in a transition from apoptotic to necrotic cardiomyocyte cell death. Moreover, TTM significantly attenuated cardiomyocyte necrosis in the 6 hours OGD group (29.6 to 17.7%) and apoptosis in the 2 hours OGD followed by 6 hours reperfusion group (1.7- to 0.7-fold increase). Interestingly, exposure to 4 hours OGD followed by 6 hours reperfusion at 37°C resulted in slightly lower caspase-3 cleavage (0.9-fold increase), but cooling to 33.5°C resulted in significantly reduced cleavage of caspase-3 (0.5-fold increase) relative to homeostasis level observed in the normoxia control.

Table 1: TaqMan® Gene Expression Assays.

Gene	Assay ID	Gene	Assay ID
IL-1β	Mm00434228_m1	iNOS	Mm00440502_m1
IL-6	Mm00446190_m1	TNF-α	Mm00443260 g1
CIRBP	Mm00483336_g1	RBM3	Mm01609819_g1
GAPDH	Mm99999915_g1		

3.2. The Effect of TTM on OGD/R-Induced Innate Immune Response in Murine Primary Cardiomyocytes. Ischemia/reperfusion injury often results inflammation of the myocardium that is primarily evidenced by an innate immune response. Therefore, we investigated the effect of TTM on various mediators of inflammation including the inducible nitric oxide synthase (iNOS) and inflammatory cytokine gene expressions in OGD/R-injured murine primary cardiomyocytes. Prolonged exposure to OGD resulted in a significant increase in iNOS expression (6 hours) (169-fold increase) compared to normoxia control that was not attenuated by cooling (142.2-fold increase), but was significantly lower at all investigated time points after reperfusion (see Figures 3(a) and 3(b), respectively). IL-1β has been observed to be markedly upregulated in the infarcted myocardium [14, 15]. We observed increasing IL-1β expression with increasing duration of exposure to OGD, while expression was generally suppressed in the reperfusion phase (see Figures 3(c) and 3(d), respectively). In contrast to IL-1β, increasing the duration of OGD had no significant effect on IL-6 expression (see Figure 3(e)). However, IL-6 gene expression tends to be

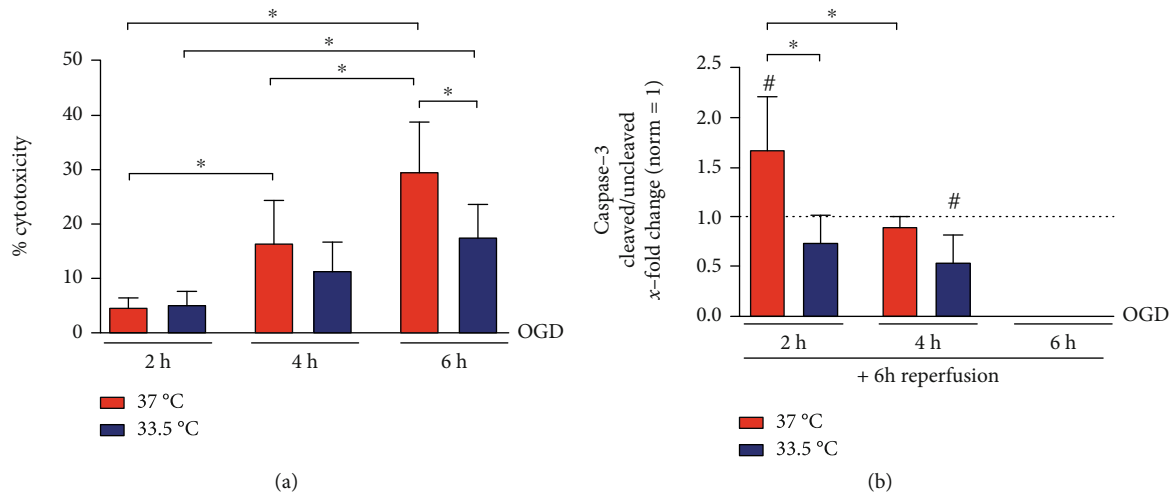

Figure 2: Increasing duration of OGD exposure resulted in a transition from apoptotic to necrotic cell death in the murine primary cardiomyocytes that could be attenuated by TTM. (a) OGD-induced toxicity as measured by LDH release at 2, 4, and 6 hours is presented as % cytotoxicity relative to positive (100%) and normoxia (0%) controls. (b) Apoptotic programmed cell death was assessed by cleavage of caspase-3 after exposure to 2, 4, or 6 hours OGD followed by 6 hours of reperfusion and is presented as x-fold change relative to normoxia control at the respective experimental time points. Data from at least 3 independent experiments are shown as mean ± SD, and statistical analysis was performed using one-way ANOVA with Tukey posttest; $^{\#}p < 0.05$ as compared to 37°C normoxia control group and $^{*}p < 0.05$ for comparisons between the two experimental groups were considered significant.

