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LPS-pretreated bone marrow stem cells as potential treatment for myocardial infraction

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1. ABSTRACT

The purpose of this study is too investigate the distribution, the number, and the survival time of rat bone marrow mesenchymal stem cells (BMSCs), implanted in rats with myocardial infarction (MI), that were initially pretreated with low dose of LPS. Adult rats were randomly divided into control group, the MI group, 0.1, 1 and 5 mg/kg LPS-pretreated groups. Following the injection of CM-Dil labeled into the mid left anterior descending coronary artery ligation in the left ventricle wall, the number and survival time of red fluorescent labeled BMSCs were determined in the myocardial infarction 48h, 1, 3, and 4 weeks post-injection.. In addition, BMSC cell survival and apoptosis as well as the expression of associated proteins were also studied. After 1 week, the number of red fluorescent cells decreased significantly in MI group and 0.1mg/kg LPS groups, and the decrease was more obvious in 5mg/kg LPS group. However, a large number of BMSCs was apparent in 1mg/kg LPS group, the survival of positive cells was higher than MI group ($P < 0.05$), part of the transplanted stem cells migrated into the injured area of the myocardial tissue, and in the injury centers more cardiac stem cells were grouped together. Following 3-4 weeks post-implantation of BMSCs, fluorescence almost disappeared in MI group , while it was still present and scattered in 1mg/kgLPS. Furthermore, while BMSCs showed low survival rate in the MI group, reduced apoptosis was observed in low-dose LPS groups.

2. INTRODUCTION

Patients with acute myocardial infarction (AMI) could develop myocardial necrosis, ventricular remodeling and resultant congestive heart failure (1). Mesenchymal stem cells (MSCs)-based regenerative therapy is currently regarded as an alternative approach to treat MI (2-4).

Bone marrow stem cells (BMSCs) represent a novel treatment modality with increasing therapeutic potential (1, 2). Bone marrow hematopoietic stem cells (HSCs) (3) and non-hematopoietic MSCs (4) have exhibited positive remodeling and regeneration of viable tissues. Recent pre-clinical and clinical studies have demonstrated that MSCs transplantation can achieve ventricular remodeling and augment cardiac function when implanted into infracted myocardium. However, the massive death of transplanted cells remains a major challenge in heart cell therapy (5). The underlying cause of unusually high cell death is multifactorial. Among various potential mechanisms, the dynamics of the early phase of cell death implicates local immune and inflammatory responses, loss of trophic factors and local tissue ischemia as the prime factors (6). However, MSC survival in such challenging conditions for some time is unclear.

Toll-like receptors (TLRs) are a class of molecules, which play an important role in the innate immune system for the recognition of

pathogen-associated molecular patterns by immune cells, thereby initiating a primary response towards invading pathogens and recruitment of the adaptive immune response (7). Cardiomyocyte Toll-like receptor 4 is involved in heart dysfunction following septic shock or myocardial ischemia (8). Lipopolysaccharide (LPS) is the antigenic component of the gram-negative bacterial cell wall and is known as the ligand of Toll-like receptor-4 (TLR4) (9, 10). LPS-induced cell death in dorsal root ganglion cell cultures depends on the TLR4 (11). Previous study showed that 1.0 μg/ml LPS protected MSCs against oxidative stress induced-apoptosis, and enhanced their survival and proliferation via TLR4 and PI3K/Akt pathway (12, 13). However, the mechanism of activation of TLR4 and LPS protecting the transplanted cells are not understood.

In the present study, MSCs were initially treated with 0.5, 1 or 5mg/kg of LPS, and subsequently transplanted into ischemic myocardium to study the survival and cardiac protective capacity of MSCs in a rat acute myocardial infarction model. Furthermore, potential underlying mechanisms, including the role of TLR4 in the signal pathway mediating LPS-induced cardiac protection were investigated.

3. MATERIALS AND METHODS

3.1. Animals preparation

Eight to teen week-old male SD rats (n=50) of 205.6±13.5g were obtained from experimental animal center of Nanjing Medical University (Nanjing, China). The procedure was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (*NIH Publication No. 85-23, National Academy Press, Washington, DC, revised 1996*).The study protocol was approved by the Animal Care and Use Committee of Nanjing Medical University.

3.2. Isolation and expansion of MSCs

Isolation of MSCs from mice was performed as previously described with minor modifications (14). Briefly, mice were sacrificed humanely and femurs were aseptically harvested. Whole marrow cells were obtained by flushing the bone marrow cavity with low glucose Dulbecco's Modified Eagle's Medium (L-DMEM, Hyclone, USA). Cells were centrifuged at 1000 × g for 5 minutes and the supernatant was removed. The cell pellet was then re-suspended with L-DMEM supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 U/ml streptomycin (Invitrogen, Carlsbad, CA), and incubated at 37°C in 5% CO2 atmosphere. After 24 and 48 hours, non-adherent cells in suspension were discarded and culture media was replaced every three days thereafter. When MSCs reached 70% of confluence, they were trypsinized with 0.25% trypsin- EDTA (Sigma-Aldrich, USA), and then re-plated in culture flasks at a density of $5 \times 10^4/cm^2$. Cells from early passages (2 and 3) only, were utilized for experiment. MSCs were confirmed by cytometry analysis of cell surface markers FITC-labeled, including CD44, CD29, CD45, CD31 and CD34.

