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Mycophenolate mofetil attenuates myocardial ischemia–reperfusion injury *via* regulation of the TLR4/NF-κB signaling pathway

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It has been well documented that the Toll-like receptor 4 (TLR4)/NF-κB signaling pathway mediates early inflammatory responses during myocardial ischemia and reperfusion (MI/R). Mycophenolate mofetil (MMF), an immunosuppressive agent, has been shown to confer protective effects against ischemia/reperfusion injury, possibly through its immunosuppressive and anti-inflammatory actions. The aim of the present study was to investigate whether MMF could modulate the TLR4/NF-κB signaling, inhibit cell apoptosis and subsequently attenuate MI/R injury. MMF (20 mg/kg) or vehicle was administered to SD rats by gavage. The rats were then subjected to MI/R injury. The results showed that after MI/R, the expressions of myocardial TLR4 and NF-κB were significantly increased, and apoptosis of cardiomyocytes was induced, as evidenced by the decreased mitochondrial membrane potential (MMP), decreased Bcl-2 protein level, and increased Bax expression. Administration of MMF attenuated MI/R injury. Further studies demonstrated that MMF inhibited the induction of TLR4, NF-κB and Bax expression, and restored the expression of bcl-2. Moreover, increased myeloperoxidase activity and serum level of tumor necrotic factor α induced by MI/R injury were also inhibited by MMF treatment. In conclusion, our results demonstrated that MMF attenuates MI/R injury through inhibition of the TLR4/NF-κB signaling pathway, which led to reduced inflammatory reaction and subsequently myocardial cell apoptosis.

1. Introduction

Myocardial ischemia and reperfusion (MI/R) injury is a complex pathophysiologic process which has been found to play an important role in serious acute and chronic heart diseases such as acute myocardial infarction and angina pectoris. Although the exact mechanism responsible for MI/R injury is poorly understood, it has been generally accepted that inflammatory response and accumulation of neutrophils play a pivotal role in the pathogenesis of MI/R injury (Steffens et al. 2009; Liang et al. 2014). Toll-like receptor 4 (TLR4), one of the pattern recognition receptors, has been found to play a crucial role in the induction of inflammatory response and its activation has been linked to the activation of nuclear factor kappa B (NF-κB) in several cell lines (He et al. 2014; Weng et al. 2013; Liu et al. 2013). Up-regulation of the TRL4/NF-κB signaling pathway stimulated the release of cytokines including tumor necrotic factor α (TNF-α) and interleukin 1 (IL-1), and also induced the infiltration of leukocytes and monocytes (Arumugam et al. 2009; Wu et al. 2013). TNF-α in turn can initiate the inflammatory reaction during MI/R (Shimamoto et al. 2006). TNF-α has a negative inotropic effect, which can impair the systolic function of the myocardium, lower the blood pressure, and also promote neutrophil adhesion to endothelial cells, thus amplifying inflammation. Excessive induction of inflammatory cytokines and activation of inflammatory cells including neutrophils have been recognized as all key elements of the inflammatory response.

It well known that MI/R injury is the acceleration of both apoptosis and necrosis of cardiomyocytes at the onset of reperfusion therapy. Further studies indicated that TLR4 has been proved to play a pathogenic role in triggering cardiomyocytes apoptosis during MI/R, and the pro-apoptotic effect of TLR4 has been considered to be mediated by release of cytokines and subsequent activated inflammatory responses (Ding et al. 2013). During MI/R injury, myocardial apoptosis also can be regulated by cytokines of TNF-α and interleukin 6 (IL-6), and further enhanced by activated NF-κB pathway, formation of oxygen free radicals, as well as accumulation of the neutrophils (Hamacher et al. 2006). TLR4 could be induced during the reperfusion injury, which may lead to the release of cytokines, accumulation of neutrophils, activated inflammation response, and finally myocardial apoptosis. Increased myocardial apoptosis in turn causes release of TNF-α, which may amplify the inflammatory response by other cytokines through synergistic interaction and further worsen the myocardial function. Considering the important role of the TLR4 pathway and myocardial apoptosis, a therapeutic approach capable of inhibiting both the TLR4 pathway and subsequent cell apoptosis might be of potential to become an effective strategy for treatment of MI/R injury.

Mycophenolate mofetil (MMF) is an immunosuppressive agent and has been successfully applied in prevention and treatment of rejection reaction after organ transplantation (Halloran et al. 1997). *In vivo*, it acts as an inhibitor of the inosine-5'-monophosphate dehydrogenase enzyme, the rate-