increased in the normothermic group during the reperfusion phase and was potentially but not significantly attenuated by cooling (see Figure 3(f)). Finally, TNF-α is another proinflammatory cytokine expressed in the myocardium after acute myocardial ischemia infarction [15]. However, we did not observed any significant changes in TNF-α expression after OGD/R-induced injury in the primary cardiomyocytes at both 37 and 33.5°C (see Figures 3(g) and 3(h), respectively). TTM tends to induced TNF-α expression in both the OGD and reperfusion phases, but did not reached significancy.

3.3. The Effect of TTM on OGD-Induced DAMP Release from Murine Primary Cardiomyocytes. Damage-associated molecular patterns or DAMPs are constituents of dying cardiomyocytes that are released from the damaged myocardium and can induce a sterile inflammatory response following myocardial infarction [16]. Therefore, we investigated the effect of TTM and OGD/R on intracellular DAMP expression and extracellular release from murine primary cardiomyocytes. Specifically, the intracellular protein expression and release of HMGB-1, Hsp70, and CIRBP were investigated. Increasing exposure to OGD (2-6 hours) had no significant effect on intracellular HMGB-1, Hsp70, and CIRBP protein expressions (see Figures 4(a), 4(c), and 4(e), respectively). Cooling also had no observable effect on intracellular HMGB-1 and CIRBP expressions during the OGD phase, but significantly induced Hsp70 expression relative to normothermic control after 2 hours OGD. Although no significant changes in intracellular DAMP expressions were observed, we did observe a significant increase in the release of HMGB-1 when exposure to OGD was prolonged from 2 hours to 6 hours (see Figure 4(b)). We also observed increasing but not significant release of Hsp70 and CIRBP into the cultured supernatants with increasing duration of OGD (see Figures 4(d) and 4(f), respectively). TTM-treated groups had generally lower release of DAMPs in the OGD phase, though not significant.

3.4. The Effect of TTM on OGD/R-Induced Expression of RBM3 and CIRBP in Murine Primary Cardiomyocytes. RBM3 and CIRBP belong to the family of cold-inducible RNA-binding proteins that have been shown to have antiapoptotic effects [4, 17]. Both RBM3 and CIRBP gene expressions were significantly suppressed by exposure to OGD for 6 hours at 37°C, with no significant changes observable in the reperfusion phase, relative to normoxic control (see Figures 5(a) and 5(b), respectively). TTM significantly attenuated RBM3 and CIRBP suppressions in the OGD phase and restored gene expression levels back to the normoxic control levels. Moreover, TTM significantly induced both RBM3 and CIRBP expressions in the reperfusion phase, and longer exposure to cooling resulted in significantly increased expressions of both RBM3 and CIRBP in a time-dependent manner in the primary cardiomyocytes.

4. Discussion

Our study confirms the cardioprotective effect of targeted temperature management during simulated ischemia/reperfusion-induced injury in murine primary cardiomyocytes. Moderate cooling to 33.5°C initiated during the OGD phase and maintained into the OGD/R phase successfully attenuates OGD-induced necrotic cell death as well as OGD/R-induced apoptotic programmed cell death. We also observed a transition from prevailing necrotic cell death with increasing duration of OGD to apoptotic programmed cell death after a short-term exposure to OGD for 2 hours followed