3.3. The labeling of BMSCs

To label MSCs with 1,1'-dioctadecyl-3,3,3'3'-testramethylindo-carbocyanine perchlorate (DiI), 2.5 mg/L of DiI was added to MSCs suspension and incubated at 37°C for 5 minutes, then subsequently at 4°C for 15 minutes with occasional mixing. MSCs labeled with DiI were washed thrice in PBS, and kept frozen in the liquid nitrogen. The activity of cells was evaluated by typan blue staining.

3.4. Myocardial infarction model

SD rats were anesthetized with ketamine (100 mg/kg) by intra-peritoneal injection and mechanically ventilated. Under a sterile condition, the heart was exposed through a left thoracotomy, and the left anterior descending coronary artery was ligated just below the atrio-ventricular border. Successful ligation was identified by cyanosis of the left ventricular anterior wall and through ST-T elevation on electrocardiogram, and rats were allowed to recover under care.

3.5. MSCs transplantation

Rats supplying LPS-preconditioned MSCs were intra-peritoneally injected with 0.5, 1, and 5mg/kg of LPS two hours prior MSC-transplantation and then collected, respectively. The normal and AMI controls were intra-peritoneally injected with L-DMEM. Fifty rats were used in our experiments, 5 of them were used as normal control group. One week after AMI, 25 rats died and the remaining 20 rats were divided into 4 groups randomly and given an intra-myocardial injection with one of the following treatments: i) 5×10^6 of 0.5mg/kg LPS-preconditioned MSCs (n=5); ii) 5×10^6 of 1mg/kg LPS-preconditioned MSCs (n=5); iii) 5×10^6 of 5mg/kg LPS-preconditioned MSCs (n=5); or iv) 5×10^6 cells for MI control group (n=5); and v) the normal control rats were given an intra-myocardial injection of 5×10^6 cells (n=5). The 5×10^6 MSCs were injected into two sites of the myocardium with a 28-gauge needle, one within the infarct area and the other into the myocardium bordering the ischemic area. At the end of the experiment, the chest was closed, and animals were weaned from the ventilator and allowed to recover. Forty-eight hours, 1, 3, or 4 weeks after cell transplantation, the rats were sacrificed and hearts from different groups were harvested for the experiments described below, respectively.

3.6. HE staining and electron microscopy analysis

Rats were sacrificed 48h, 1, 3 or 4 weeks after cell transplantation,, and heart muscle samples were collected, fixed in 2% para-formaldehyde, cut transversely, embedded in paraffin, stained with hematoxylin and eosin and analyzed under light microscopy. For electron microscopy analysis, frozen tissue sections were analyzed by fluorescence microscope for CD-Dil labeled donor cells. Myocardial tissues (1 mm^3) from AMI area and normal area were fixed in 4% glutaraldehyde for 2 hours, washed thrice in PBS, dehydrated in alcohol ascending concentrations, soaked with propylene oxide for 20 min,

and embedded and polymerized with the epoxy resin (60 □). Ultrathin cryo-sections were cut at -110□ using a diamond knife (Diatome) in an ultra-cryomicrotome (Leica) and double stained with uranyl acetate and lead citrate. Images were acquired with Philips CM120 electron microscope; these morphometric studies were performed by two independent examiners, who were blinded to treatment assignment.

3.7. TUNEL assay

Rats from each group were sacrificed 48h, 1, 3 or 4 weeks after cells transplantation., and apoptotic cardiomyocytes were evaluated in the infracted heart by TUNEL assay, using *in situ* Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. Apoptotic cells were identified through the brown color in their nuclei. Tissue sections were examined microscopically and the percentage of apoptotic cells per total cells was determined in five randomly chosen fields at the magnification of 400. The apoptotic index (AI) =□the number of apoptotic cardiomyocytes/the number of total cardiomyocytes×100%.

3.8. Semi-quantitative RT-PCR

Total cellular RNA was isolated from MSCs by Trizol Reagent (Invitrogen, Carlsbad, CA). The oligo (dT)-primed first-strand cDNA was synthesized from 1 μg total RNA using the Superscript II kit (Invitrogen, Carlsbad, CA). The following specific primers were used in the experiments: TLR4 (374 bp) forward primer 5'-AGAAGAAGGAGTGCCCCGCT-3' and TLR4 reverse primer 5'-AGGCTTTTCCATCCAACAGG-3'; NF-kB (523bp) forward primer 5'-CAACACAGGCATCACCCAT-3', and NF-kB reverse primer 5'-CAGCAAGTCCTCCACCACA-3'; and MyD88 (646bp) forward primer 5'CGGTTCATCACTATCTGCGACTA-3' and NF-kB reverse primer 5'-TACCTCATGCAAAGGCACTTC-3'. GAPDH of 112 bp fragment was amplified as an internal control, GAPDH forward primer 5'- GTGAAGGTCGGAGTCAACG -3' and GAPDH reverse primer 5'- TGAGGTCAATGAAGGGGTC -3'. The optimal PCR conditions involved 40 cycles of 94°C predenaturation for 3 mins, 94°C denaturation for 30 seconds, , 60°C annealing for 40 seconds, and 72°C extension for 30 seconds, with a final extension at 72°C for 10 minutes.