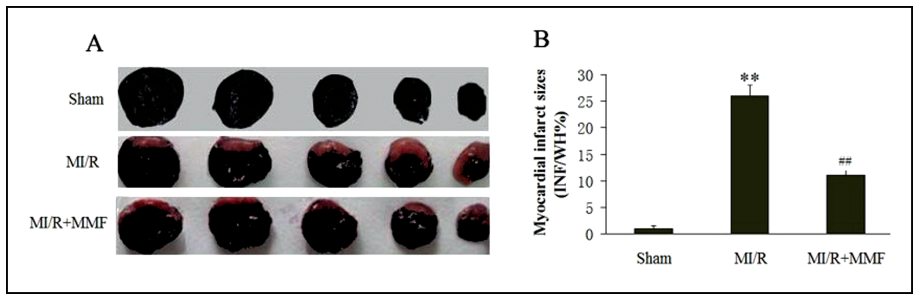

Fig. 1: Effect of MMF treatment on myocardial infarct sizes (INF/WH %) in each group. A. Representative NBT staining of samples from rat ventricles subjected to different treatment groups. B. Quantitative densitometric analysis of myocardial infarct sizes (INF/WH%). Values are presented as mean ± S.E.M.; ** $P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

controlling enzyme in the *de novo* biosynthesis of guanosine and deoxyguanosine nucleotides. MMF administration led to reduced lymphocyte proliferation and the transfer of cell adhesion molecules, which involved in mediating the inflammatory response. MMF has a variety of physiologic effects in addition to its well known immunosuppressive activity (Allison et al. 2005), which may confer a beneficial effect on ischemia reperfusion injury (Farivar et al. 2005; Liu et al. 2009). In rat model of ischemia-reperfusion injury, MMF treatment effectively prevented the deterioration of renal function and interstitial fibrosis. Moreover, the potential mechanisms underlying the above protective effects of MMF has been associated with decreased expressions of transforming growth factor-β1, monocyte chemotactic protein 1 in macrophages (Jiang et al. 2012; Yang et al. 2007; Chávez et al. 2007). A previous study has demonstrated that TLR4 signaling was involved in the development of renal ischemia/reperfusion injury, and MMF treatment was associated with down-regulated TLR4 signaling, reduced inflammatory responses and improved renal function (Zhang et al. 2013). As to its effects in the cardiovascular system, it has been demonstrated that MMF could be used as a powerful immunosuppressant in cases of heart transplantation because it could postpone the onset of cardiac allograft vasculopathy (CAV) and reduce its progression (Kaczmarek et al. 2006; Dandel et al. 2010). Interestingly, TLR4 signaling and myocardial apoptosis were also involved in the pathogenesis of MI/R injury (Tian et al. 2010; Hua et al. 2007). Therefore we hypothesized that MMF may have a beneficial effect on MI/R injury *via* regulation of the TLR4 pathway and subsequent attenuation of the myocardial apoptosis.

2. Investigations, and results

2.1. MMF reduced the area of myocardial infarction induced by MI/R

The effect of MMF treatment on sizes of myocardial infarction was determined by NBT staining. As shown in Fig. 1, the area of light red represents the infarction region and the dark blue area represents normal myocardial tissue. Compared with the MI/R group, MMF treatment significantly reduced the sizes of myocardial infarction ($p < 0.01$).

2.2. MMF attenuated the morphological disorders in myocardium induced by MI/R

Myocardium from the sham group was normal in morphology, with cell membranes remaining integrated, and no characteristics of apoptosis, necrosis, or other pathological changes. The myocardium from the MI/R group showed local swelling, myocardial necrosis, distorted cardiac muscles and breaked fibers; whereas treatment with MMF attenuated the above morphological disorders, as represented by well-arranged myocardial fibers and integrated structure (Fig. 2).

2.3. The effect of MMF on mitochondrial membrane potential (ψ)

The mitochondrial membrane potential (MMP) was determined as an index of early myocardial apoptosis by JC-1 assay. JC-1 is a cationic dye that accumulates in mitochondria. Monomers of JC-1 dye fluoresce in the green range, whereas JC-1 aggregates fluoresce in the red range. Therefore, a decrease in red fluorescence intensity represents mitochondrial swelling. Dominance of red fluorescence over green fluorescence is a feature of normal myocardial cells, suggesting preservation of functional integrity of mitochondria. In the present study a significantly decreased ratio of red fluorescence to green fluorescence was observed in the MI/R group compared with the sham group. Whereas the ratio was markedly increased in the MMF treated group compared with MI/R group, indicating that MMF could partially restore the reduced MMP, therefore inhibiting early myocardial apoptosis (Fig. 3).

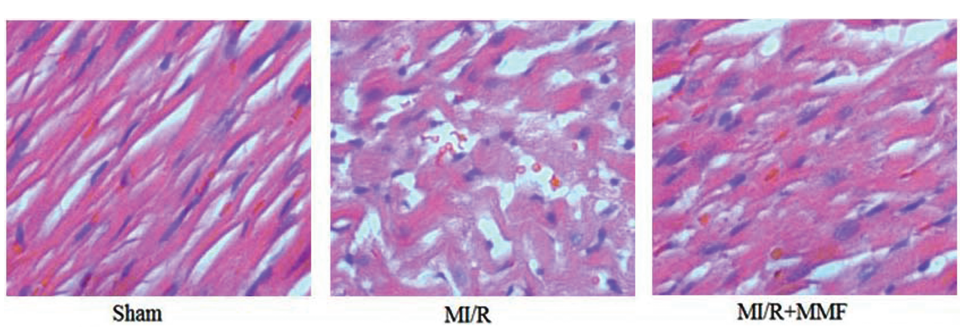

Fig. 2: Effect of MMF treatment on histopathological changes in heart tissue of different groups. Sham group: The myocardial fibers are well arranged with normal staining of the nuclei. MI/R group: myocardial fibers are partially ruptured and lysed. Several red blood cells are observed. MI/R + MMF group: Morphological change significantly improved with only slight edema in the interstitial tissues.