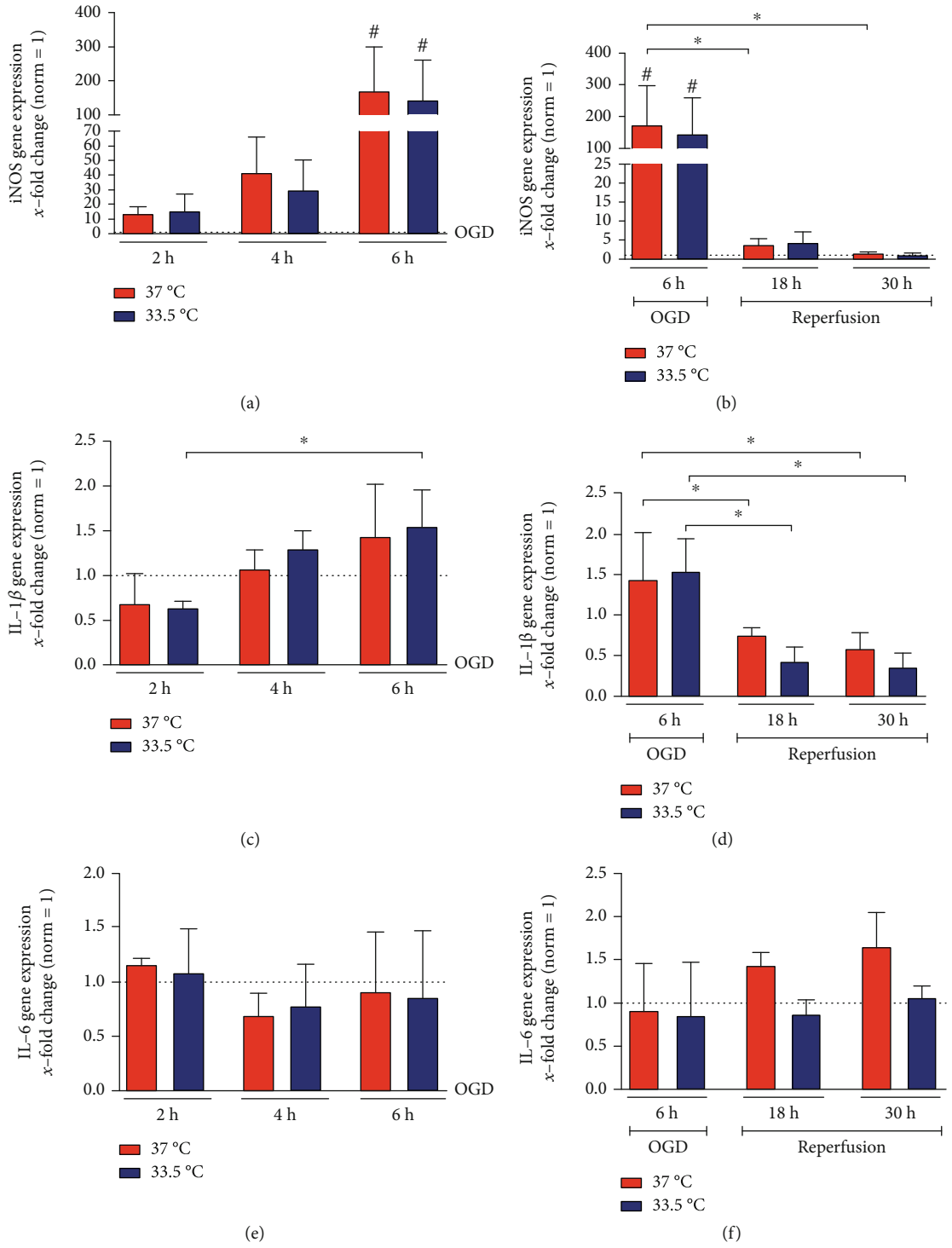

Figure 3: Continued.