3.9. Western blotting analysis

Myocadial tissue samples were directly lysed in Triton buffer (20 mM Hepes and 0.5% Triton X-100 at pH 7.6). Aliquots of protein extracts (50 mg) were separated on a 10% SDS-PAGE. Subsequently, the protein was electrophoretically transferred onto a PVDF membrane (Bio-Rad). After TBS-Tween 20 (TBST) containing 5% skim milk pretreatment, membranes were incubated with the following primary antibodies, anti-rat TLR4 (Concentration) antibody (Abacm), anti-rat NFkB (Concentration) antibody (Abcam) , and anti-rat MyD88 (Concentration) antibody (Cell signalling) over night at 4°C, respectively, followed by incubation with appropriate secondary antibodies diluted at 1:10000 in TBST for 1 h. Finally, blots were developed with chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA). For equal protein loading, blots were re-stained using mouse anti-rat GAPDH antibody (dilution 1:5000) as loading control.

3.10. Statistical analysis

All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as mean±Std.Deviation. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test were applied when the homogeneity of variance assumption had been satisfied, otherwise Games-Howell test was used instead. P values <0.05 were considered statistically significant.

4. RESULTS

4.1. Characteristics of BMSCs in primary and passaged cultures

While few primary cells cultured for 12 hours adhered to the culture bottle, a great number of these adherent cells was observed after 24 hours of culture, and exhibited diverse morphological characteristics (e.g. spindle shape), and few of them began to differentiate into fibroblasts. After 72 hours, cells adhered in local colony-growing type. After subculture, the cells had spindle shape, their growth capacity enhanced, and their cell density was uniform. Flow cytometry analysis showed that the percentage of cells expressing CD29 was 96.08%, CD34 was 5.46%, CD31 was 8.26%, CD44 was 89.37% and CD45 was 32.56% (Figure 1).

4.2. HE staining and TEM analysis

HE results showed that heart muscle exhibited focal coagulative necrosis, congesting myocardial interstitial, and edema after 48 hours. For 1 to 3 weeks post-injection, the necrotic tissue was gradually absorbed, leading to fibrosis. After 4 weeks, infracted areas began scaring (Figure 2).

Electron microscopy revealed no visible abnormal changes in the myocardial cell ultrastructure of normal control rat group, showing membrane structure integrity, and muscle filaments in orderliness, Z lines were clear and the structure of mitochondria was normal with dense cristae. In MI group, 0.1mg/kg and 5mg/kg LPS- preconditioned MSCs groups, there was focal myocardial myofilament dissolution, rupture of Z line into abnormal contracting bands, and a large number of apoptotic cells with condensed chromatin, and formation of nuclear crescent bodies. Hyperplasia was observed, accompanied by moderate to severe swelling of the chamber, occasional ruptured (Figure 3).

4.3. Analysis of apoptosis in the Myocardium

The percentage of TUNEL-positive nuclei, detectable in both control and experimental infracted heart muscle cells (Fig 2), was markedly increased in MI control group (62.8 ± 5.6% versus 15.3± 1.6%). The cell percentage in 1mg/kg LPS-preconditioned MSC group (22.8 ±2.6%) decreased significantly when compared to 0.1mg/kg (45.2±4.6%) or 5 mg/kg (70.6 ±9.2%) LPS-preconditioned MSC groups, respectively (P <0.05); these results suggest that small dose LPS-