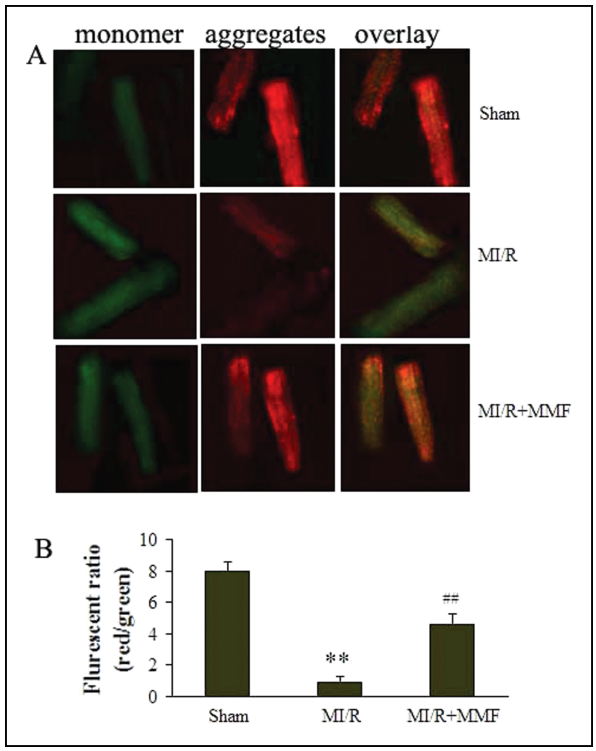

Fig. 3: Effect of MMF on MMP of the cardiomyocytes in each group. A: the fluorescence of JC-1 monomers (green) and JC-1 aggregates (red) B: Ratio of red to green fluorescence. Values are presented as mean ± S.E.M.; $^{**}P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

2.4. The effect of MMF on Bcl-2 and Bax expression

The Bcl-2 family consists of pro-apoptotic (e.g. Bax) and anti-apoptotic (e.g. Bcl-2) members. The interactions between pro-apoptotic and anti-apoptotic proteins regulated the possibility of cells to survive or undergo apoptosis after a certain stimulus or injury. So in the present study, expressions of Bax and Bcl-2 in the myocardium were also determined as another index of myocardial apoptosis by western blot. Results of our current study showed that myocardial Bax expression was significantly increased, while Bcl-2 expression was significantly decreased in the MI/R group as compared with the sham group (Fig. 4). MMF administration increased the expression of Bcl-2 while decreased the expression of Bax compared with those in myocardium from the MI/R group, indicating that MMF may reduce myocardial apoptosis subjected to MI/R injury by regulation of Bcl-2 family proteins.

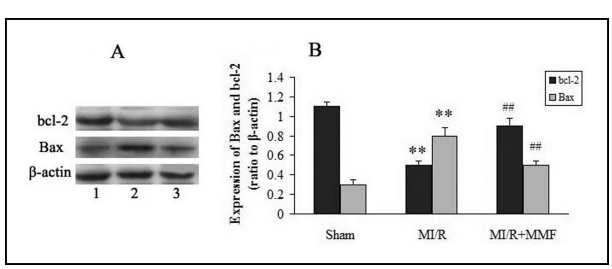

Fig. 4: Effect of MMF treatment on expressions of Bax and bcl-2 proteins in each group as detected by western blot. A. Representative Immunoblots of samples from rat ventricles subjected to different treatment groups. B. Quantitative densitometric analysis of Bax and bcl-2 with β-actin as an internal standard. 1: sham group; 2: MI/R group; 3: MI/R + MMF group. Values are presented as mean ± S.E.M.; $^{**}P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

2.5. MMF reduced TLR4 expression in the myocardium subjected to MI/R

Protein level of TLR4 was determined by immunohistochemistry and western blot in the current study. As shown in Fig. 5, TLR4 expression was significantly increased in the myocardium of MI/R group as compared with that from the sham group. Interestingly, treatment with MMF reduced the protein level of TLR4 in the myocardium as proved by both the immunohistochemistry and western blot, which suggested that inhibition of TLR4 may be involved in the protective effects of MMF to MI/R.

2.6. MMF reduced NF-κB in the myocardium subjected to MI/R

To further investigate the effect of MMF on TLR4 mediated signaling pathway, NF-κB, a downstream molecule of the TLR4 signaling pathway was determined. The results from western blotting analyses showed that the expression of NF-κB was up-regulated in the MI/R group compared with sham group, whereas administration of MMF sigficantly decreased the expression of NF-κB. These results demonstrated that inhibition of TLR4/NF-κB signaling pathway may be an important mechanism underlying the potential protective effects of MMF to MI/R (Fig. 6).