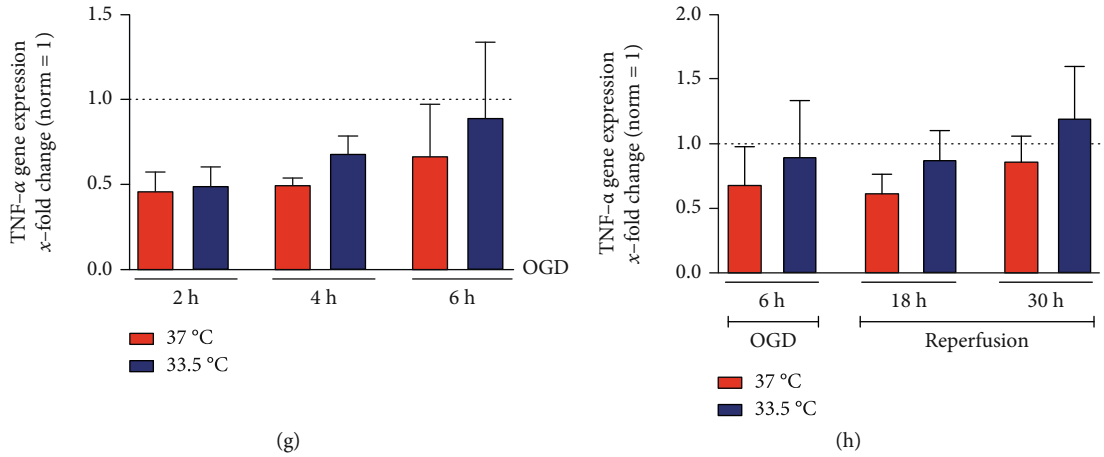

Figure 3: RT-qPCR gene expression analysis of OGD/R-induced innate immune response of (a, b) iNOS inflammatory enzyme and proinflammatory cytokines (c, d) IL-1β, (e, f) IL-6, and (g, h) TNF-α in murine cardiomyocytes after exposure to 2, 4, and 6 hours OGD followed by 12 and 24 hours reperfusion (18 hours and 30 hours after experimental start, respectively) at 37°C and 33.5°C. Data from at least 3 independent experiments are shown as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey posttest; $^{\#}p < 0.05$ as compared to 37°C normoxia control group and $^{*}p < 0.05$ comparison between the two experimental groups were considered significant.

by reperfusion for 6 hours. This transition in cell death mechanism can be attributed to a decrease in intracellular ATP content that has been observed during ischemia, as well as energy-dependent crucial activation steps of apoptosis that predominantly proceed after the restoration of energy during the reperfusion phase [18]. Moreover, the intensity of the initial damage in the sense of prolonged exposure to OGD is another crucial factor in determining the resulting cell death mechanism, as previously demonstrated by Bonfoco et al., in cortical neurons [19]. Therefore, the decrease in ATP concentrations due to increasing duration of OGD results in compensatory mechanisms failure, cell rupture, and ultimately necrosis, whereas short-term OGD-induced damage leads to the induction of apoptosis in the following reperfusion phase.

TTM protects cardiomyocytes from OGD/R-induced injury by attenuating necrosis after prolonged exposure to OGD, as demonstrated by a decreased LDH release into the cultured supernatant. TTM also attenuated apoptosis in the reperfusion phase after a shorter exposure to OGD, thus increasing cell viability in general. The antiapoptotic effect of cooling, as assessed by the attenuation of caspase-3 activation has so far been mostly observed in neurons [20–22], but not extensively investigated in cardiac cells. Our experimental study shows that the antiapoptotic effect of TTM is also implemented by the attenuation of caspase-3 activation in primary murine cardiomyocytes. However, since caspase-3 is the common end line effector for both the intrinsic and extrinsic apoptotic pathways, no conclusions can be drawn about the origin of the initiating stimulus.

We also observed a significant increase in gene expression of the antiapoptotic cold-shock proteins, CIRBP and RBM3, during reperfusion in the cooled experimental groups. Even though no significant increases in the gene expressions of RBM3 and CIRBP were observable during OGD, cooling effectively preserved homeostasis gene expression levels comparable to the normoxic control group, whereas CIRBP and RBM3 gene expressions were significantly decreased in the uncooled OGD groups. To date, these cold-shock proteins have been described to exert antiapoptotic effects in neuronal cells, and specifically, RBM3 has been discussed as a potential therapeutic approach in neurodegenerative diseases due to its association with preserving neuroplasticity [23]. Even though the cytoprotective mechanisms are not fully understood, *in vitro* studies suggest that hypothermia-induced upregulation of RBM3 leads to the suppression of poly(ADP-ribose) polymerase 1 (PARP) cleavage, a crucial step in caspase-dependent apoptosis [17], while CIRBP interferes with the mitochondrial apoptotic signaling pathways [4]. A study conducted by Long et al. demonstrated that overexpression of CIRBP further promotes cell proliferation and reduces ROS production through the inhibition of the NF-κB signaling pathway in H9C2 cardiomyocytes subjected to simulated ischemia. CIRBP knockdown, however, leads to opposite effects including decreased cell viability and enhanced apoptosis [24]. This is in accordance with our results that show antiapoptotic RBM3 and CIRBP are not only expressed in neuronal cells but also in cardiomyocytes, suggesting a crucial mechanism in the cardioprotective effects induced by TTM. Our data suggests that TTM not only increases cell viability by attenuating necrosis during ischemia and apoptosis in the early reperfusion phase by reducing caspase-3 activation but could possibly have a sustained protective effect in the late reperfusion phase by the upregulation of the antiapoptotic cold-shock proteins. However, the exact regulatory mechanisms exerted by RBM3 and CIRBP in primary cardiomyocytes remain to be elucidated.