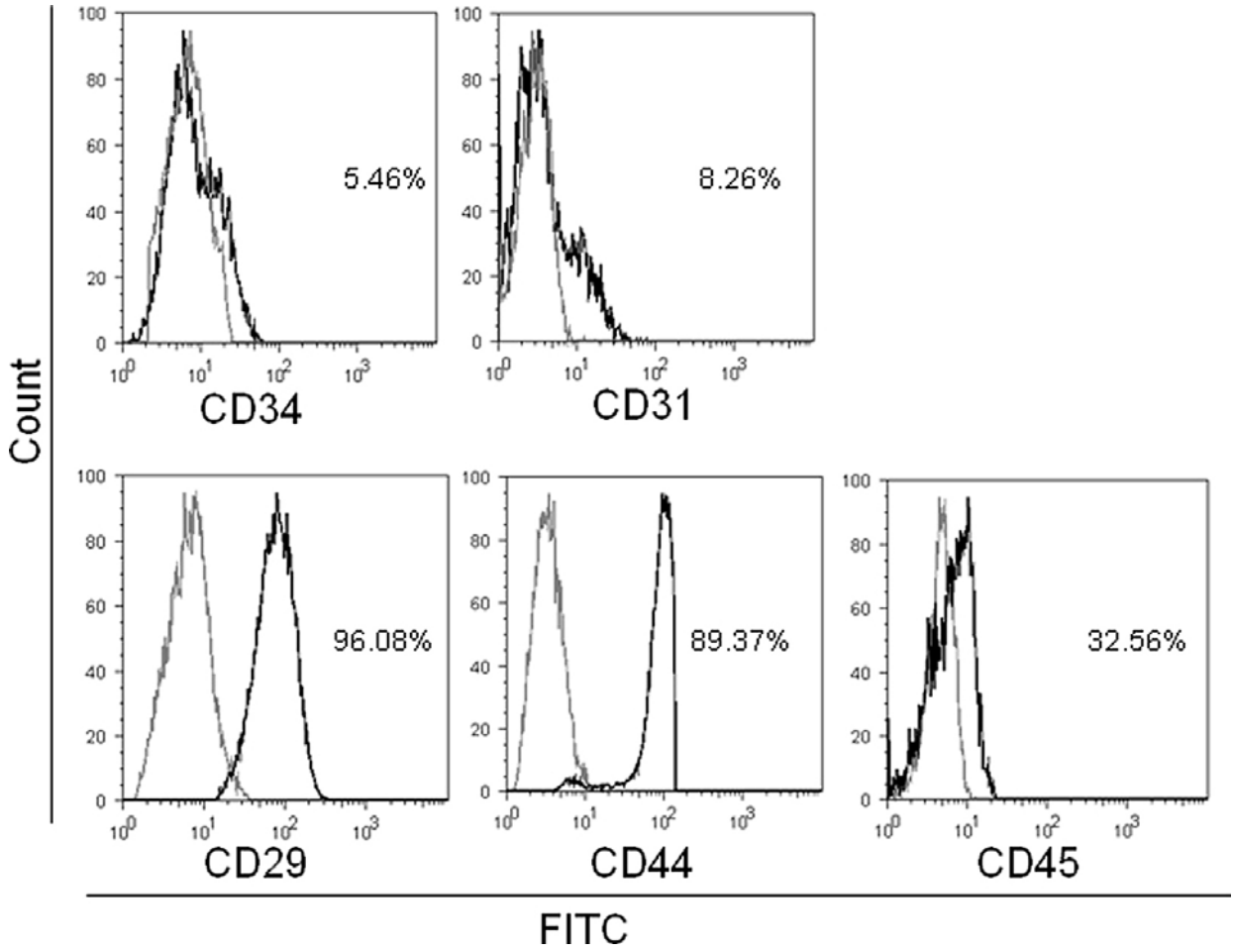

Figure 1. Flow cytometry analysis different marker expression on MSCs. Isolated MSCs from rat were cultured until reached 70% of confluence, then collected and stained with FITC-CD34, CD31, CD29, CD44 and CD45. Representative results of three independent experiments are shown.

preconditioned MSC transplantation could reduce apoptosis in the myocardium (Figures 4).

4.4. Analysis of DiI-labeled MCSs

Nearly 100% engrafted MSCs were detected along the track of needle injection by the presence of DiI-labeled cells. Typan blue staining revealed that cell activity was larger than 95%. DiI-positive cells around cell transplantation area were evenly colored red fluorescent. In the MI group after 48 hours, MSCs with red fluorescence were found in the infracted myocardium. Oval shaped-nuclei MSCs, were gathered at the edge of infarction, and the number was not different between controls and experimental groups ($P>0.05$). After 1 week, red fluorescent cells in both MI and control groups were significantly decreased. However, MSCs in 1 mg/kg LPS-preconditioned MSCs groups were observed with slightly decreased fluorescencein, and the number of DiI-positive cells significantly larger than MI group ($P<0.05$). In 1 mg/kg LPS-preconditioned MSCs groups, few transplanted MSCs migrated to the injured area of the myocardial tissue, in the center of which had gathered many MSCs. After 3 and 4 weeks, while few DiI-positive cells were observed in MI group, few scattered fluorescent cells, fused with myocardial cells, were observed ($P<0.05$) in 1 mg/kg LPS-preconditioned MSCs groups. The filaments in induced MSCs were observed by transmission electron microscopy (Figure 5).

4.5. Expression levels of TLR4, NFkB and MyD88

After 48 hours of transplantation, TLR4 mRNA levels were significantly increased in MI control group as well as all the three LPS-preconditioned MSCs groups, when compared to normal control group ($P<0.05$). However, while mRNA expression levels of NFkB and MyD88 were also increased in MI control group, 0.5 and 5mg/kg LPS-preconditioned MSC groups, they were significantly decreased in the 1mg/kg LPS-preconditioned MSCs groups ($P<0.05$), with a maximal peak observed one week after MI. After 3 and 4 weeks, changes in the three mRNA expression levels were not significant ($P>0.05$) (Figure 6).

The pattern of protein expression levels paralleled the same patterns of mRNA expression described in figure 6 (Figure 7).

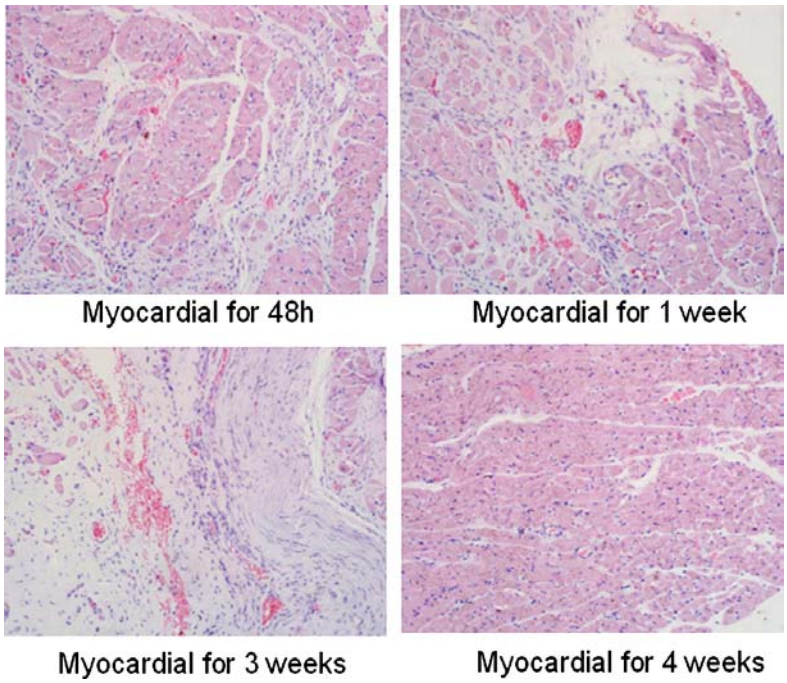

Figure 2. Heart tissue was necrosis focally along different time points. Histopathological analysis of heart specimens obtained from myocardial rat on days 48h, 1,3 and 4 weeks. Representative results of three independent experiments are shown. (magnification, x 400).

