Protective Effects of Trimetazidine on Bone Marrow Mesenchymal Stem Cells Viability in an ex vivo Model of Hypoxia and in vivo Model of Locally Myocardial Ischemia*

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Summary: Bone marrow mesenchymal stem cells (MSCs) have shown potential for cardiac repair following myocardial injury, but this approach is limited by their poor viability after transplantation. The present study was to investigate whether trimetazidine (TMZ) could improve survival of MSCs in an ex vitro model of hypoxia, as well as survival, differentiation, and subsequent activities of transplanted MSCs in rat hearts with acute myocardial infarction (AMI). MSCs at passage 3 were examined for their viability and apoptosis under a transmission electron microscope, and by using flow cytometry following culture in serum-free medium and exposure to hypoxia (5% CO₂, 95% N₂) for 12 h with or without TMZ. Thirty Wistar rats were divided into 3 groups (n=10 each group), including group I (AM1 control), group II (MSCs transplantation alone), and group III (TMZ+MSCs). Rat MSCs (4×10⁷) were injected into peri-infarct myocardium (MSCs group and TMZ+MSCs group) 30 min after coronary artery ligation. The rats in TMZ+MSCs group were additionally fed on TMZ (2.08 mg•kg⁻¹•day⁻¹) from day 3 before AMI to day 28 after AMI. Cardiac structure and function were assessed by echocardiography at 28th day after transplantation. Blood samples were collected before the start of TMZ therapy (baseline), and 24 and 48 h after AMI, and inflammatory cytokines (CRP, TNF-α) were measured. Then the survival and differentiation of transplanted cells in vitro were detected by immunofluorescent staining. The cellular apoptosis in the peri-infarct region was detected by using TUNEL assay. Furthermore, apoptosis-related proteins (Bcl-2, Bax) within the post-infarcted myocardium were detected by using Western blotting. In hypoxic culture, the TMZ-treated MSCs displayed a two-fold decrease in apoptosis under serum-free medium and hypoxia environment. In vivo, cardiac infarct size was significantly reduced, and cardiac function significantly improved in MSCs and TMZ+MSCs groups as compared with those in the AMI control group. Combined treatment of TMZ with MSCs implantation demonstrated further decreased MSCs apoptosis, further increased MSCs viability, further decreased infarct size, and further improved cardiac function as compared with MSCs alone. The baseline levels of inflammatory cytokines (CRP, TNF-α) had no significant difference among the groups. In contrast, all parameters at 24 h were lower in TMZ+MSCs group than in MSCs group. Furthermore, Western blotting indicated that the expression of anti-apoptotic protein Bcl-2 was up-regulated, while the pro-apoptotic protein Bax was down-regulated in the TMZ+MSCs group, compared with that in the MSCs group. It is suggested that implantation of MSCs combined with TMZ treatment is superior to MSCs monotherapy for MSCs viability and cardiac function recovery.

Key words: trimetazidine; bone marrow mesenchymal stem cells; viability; myocardial ischemia

It has been known that myocardial infarction leads to irreversible loss of tissue and deficits in cardiac performance. The remaining myocytes in infarcted tissues undergo progressive replacement by fibroblasts, forming scar tissue. Fortunately, recent experimental results have suggested the possibility of regenerating damaged myocardium using adult bone marrow mesenchymal stem cells (MSCs). Although MSCs represent a suitable source of autologous cells in such cell therapy, MSCs therapy is limited by poor cell viability after transplantation. More recently, there have been several attempts to improve the viability of MSCs by in vitro expansion and/or in vivo genetic engineering. Trimetazidine (TMZ) has cytoprotective effects on ischemic cardiac tissue, whose cytoprotective mechanisms are mainly related to the TMZ-induced “metabolic shift” from lipid β-oxidation to glucose aerobic oxidation. TMZ also limits membrane damage induced by reactive oxygen species (ROS) and protects tissue from free radicals with its antioxidant effects. It has been suggested that ROS- and nitric oxide (NO)-mediated damage en-
hances the release of proinflammatory mediators such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-8 from macrophages both in inflammation and ischemia[8,9]. Furthermore, TMZ limits intracellular acidosis[8,9] and accumulation of inorganic phosphate, Na+ and Ca2+[10] in cardiac tissue. These changes are independent of oxygen supply or demand alterations. Therefore, we proposed that TMZ administration may improve the transplanted MSCs viability through microenvironmental amelioration of peri-infarct and infarct region, thus improving treatment of peri myocardial infarction and end-stage cardiac failure.