2.7. MMF inhibited the infiltration of the neutrophilic cells in the myocardium subjected to MI/R

The activity of MPO in the myocardium reflects, at least in part, the infiltration of neutrophilic cells. Results of our study indicated that the MPO activity in the myocardium from the MI/R group was dramatically higher as compared with that from the sham group. Moreover, MMF treatment significantly decreased myocardial MPO activity, suggesting that MMF can inhibit the infiltration neutrophilic cells in MI/R (Fig. 7).

2.8. MMF inhibited TNF-α level in the serum subjected to MI/R

In order to further verify the effect of MMF on the TLR4/NF-κB signaling, serum TNF-α levels were determined in rats from each groups. As shown in Fig. 8, MMF significantly reduced the serum level of TNF-α as compared with the MI/R group, demonstrating that treatment with MMF may confer its cardioprotective effect by reducing TNF-α in MI/R injury.

3. Discussion

In the present study, we provided the evidence for the first time that administration of MMF before MI/R attenuated the area of myocardial infarction, improved morphological disorders in myocardial issues subjected to MI/R injury. Furthermore, this potential cardioprotective effect of MMF may be related to the inhibition of TLR4 and NF-κB signaling, reduction of myocardial cell apoptosis, attenuation of neutrophilic cell accumulation, and inhibition of serum TNF-α in MI/R injury. These results indicated that MMF treatment has protective effect against MI/R injury probably *via* inhibition of the TLR4/NF-κB signaling pathway, and subsequent myocardial apoptosis and inflammatory response.

The most effective therapy for ischemia-induced myocardium damage is to restore the blood supply of the myocardium immediately. However, reperfusion therapy itself can cause injury to the myocardium, probably *via* a robust local and systemic inflammatory response which may deteriorate tissue injury and

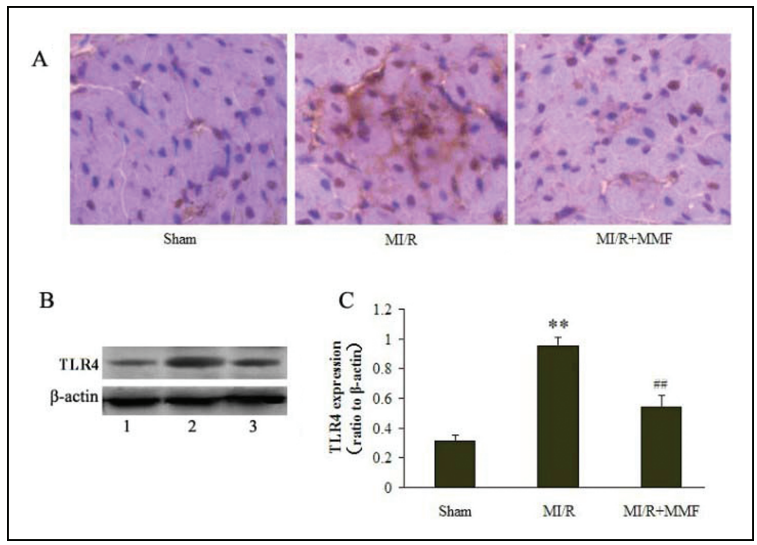

Fig. 5: Effect of MMF treatment on myocardial TLR4 expression in each group. A: Representative immunohistochemistry of TLR4 from rat ventricles. The brown in the cytoplasm repents the expression of TLR4. B. Representative immunoblots of samples from rat ventricles subjected to different treatment groups. C. Quantitative densitometric analysis of TLR4 protein with β-actin as an internal standard. 1: sham group; 2: MI/R group; 3: MI/R + MMF group. Values are presented as mean ± S.E.M.; $^{**}P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

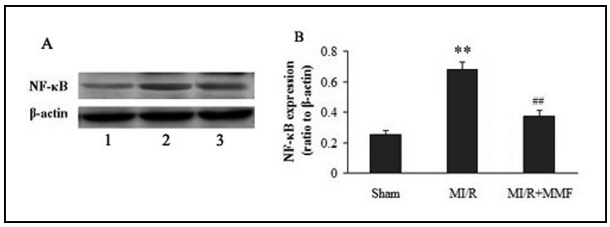

Fig. 6: Effect of MMF treatment on myocardial NF-κB expression in each group. A. Representative immunoblots of samples from rat ventricles subjected to different treatment groups. B. Quantitative densitometric analysis of NF-κB protein with β-actin as an internal standard. 1: sham group; 2: MI/R group; 3: MI/R + MMF group. Values are presented as mean ± S.E.M.; $^{**}P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