NO has both antioxidative and anti-inflammatory effects under normoxic conditions [25], but it can also generate reactive nitrogen species free radicals under hypoxia [26, 27]. In cardiomyocytes, endothelial NOS (eNOS) is constitutive, and iNOS is mostly transcriptionally regulated and expressed after stimulation [27]. iNOS gene expression

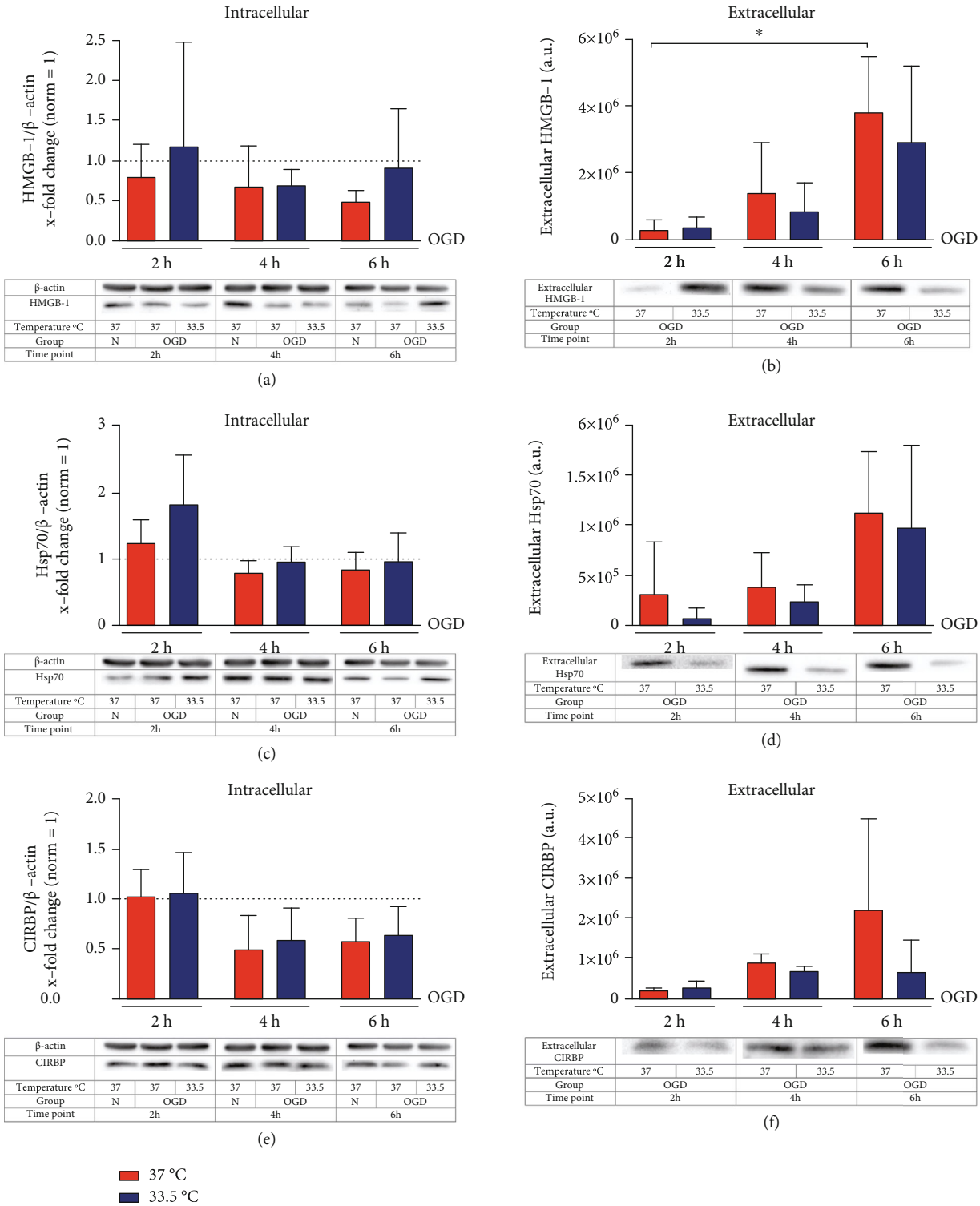

FIGURE 4: Western blot analysis of intracellular expression and extracellular release of the following DAMPs: (a, b) HMGB-1, (c, d) Hsp70, and (e, f) CIRBP after exposure to 2, 4, and 6 hours OGD at 37°C and 33.5°C, respectively. Data from at least 3 independent experiments are shown as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey posttest; $^{\#}p < 0.05$ as compared to 37°C normoxia control group and $^{*}p < 0.05$ comparison between the two experimental groups were considered significant.