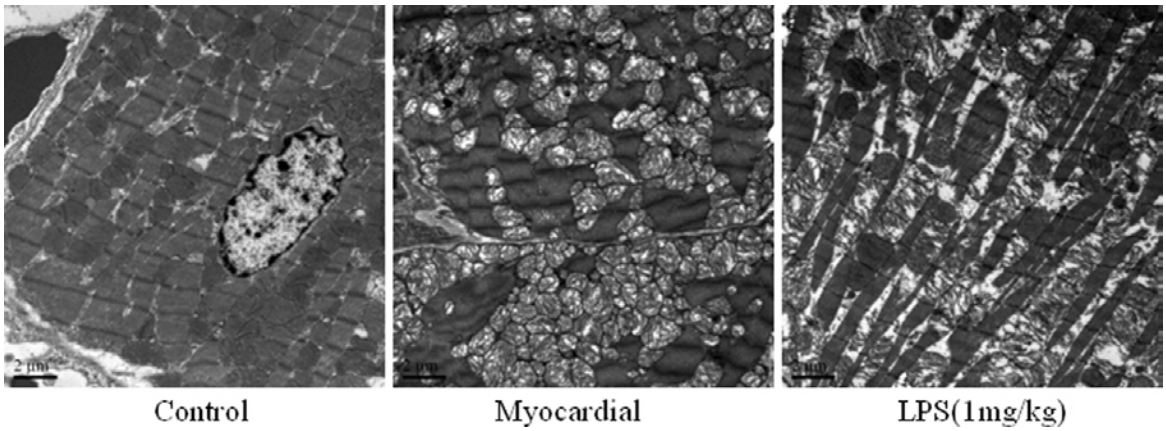

Figure 3. Electron microscopy analysis of Normal control, myocardial group and 1mg/kg LPS pretreatment group. Ultrastructure of myocardial cells in normal different groups were examind. The three pitures shown are from representative results of three independent experiments are shown. (magnification, x5000).

5. DISCUSSION

In the present study, using mouse model of AMI, survival of MSCs in myocardial infarction rat with 1mg/kg LPS pretreatment was improved and cell apoptosis was decreased. In addition, after 48h of MI, TLR4, NFkB and MyD88 mRNA as well as protein expression levels were remarkably decreased in 1mg/kg LPS pretreatment group. These findings suggest that a dose of LPS preconditioning as low as 1mg/kg improves survival of MSCs, and reduce apoptosis in infracted myocardium, most likely though inhibition of TLR4/MyD88/NFkB signaling pathway.

Although few reports have quantified stem cell survival after transplantation, donor stem cell survival may be as low as 1-5% 48 hours after transplantation (2,15), possibly due to a hostile, nutrient-deficient, inflammatory environment within damaged myocardium. Low cell survival may limit the sustained reparative capacity of stem cells *in vivo*. In present the study, survival of transplanted MSCs was elevated in infracted myocardium after LPS preconditioning by DiI-labeled MCSs analysis. For CM-DiI labeled transplanted BMCSs, cell labeling efficiency was 100%, mainly concentrated in the cell membrane, and no extravasation phenomenon, nor morphological or biological