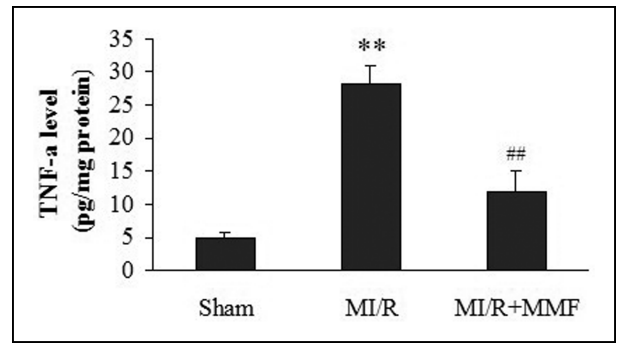

Fig. 8: Effect of MMF treatment on serum TNF-α level in each group. MMF treatment significantly deduced the TNF-α level subjected to MI/R injury. Values are presented as mean ± S.E.M.; $^{**}P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

adversely affect left ventricular recovery (Frangogiannis et al. 2002). Toll-like receptor 4 (TLR4), has been recognized as one of the pattern recognition receptors not only participating in the immune response, but also contributing to the inflammatory response of I/R injury (Ha et al. 2010; Li et al. 2010; Kaczorowski et al. 2009). TLR4-deficient mice have been found to have significantly smaller infarctions, fewer neutrophilic infiltrations and fewer lipid peroxides and less complement deposition compared with control mice after MI/R (Oyama et al. 2004), which is consistent with the notion that besides its role in innate immune responses, TLR4 serves as a proinflammatory agent in murine myocardial ischemia-reperfusion injury. It has also been reported that TLR4 deficiency led to activated ERK and PI3K pathways, which further attenuated I/R induced p38 and JNK signaling activation and eventually conferred protective effects to myocardium from I/R injury (Hua et al. 2007; Zhao et al. 2009). In addition, deletion of TLR4 abrogated both systemic and intragraft inflammatory responses induced by cold I/R (Kaczorowski et al. 2007). Also, studies have indicated that the effect of TLR4 in regulation of inflammatory response in MI/R injury was related to activation of NF-κB and other pro-inflammatory cytokines including TNF-α and IL-1β, et al. (Yang et al. 2011; van and Bogers 2008; Ding et al. 2012). In our study, enhanced expressions of TLR4 and NF-κB were observed during MI/R injury, which were consistent with these previous observations. Interestingly, administration of MMF before MI/R injury markedly reduced the expressions of TLR4 and NF-κB, in parallel with a reduced inflammatory response as indicated by lowered TNF-α and MPO levels, which may underlying its effects of reduced infarction area and improved morphological change. These results demonstrated that MMF may confer a protective effect to MI/R probably due to its anti-inflammatory properties.

Another main component of MI/R injury is myocardial apoptosis, which has been found to be initiated during ischemia while largely executed during reperfusion (Zhao et al. 2000). Using a gene defective mice model, Ding et al. (2013) demonstrated that TLR4 and its related pathway contributed to the development of

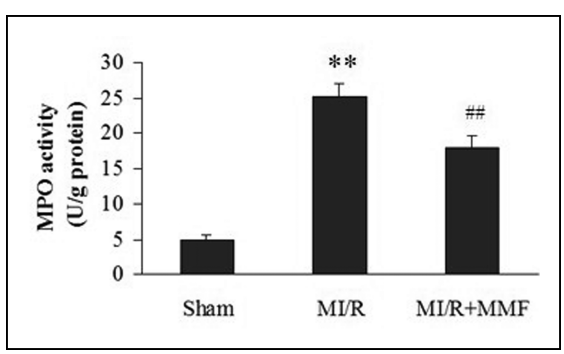

Fig. 7: Effect of MMF treatment on MPO activity in the myocardium from rats in each group. MMF treatment significantly deduced the MPO activity subjected to MI/R injury. Values are presented as mean ± S.E.M.; $^{**}P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. MI/R group.

myocardial apoptosis. In order to further investigate the mechanism of MMF on MI/R injury, we examined mitochondrial status and the expressions of Bax and Bcl-2, two important pathophysiologic processes involved in the regulation of myocardial apoptosis. Apoptosis can be initiated *via* two alternative signal pathways: the extrinsic pathway, which acts through death receptors on cell surfaces, and the intrinsic pathway, which acts through the mitochondria (Cohen 1997). Disruption of MMP was associated with early apoptosis resulting from MI/R injury (Wang et al. 2013). The mitochondria-dependent pathway is regulated by members of the Bcl-2 family proteins which including both the pro-apoptotic proteins (Bax) and the anti-apoptotic proteins (Bcl-2). As shown in the current study, the ratio of JC-1 aggregates to monomers decreased, and pro-apoptotic (Bax) protein expression increased while anti-apoptotic (Bcl-2) protein expression decrease in the MI/R group. However, MMF treatment not only inhibited the TLR4/NF-κB pathway, but also attenuated myocardial apoptosis, as confirmed by increased MMP, Bcl-2 expressions and decreased Bax expression. It has been demonstrated that apoptosis can also be induced by inflammatory cytokines such as TNF-α (Xu et al. 2011) and IL-6, which can be enhanced by TLR4 and its-related pathway (Ding et al. 2013). Excessive TNF-α after I/R interaction with TNF-α receptor type 1 (TNFR1) could induce cell apoptosis (Chen et al. 2007; Shen et al. 2006), which in turn amplify the inflammatory response. Subsequently, augmented inflammatory response and apoptosis work in concert to worsen the cardiac function. It is noteworthy that results from our current study revealed the both TNF-α level and apoptotic regulatory proteins were dramatically reduced by administration of MMF compared with that of MI/R group.