increases during the OGD phase and is significantly increased in both the normothermic and hypothermic groups after 6 hours exposure to OGD, which correlates with the observed amount of necrotic cell death. No significant increase in iNOS was observed after reperfusion. Moderate TH tends to reduce the expression of iNOS in primary cardiomyocytes during OGD. Contrary to our observations in the primary cardiomyocytes, Han et al. observed a reduction in

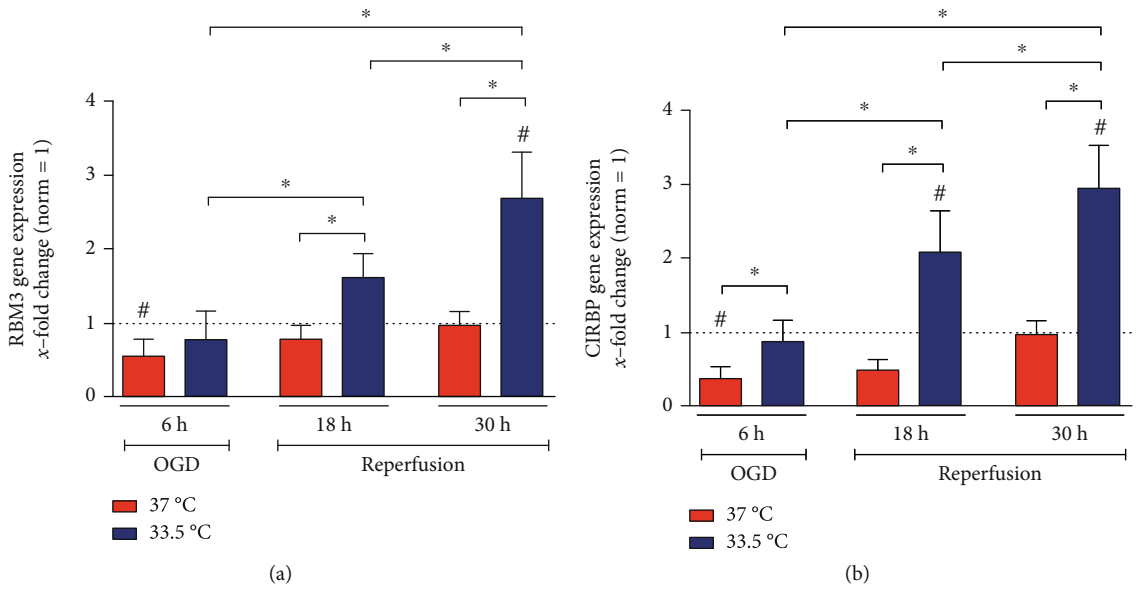

Figure 5: RT-qPCR gene expression analysis of the cold-inducible RNA binding proteins (a) RBM3 and (b) CIRBP after exposure to 6 hours OGD followed by 12 and 24 hours reperfusion (18 hours and 30 hours after experimental start, respectively) at 37°C and 33.5°C. Data from at least 3 independent experiments are shown as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey posttest; $^{\#}p < 0.05$ as compared to 37°C normoxia control group and $^{*}p < 0.05$ comparison between the two experimental groups were considered significant.

NO and iNOS protein expression after acute ischemia in a rat model of cerebral artery occlusion, as well as in a microglia culture stimulated with lipopolysaccharides (LPS) and cooled to 33°C [28]. The importance of iNOS in the context of I/R-induced injury in the literature is still controversial [29], as there are studies suggesting both damaging [30] and cardioprotective effects [31] induced by iNOS gene expression. Ultimately, the ratio between protective NO and reactive nitrogen compounds that arise during NO degradation seems to be decisive balance for either the protective or harmful effect of iNOS [29] and warrants further investigation. Moreover, iNOS gene expression is dependent on NF-κB activity and is involved in the innate immune response to proinflammatory cytokines.