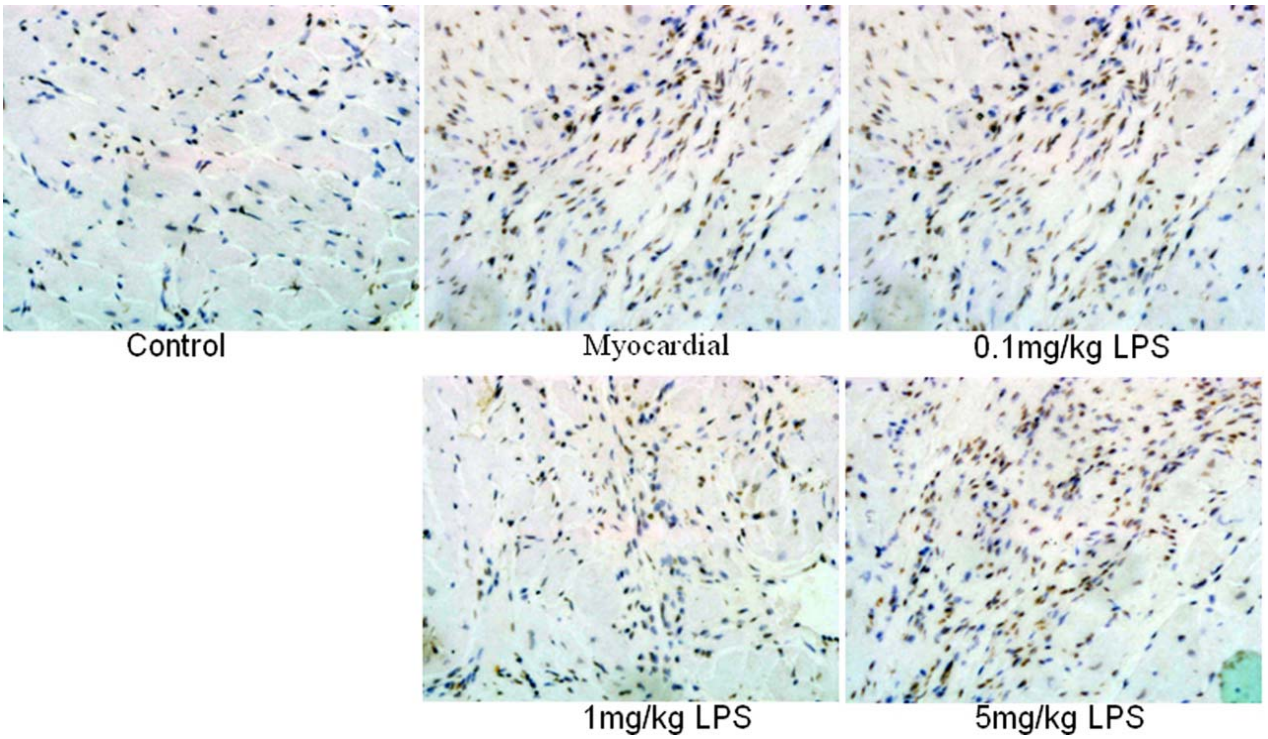

Figure 4. Myocardium apoptosis analysis. The apoptosic cells containing DNA fragments were showed violet blue while nucleus of normal cells is pink. In MI group, the percentage of apoptosis cells markedly increased compared with normal control group ($P < 0.05$). The apoptosis cell percentage in 1mg/kg LPS- preconditioned MSCs group decreased compared with 0.1mg/kg and 5 mg/kg LPS- preconditioned MSCs group ($P < 0.05$). Representative results of three independent experiments are shown.

activity was affected by the dye. 48h after transplantation, there is no significant changes in cell morphology, and immunofluorescence results showed that transplanted MSCs were mainly distributed in the surrounding damaged myocardial region. After 1 week post-transplantation, the number of red fluorescent cells was significantly decreased in MI group, inflammation was higher in injured myocardium, (maximum peak). CM-Dil-positive cells reduction might be related to a strong inflammatory reaction following the clearance by phagocytic cells around the necrotic myocardium, ,leading to early apoptosis of transplanted stem cells. While in the low-dose LPS pretreatment group, ,though the fluorescence level decreased slightly, the number of surviving positive cells was significantly higher than MI group.

Stem cells formed long, fibroblast-like, closely arranged and gradually gathered cells to the injured central area. After 3 to 4 weeks, no red fluorescence was detected in the myocardial infarction group, while in the low-dose LPS pretreatment the fluorescence was still scattered, some have been fused with myocardial cells, showing myocardial-like changes (e.g. myofilament-like structures and spontaneous slower rhythm cell pulse). Transplanted cells in MI had a very low survival rate, one of the important reason is that after the MI, the changed microenvironment (myocardial ischemia, hypoxia and inflammation after myocardial necrosis) promoted apoptosis. Small doses of LPS pretreatment could reduce the local inflammatory response and improve the cardiac micro-environment, thus reducing cell apoptosis after transplantation. In fact, even after 4 weeks post-transplantation, BMSCs were still present, promoting transplanted cells to further differentiate into cardiomyocytes, which significantly increased stem cell transplantation in clinical application in ischemic heart disease.

Previous study found that Role of LPS/CD14/TLR4-mediated inflammation could be a therapeutic target in necrotizing enterocolitis (16). In *in vitro* study, Chao *et al*, have already showed that small doses of LPS pretreatment cardiac cells decrease myocardial apoptosis in serum deprivation, and improved myocardial function through TLR4/PI3K/Akt signaling pathway, (17), However, they potential inflammatory factors in the infarct zone were not analyzed and animal experiments are lacking. Ischemia after MI in the microenvironment can lead to the same or different signal transduction pathways induced by TLR activation. Activation of gene expression control, the various effects of the transcription factor NF-kB (nuclear factor kappaB,), and inhibition of systemic inflammation in the adaptive immune response, play an important regulatory role (18-19). In addition, Toll-like receptor-mediated MyD88 (myeloid differentiation factor 88)-dependent activation of NFkB pathway is the major intracellular signaling pathways (20). MyD88 knockout mice lost IL-1 and IL-8, and the ability to respond to peptidoglycan, showing that TLR4 signaling pathway is dependent on MyD88. MyD88