Despite the fact that our study provide the first evidence that MMF treatment may protect the myocardium form MI/R by inhibition of TLR4/NF-κB signaling pathway, and subsequent myocardial apoptosis and inflammation, our study also have limitations. We measured the effect of MMF on inflammatory response and apoptosis just using pharmacological method in a rat model, which cannot indicate a causative relationship between MMF administration and reduced inflammatory response or attenuated apoptosis. Therefore, further study using signaling inhibitors or small interfering RNAs were still needed. In conclusion, our study demonstrated that the administration of MMF before MI/R confers beneficial effect to the heart, and the mechanism may involve in the down regulation of TLR4/NF-κB pathway, and reducing of myocardial apoptosis. MMF, a powerful immunosuppressant capable of delay the onset of cardiac allograft vasculopathy (CAV) and reduce its progression (Kaczmarek et al. 2006; Dandel et al. 2010), also have cardioprotective effect in a rat model of MI/R. These results provide the theoretical basis for that use of MMF as part of an immunosuppressant regimen may also confer potential protective effects against MI/R injury.

4. Experimental

The experimental protocols were approved by Committee of Shangdong University for the Use of Experimental Animals for Research and Teaching.

4.1. Animal model of myocardial ischemia–reperfusion and experimental protocols

Male SD rats (250–300 g) were subjected to 30 min of myocardial ischemia and 2 h of reperfusion, as described previously (Hadi et al. 2013). Briefly, rats were anesthetized with pentobarbital sodium (45 mg/kg, IP). Ischemia was induced by exteriorizing the heart with a left thoracic incision, and followed by a slipknot (5–0 silk) around the left anterior descending coronary artery (LAD). After 30 min of ischemia, the slipknot was released and the animal received 120 min of myocardial reperfusion.

Rats were randomly assigned to one of the following three experimental groups: (1) sham group, for which the silk was drilled underneath the LAD but the LAD was not ligated; (2) MI/R group, for which LAD was ligated for 30 min and followed by 120 min of reperfusion; (3) MI/R + MMF group, for which MMF (20 mg/kg, dissolved in 0.5% sodium carboxymethylcellulose) was daily administered by gavage 5 days prior to the induction of ischemia until the animals were killed (Liu et al. 2009).

4.2. Determination of the size of myocardial infarction

At the end of reperfusion, size of myocardial infarction was determined by NBT staining technique with a digital imaging system. The heart tissues were quickly removed to a −20°C refrigerator for cryopreservation, before they were cut into 1 mm slices. The tissue sections were placed in 1% NBT solution and incubated for 15 min, and then placed in 4% formaldehyde solution overnight. NBT stained area (dark blue after staining, non-infarct area) and non-NBT stained area (light red area after staining, infarct area) were analyzed with a digital imaging system by computer. Proportions of infarct myocardial to the whole myocardial tissues (infarct area/whole heart area %) were then calculated.

4.3. HE staining and immunohistochemical evaluation

Myocardial tissues were fixed in 4% formaldehyde solution overnight before they were gradiently dehydrated with ethanol. After 24 h, tissues were embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin-eosin (HE) staining to evaluate morphological changes. For immunohistochemical analyses of TLR4, paraffin was removed, and 10% rabbit serum was used to block non-specific binding sites within these slices. After being rinsed in PBS once, the sections were treated with TLR4 primary antibody, and then incubated in 4 °C overnight. The sections were rinsed in PBS three times, treated with secondary antibody, and incubated in 37 °C for another 1 h. The slices were subsequently rinsed in PBS for three times, treated with DAB, washed with PBS, counterstained with hematoxylin, and mounted for observation under light microscopy.

4.4. Western blot

The protein concentrations of the samples were determined by the Bradford method. After boiling the samples for 5 min, the protein samples were fractionated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked with milk powder at room temperature for 2 h, before they were incubated with primary antibodies of TLR4, NF-κB, Bax and Bcl-2 at 4 °C overnight. Following primary antibody incubations, membranes were further incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgGs). Detection of the bands was performed with enhanced chemiluminescence reagents (ZSGB-BIO, China).

4.5. Isolation of the cardiomyocytes

After reperfusion for 2 h, the heart was quickly excised, mounted on a perfusion system, and perfused *via* the aorta with Tyrode solution under constant flow conditions (10 ml/min) for 5 min, which was followed by perfusion with nominally Ca^{2+}-free Tyrode for another 8 min. Tissue digestion was initiated by adding 18 mg type II collagenase into 50 ml of Ca^{2+}-free Tyrode, which contained 20 mg BSA. After 10 min, the type II collagenase was washed out by 5 min perfusion with Ca^{2+}-free Tyrode. All the solutions were inflated with 95% O_2 and 5% CO_2. The right ventricle was cut off first, and then the interventricular septum followed by the left ventricle. Separate ventricular parts were dispersed mechanically, and cardiomyocyte solutions were adjusted to the same cell density and transferred to culture medium.

4.6. Assessment of mitochondrial membrane potential (ψ)

Cardiomyocytes were incubated with of 2 μM JC-1 at 37 °C for 20 minutes, and centrifuged at 600 g for 4 min. After the supernatant was aspirated, the cells with JC-1 buffer solution were resuspended. The above centrifugation, aspiration, and resuspension were repeated. Thereafter, labeled cells were analyzed and quantified by fluorescence microscope (Leica. DMI3000 B, Germany).