The inflammatory response occurring in the context of AMI is another process that can be modulated by cooling. It has been shown *in vivo* that moderate TH inhibits the activation of microglia and weakens the inflammatory response by inhibiting the TLR-4 signaling pathway [32]. TLR-4 and other pattern recognition receptors (PRR) lead to activation of NF-κB, which enhances iNOS and cytokine production and the inflammatory response and can therefore be attenuated by cooling [33–35]. In this study, we investigated the cytokine dynamics of proinflammatory IL-1β, IL-6, and TNF-α during OGD and after reperfusion. In an *in vivo* rat model, Shi et al. demonstrated the anti-inflammatory effect of moderate TH (32°C) initiated 2 minutes after coronary artery occlusion, as measured by significant reductions in proinflammatory cytokines IL-6 and TNF-α expressions [36]. In our experimental study, gene expression levels of the investigated proinflammatory cytokines were not significantly increased during OGD/R compared to normoxic levels and also not significantly affected by TTM. These findings are not generally conflicting with the observations by Shi et al., as their *in vivo* rat model investigated the whole myocardium and measured the increase in overall inflammatory response from all cells involved [36]. However, cardiomyocytes amount to only 30-35% of the entire myocardium [37]. Additionally, nuclear translocation of the transcription factor NF-κB, which is responsible for cytokine induction, is mainly observed in interstitial cells of the myocardium and not in cardiomyocytes [38], thus correlating with the lack of immunoresponse observed in our experiments. Although primary cardiomyocytes are essentially nonimmunocompetent cells, they are generally perceptive to other detrimental stimuli such as DAMPs. Mathur and colleagues have shown that Hsp70, which has been previously described as a DAMP and has also been investigated by our group, is able to bind to the TLR-2 and cause contractile dysfunction in HL-1 cardiomyocytes [39]. This shows that the lack of immune response in the cardiomyocytes is not due to the absence of PRR but is rather based on differing intracellular signaling pathways. However, we have demonstrated that primary cardiomyocytes can nevertheless contribute to the initiation of the sterile inflammatory response through the release of DAMPs, which can potentially be attenuated by cooling (see Figure 6). This is in correlation with our previous observations of the cytoprotective properties of TTM in cardiomyocytes, especially when initiated during the OGD phase [9, 10].

This is especially interesting in the case of the antiapoptotic CIRBP, which has just recently been described as potential DAMP when secreted to the extracellular matrix [40]. Moreover, CIRBP has been shown to increase serum levels of TNF-α, IL-6, and HMGB-1 via activation of the TLR4-MD2-complex when given to healthy rats [40, 41]. This

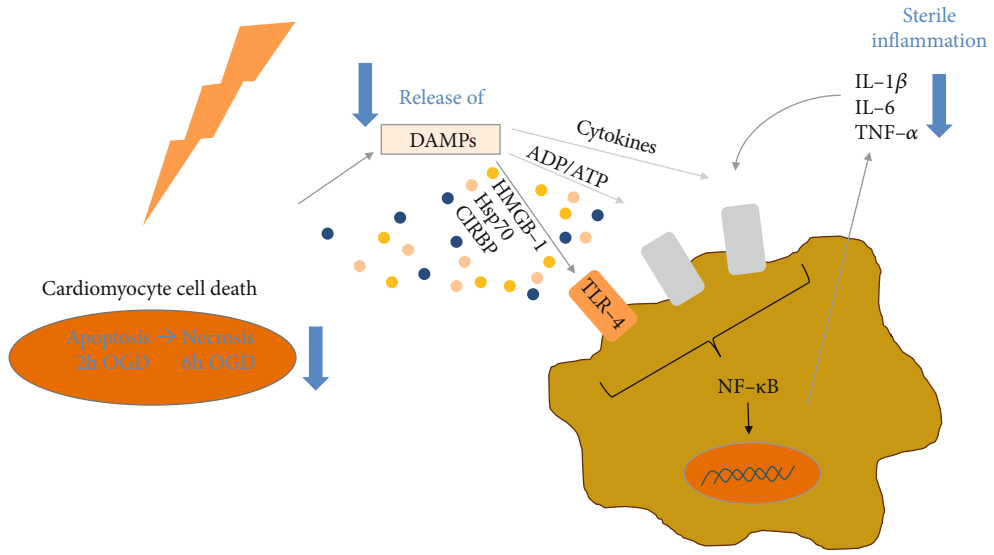

Figure 6: Synopsis of ischemia/reperfusion-induced DAMP release and the sterile inflammatory response in murine primary cardiomyocytes.