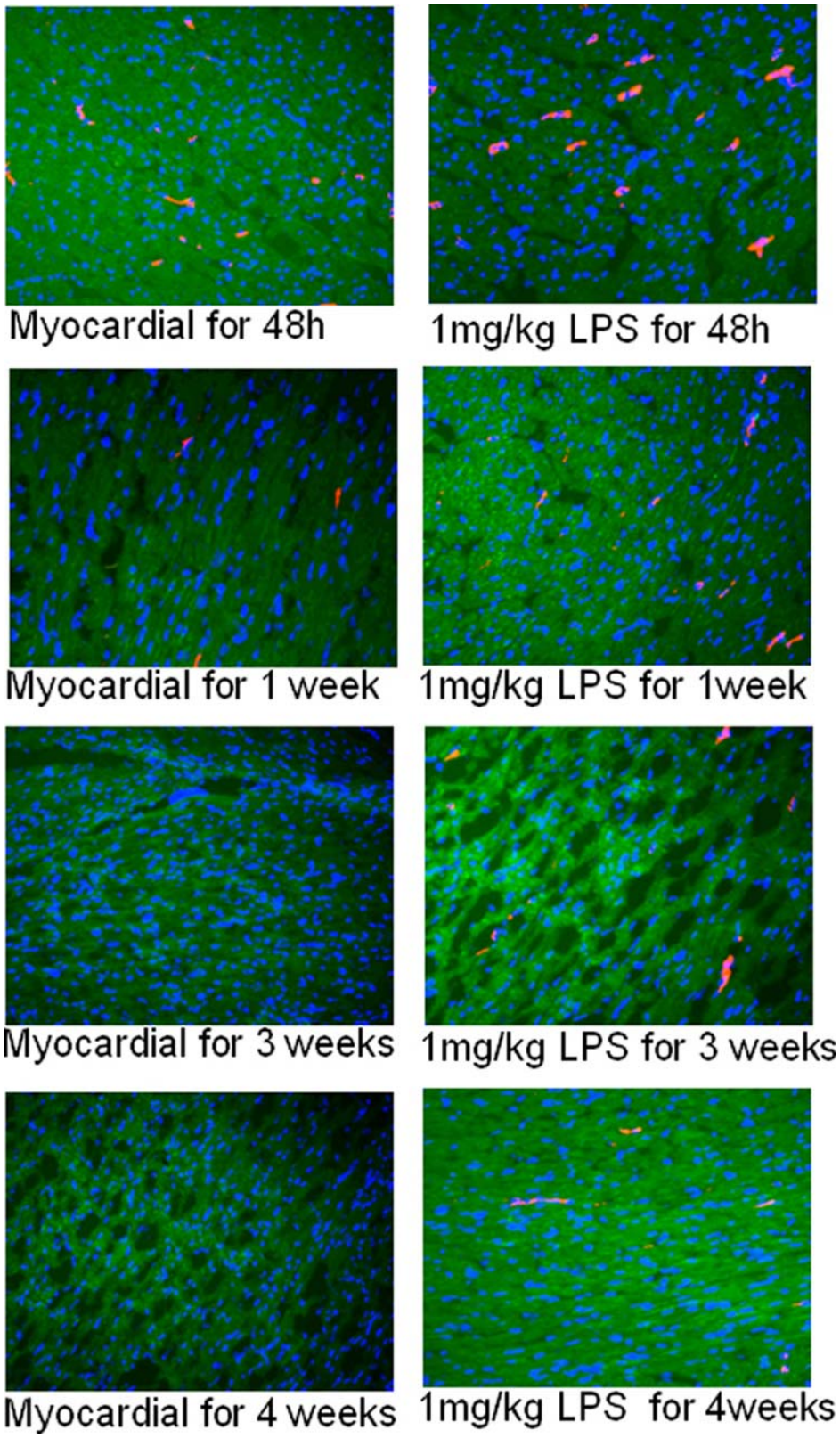

Figure 5. The distribution of transplanted BMSCs. Heart tissue from Myocardial and 1mg/kg LPS group were collected to observe the specific role of low doses LPS (1mg/kg) on survival and distribution of transplanted BMSCs. (magnification, □ 400). Representative results of three independent experiments are shown.

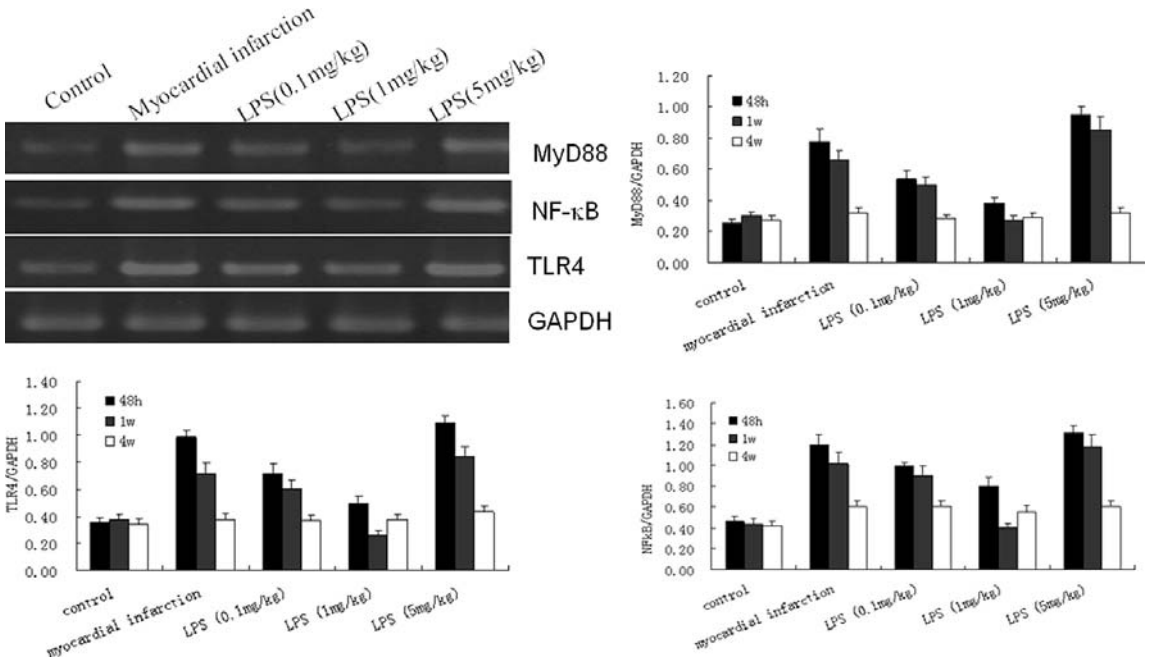

Figure 6. mRNA expression levels of TLR4, NFkB and MyD88 after transplantation. After 48 hours of transplantation, TLR4 mRNA levels were significantly increased in all groups when compared to normal control group ($P<0.05$).. However, while mRNA expression levels of NFkB and MyD88 were also increased in MI, 0.5, and 5mg/kg LPS-preconditioned MSCs groups, NFkB and MyD88 mRNA levels were decreased in 1mg/kg LPS-preconditioned MSCs groups ($P<0.05$), peaking one week after MI. After 3 and 4 weeks, mRNA expression levels of the three transcripts were not significantly different ($P>0.05$). Representative results of three independent experiments are shown.