4.7. Detection of levels of myeloperoxidase

Each neutrophilic cell contains a certain amount of MPO. The characteristics of hydrogen peroxide reduction can be used to analyze the activity of the enzyme and quantitatively determination of the amount of the neutrophilics. Therefore, the activities of MPO may reflect the degrees of neutrophilic infiltration in the myocardium. After reperfusion, the myocardial tissue was placed at −20°C refrigerator for preservation. MPO test kits were applied to determine the activities of MPO in the myocardial tissue.

4.8. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected at the time of heart harvest, and the serum was prepared and assayed for TNF-α by ELISA kit following the manufacturers' instructions.

4.9. Statistic analysis

All data are expressed as mean ± standard errors (SEM). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test. Differences were considered as statistically significant if $p < 0.05$.

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References

Allison AC (2005) Mechanism of action of mycophenolate mofetil. Lupus 14: s2-s8.

Arumugam TV, Okun E, Tang SC, Thundyil J, Taylor SM, Woodruff TM (2009) Toll-like receptors in ischemia–reperfusion injury. Shock 32: 4–16.

Chávez-Velásquez M, Pons H, Medina M, Quiroz Y, Parra G, Herrera J (2007) Effects of mycophenolate mofetil in ischemic acute renal failure in rats. Nefrologia 27: 448–458.

Chen CC, Young JL, Monzon RI, Chen N, Todorovic V, Lau LF (2007) Cytotoxicity of TNFalpha is regulated by integrin-mediated matrix signaling. EMBO J 26: 1257–1267.

Cohen GM (1997) Caspases: The executioners of apoptosis. Biochem J 326(Pt 1): 1–16.

Dandel M, Hetzer R (2010) Impact of immunosuppressive drugs on the development of cardiac allograft vasculopathy. Curr Vasc Pharmacol 8: 706–719.

Ding HS, Yang J, Gong FL, Yang J, Ding JW, Li S, Jiang YR (2012) High mobility group box 1 mediates neutrophil recruitment in myocardial ischemia-reperfusion injury through toll like receptor 4-related pathway. Gene 509: 149–153.

Ding HS, Yang J, Chen P, Yang J, Bo SQ, Ding JW (2013) The HMGB1-TLR4 axis contributes to myocardial ischemia/reperfusion injury via regulation of cardiomyocyte apoptosis. Gene 527: 389–393.

Farivar AS, MacKinnon-Patterson B, Barnes AD, Mulligan MS (2005) The effect of anti-inflammatory properties of mycophenolate mofetil on the development of lung reperfusion injury. J Heart Lung Transplant 24: 2235–2242.

Frangogiannis NG, Smith CW, Entman ML (2002) The inflammatory response in myocardial infarction. Cardiovasc Re 53: 31–47.

Ha T, Liu L, Kelley J, Kao R, Williams D, Li C (2011) Toll-like receptors: new players in myocardial ischemia/reperfusion injury. Antioxid Redox Signal 15: 1875–1893.

Hadi RR, Al-amran F, Yousif M, Zamil ST (2013) Antiapoptotic effect of simvastatin ameliorates myocardial ischemia/reperfusion injury. ISRN Pharmacol 2013: 815094.

Halloran P, Mathew T, Tomlanovich S (1997) Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. Transplantation 63: 39–47.

Hamacher-Brady A, Brady NR, Gottlieb RA (2006) The interplay between pro-death and pro-survival signaling pathways in myocardial ischemia/reperfusion injury: apoptosis meets autophagy. Cardiovasc. Drugs Ther 20: 445–462.

He W, Wang Z, Zhou Z, Zhang Y, Zhu Q, Wei K, Lin Y, Cooper PR, Smith AJ, Yu Q (2014) Lipopolysaccharide Enhances Wnt5a Expression through Toll-like Receptor 4, Myeloid Differentiating Factor 88, Phosphatidylinositol 3-OH Kinase/AKT and Nuclear Factor Kappa B Pathways in Human Dental Pulp Stem Cells. J Endod 40: 69–75.

Hua F, Ha T, Ma J, Li Y, Kelley J, Gao X, Browder IW, Kao RL, Williams DL, Li C (2007) Protection against myocardial ischemia/reperfusion injury in TLR4-deficient mice is mediated through a phosphoinositide 3-kinase-dependent mechanism. J Immunol 178: 7317–7324.

Jiang S, Tang Q, Rong R, Tang L, Xu M, Lu J, Jia Y (2012) Mycophenolate mofetil inhibits macrophage infiltration and kidney fibrosis in long-term ischemia-reperfusion injury. Eur J Pharmacol 688: 56–61.

Kaczmarek I, Ertl B, Schmauss D, Sadoni S, Knez A, Daebritz S, Meiser B, Reichart B (2006) Preventing cardiac allograft vasculopathy: long-term beneficial effects of mycophenolate mofetil. J Heart Lung Transplant 25: 550–556.