emphasizes the balancing act between induction of intracellular protective proteins and prevention of their release into the extracellular matrix via active or passive secretion mechanisms that can potentially result in damaging effects. In order to augment the potential cardioprotective effects of TTM and reduce the detrimental effects of the release of DAMPs, which include initiation of a sterile inflammatory response or worsening of clinical outcome as previously described for CIRBP, the application of DAMP-neutralizing antibodies or filters is conceivable. Godwin et al. showed significantly increased survival rates (75% vs. 37.5%) and reduced markers for hepatic damage when treated with a CIRBP-neutralizing antibody after hepatic I/R injury [42].

5. Conclusion

Targeted temperature management initiated 1 hour after exposure to OGD significantly increases murine primary cardiomyocyte viability, as observed by significant reduction in apoptotic caspase-3 activation and LDH release to the cultured supernatant. Interestingly, the underlying cell death mechanisms induced by exposure to OGD/R are dependent on the duration of the inflicted cell injury and shift from apoptosis to necrosis with increasing durations of OGD. Additionally, the release of DAMPs correlated with observed necrotic cell death and can potentially be attenuated by TTM. Our findings suggest that cardiomyocytes, although not primarily immunocompetent cells, are still able to contribute to the innate immune response occurring after AMI. However, cardiomyocytes do not contribute to the inflammatory response by directly producing proinflammatory cytokines. Therefore, the anti-inflammatory and cardioprotective effect of TTM could possibly be augmented by targeting the attenuation of DAMP release. Further mechanisms of cardioprotection initiated by TTM include the expression of cold-shock proteins RBM3 and CIRBP in the late reperfusion phase, indicating that the antiapoptotic effects of TTM are lasting beyond the acute injury phase. Our findings suggest that TTM is a promising therapeutic approach for cardioprotection and, therefore, confirm the results of many previous *in vivo* and *in vitro* studies. In order to further investigate the effects of TTM, the experimental protocol needs to be transferred to a coculture model including immunocompetent cells of the myocardium, such as macrophages. Eventually, more clinical studies will be necessary to elaborate an optimal time-temperature protocol for the induction of TTM in patients after AMI.

Abbreviations

APACHE II:	Acute Physiology And Chronic Health Evaluation II
AMI:	Acute myocardial infarction
AKT:	Protein kinase B
ATP:	Adenosine triphosphate
Bax:	Bcl-2-associated X protein
Bcl-2:	B-cell lymphoma 2
BSA:	Bovine serum albumin
CIRBP:	Cold-inducible RNA-binding protein
DAMPs:	Damage-associated molecular patterns
DMEM:	Dulbecco's modified eagle medium
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
ERK1/2:	Extracellular signal-regulated protein kinases 1 and 2
FBA:	Fetal bovine serum
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HIE:	Hypoxic/ischemic encephalopathy
HMGB-1:	High-mobility group box-1
Hsp70:	Heat shock proteins 70
IL-1β/-6/-18:	Interleukin-1β/-6/-18
iNOS:	Inducible nitric oxide synthase
IOTH:	Intra-OGD therapeutic hypothermia
I/R:	Ischemia-reperfusion

JAK-STAT: Janus kinase/signal transducers and activators of transcription
LPS: Lipopolysaccharides
Mcl-1: Myeloid cell leukemia-1
MCP-1/CCL2: Monocyte chemoattractant protein-1/CC-chemokine ligand 2
(m)RNA: (Messenger) ribonucleic acid
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B-cells
NO: Nitric oxide
OGD/R: Oxygen-glucose deprivation/reperfusion
PARP: Poly(ADP-ribose) polymerase 1
PRRs: Pattern recognition receptors
PVDF: Polyvinylidene difluoride
qPCR: Quantitative polymerase chain reaction
RBM3: RNA binding motif 3
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RIPA buffer: Radioimmunoprecipitation assay buffer
SDS: Sodium dodecyl sulfate
SOCS-3: Suppressor of cytokine signaling 3
SOFA: Sequential organ failure assessment
STAT3: Signal transducer and activator of transcription 3
TH: Therapeutic hypothermia
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor-alpha
TTM: Targeted temperature management.

Data Availability

The (experimental) data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Giang Tong and Phuong D. Lam contributed equally to the drafting of the manuscript.

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