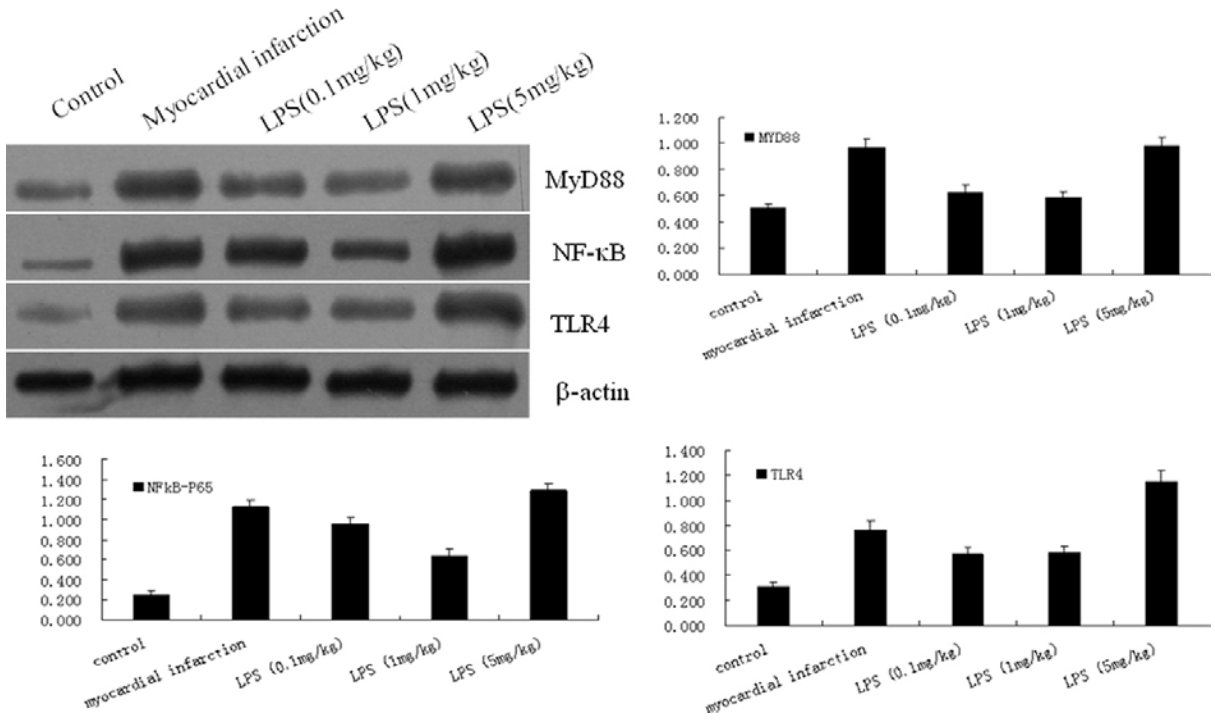

Figure 7. Expression levels of TLR4, NFkB and MyD88 proteins. of the patterns of protein expression levels of the 3 proteins followed similar patterns of mRNA expression levels as described in figure 6. Representative results of three independent experiments are shown.

and the downstream kinase binds and phosphorylates IRAK, leading to inhibition of NFkB protein IkBα degradation, and activation of NFkB. Previous study found that LPS could regulate TLR4 signal transduction in nasopharynx epithelial cell line 5-8F *via* NFκB and MAPK signaling pathways (21). In this study, in relatively small dose of LPS pretreatment group, only a small number of mitochondria blurred and the percentage of apoptotic cells was only 22.8%, when compared to MI and other LPS pretreatment groups. Western Blot and RT-PCR analyses showed that after myocardial infarction, both TLR4, NFkB, and MyD88 mRNA and protein expression levels were significantly increased, however, those in the low dose LPS pretreatment group declined. These results put together suggest that low-dose LPS pretreatment might inhibit cardiac TLR4-MyD88-NFkB signaling pathway to suppress NF-kB activation, reduce apoptosis of BMSCs in MI and promote their further differentiation into cardiomyocytes.

In conclusion, low-dose LPS pretreatment of MI rat can improve the survival rate of transplanted cells, reduce apoptosis of myocardial cells after MI, suggesting that by inhibiting TLR4-MyD88-NFkB signaling pathway to reduce inflammation, low-dose LPS pretreatment could reduce the risk for myocardial cell damage.

6. ACKNOWLEDGEMENTS

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Abbreviations: BMSCs, bone marrow mesenchymal stem cells; MI, myocardial infarction; AMI, Acute myocardial infarction; MSCs, Mesenchymal stem cells; HSCs, hematopoietic stem cells; TLRs, Toll-like receptors; LPS, Lipopolysaccharide; TLR4, Toll-like receptor-4

Key Words: Myocardial Infarction, TLR4, LPS, MSCs

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