Kaczorowski DJ, Nakao A, Mollen KP, Vallabhaneni R, Sugimoto R, Kohmoto J (2007) Toll-like receptor 4 mediates the early inflammatory response after cold ischemia/reperfusion. Transplantation 84: 1279–1287.

Kaczorowski DJ, Nakao A, McCurry KR, Billiar TR (2009) Toll-Like Receptors and Myocardial Ischemia/Reperfusion, Inflammation, and Injury. Curr Cardiol Rev 5: 196–202.

Li Y, Si R, Feng Y, Chen HH, Zou L, Wang E (2011) Myocardial ischemia activates an injurious innate immune signaling via cardiac heat shock protein 60 and Toll-like receptor 4. J Biol Chem 286: 31308–31319.

Liang X, Huang J, Lin X, Qin F, Wen Q, Chen C (2014) The Effect of 17-Methoxyl-7-Hydroxy-Benzene-Furanchalcone on NF-κB and the Inflammatory Response During Myocardial Ischemia Reperfusion Injury in Rats. J Cardiovasc Pharmacol 63: 68–75.

Liu H, Chen K, Feng W, Wu X, Li H (2013). TLR4-MyD88/Mal-NF-κB Axis Is Involved in Infection of HSV-2 in Human Cervical Epithelial Cells. PLoS One 8: e80327.

Liu YX, Jin LM, Zhou L, Xie HY, Jiang GP, Wang Y, Feng XW, Chen H, Yan S, Zheng SS (2009) Mycophenolate mofetil attenuates liver ischemia/reperfusion injury in rats. Transpl Int 22: 747–756.

Oyama J, Blais C Jr, Liu X, Pu M, Kobzik L, Kelly RA (2004) Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. Circulation 109: 784–789.

Shimamoto A, Chong AJ, Yada M, Shomura S, Takayama H, Fleisig AJ (2006) Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia–reperfusion injury. Circulation 114(1 Suppl):1270–1274.

Steffens S, Montecucco F, Mach F (2009) The inflammatory response as a target to reduce myocardial ischemia and reperfusion injury. Thromb Haemost 102: 240–247.

Shen HM, Pervaiz S (2006) TNF receptor superfamily-induced cell death: redox-dependent execution. FASEB 20: 1589–1598.

Tian Y, Zhang W, Xia D, Modi P, Liang D, Wei M (2011) Postconditioning inhibits myocardial apoptosis during prolonged reperfusion via a JAK2-STAT3-Bcl-2 pathway. J Biomed Sci 18: 53.

Van der Kaaij NP, Bogers AJ (2008) Cytokines link Toll-like receptor 4 signaling to cardiac dysfunction after global myocardial ischemia. Invited commentary. Ann Thorac Surg 85: 1685.

Wang Y, Li X, Wang X, Lau W, Wang Y, Xing Y, Zhang X, Ma X, Gao F. Zhang X, Ma X, Gao F (2013) Ginsenoside Rd attenuates myocardial ischemia/reperfusion injury via Akt/GSK-3β signaling and inhibition of the mitochondria-dependent apoptotic pathway. PLoS One 8: e70956.

Weng H, Deng Y, Xie Y, Liu H, Gong F (2013) Expression and significance of HMGB1, TLR4 and NF-κB p65 in human epidermal tumors. BMC Cancer 13: 311.

Wu Y, Liu Y, Huang H, Zhu Y, Zhang Y, Lu F, Zhou C, Huang L, Li X, Zhou C (2013) Dexmedetomidine inhibits inflammatory reaction in lung tissues of septic rats by suppressing TLR4/NF-κB pathway. Mediators Inflamm 2013: 562154.

Xu H, Yao Y, Su Z, Yang Y, Kao R, Martin CM, Rui T (2011) Endogenous HMGB1 contributes to ischemia-reperfusion-induced myocardial apoptosis by potentiating the effect of TNF-α/JNK. Am J Physiol Heart Circ Physiol 300: H913–921.

Yang B, Harris KP, Jain S, Nicholson ML (2007) Caspase-7, Fas and FasL in long-term renal ischaemia/reperfusion and immunosuppressive injuries in rats. Am J Nephrol 27: 397–408.

Yang J, Zhang XD, Yang J, Ding JW, Liu ZQ, Li SG, Yang R (2011) The cardioprotective effect of fluvastatin on ischemic injury via down-regulation of toll-like receptor 4. Mol Biol Rep 38: 3037–3044.

Zhang YX, Zhang JR, Wang ZG (2013) Mycophenolate mofetil affects monocyte Toll-like receptor 4 signaling during mouse renal ischemia/reperfusion injury. Chin Med J (Engl) 126: 1224–1229.

Zhao P, Wang J, He L, Ma H, Zhang X, Zhu X, Dolence EK, Ren J, Li J (2009) Deficiency in TLR4 signal transduction ameliorates cardiac injury and cardiomyocyte contractile dysfunction during ischemia. J Cell Mol Med 13: 1513–1525.

Zhao Z-Q, Nakamura M, Wang N-P, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J (2000) Reperfusion induces myocardial apoptotic cell death. Cardiovascular Research 45: 651–656.