1 MATERIALS AND METHODS

1.1 Main Reagents

Annexin V/propidium iodide (PI) kit was purchased from Biovision, USA. TNF-α cytoscreen ELISA kit was a product of Biosource, USA. Anti-Troponin T monoclonal antibody (MAb) was from Maxim Pharmaceuticals, USA. Anti-factor VIII related Ag MAb was a product of Santa Cruz Biotechnology Inc., USA. Antibody (Ab) conjugated to Cy3 was from Santa Cruz Biotechnology Inc., USA. Tunel kits were from Roche Diagnostics Limited., Hong Kong, SAR, China. Bcl-2 and Bax were procured from NeoMarkers, Lab vision Co., USA. Anti-mouse IgG was from KPL Inc., Europe. Tzm was from Servier, France.

1.2 Main Instruments

Main instruments used included the following: anaerobic chamber (5% CO2, 95% N2, Thermo Forma Anaerobic System Model 1025, USA), flow cytometry (Becton-Dickinson, USA), particle-enhanced immuno-nopelometry (N Latex CRP mono, Behring Diagnostics, Germany), echocardiography (sequia 512 Acuson, Mountain View, USA), confocal microscope (TCS SP-2, Leica, Germany).

1.3 MSCs Preparation

MSCs were isolated from the femoral and tibial bones of rats[11]. We collected bone marrow-derived MSCs from aspirates of the femurs and tibias of 4-week-old male Wistar rats. Mononuclear cells recovered from the interface after centrifugation in Percoll. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. After 48 or 72 h, non-adherent cells were discarded. Fresh complete medium was added and replaced every 3 or 4 days for about 10 days. MSCs at passage 3 were examined.

To generate hypoxic conditions, the cells were incubated anaerobically for 12 h in deoxygenated serum-free DMEM with or without TMZ (5×10-4 mol/L) in an anaerobic chamber (5% CO2, 95% N2).

1.4 Morphological Profiles of Apoptosis Cells under Transmission Electron Microscopy

For the electron microscopy, the cells were washed and fixed overnight at 4°C with 2% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer, dehydrated in a graded series of buffers, embedded in resin, and thinly sectioned. The sections were post-stained in uranyl acetate and lead citrate and observed under a transmission electron microscope.

1.5 Apoptosis Analysis Using Annexin V/PI Staining and Flow Cytometry

Apoptotic cell death was measured using FITC-conjugated Annexin V/PI assay by using flow cytometry.

1.6 MSCs Labeling

To label viable MSCs with DAPI, sterile DAPI solution was added to the culture medium on the day of implantation at a final concentration of 50 μg/mL for 30 min. The cells were rinsed 6 times in PBS to remove unbound DAPI, detached with 0.25% (v/v) trypsin and suspended in serum-free medium (at a density of 4×107 cells/100 μL) for grafting.

1.7 AMI Generation, Experimental Groups and MSCs Transplantation

The experiments were performed in 8-week-old male Wistar rats (about 250 g). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the protocol was approved by the Institutional Animal Care Committee from Wuhan University. Transient focal infarcts were surgically induced by ligating the left anterior descending coronary artery.

Thirty rats were randomly divided into the following three groups of 10 rats each: I, AMI control group; II, MSCs transplantation group; III, TMZ +MSCs group. The number of rats dying in groups I, II and III were three, two, and two respectively prior to the 28-day sacrifice.

For cell transplantation, MSCs (4×107 cells) were suspended in 100 μL serum-free medium and injected into four sites bordering the infarct area (each injection 107 cells/25 μL of MSCs suspension) with a Hamilton syringe and a 30-gauge needle 30 min after AMI. For AMI control rats, they were injected with an equivalent volume of the serum-free medium.

For medicine administration, the rats in TMZ+MSCs group were additionally fed on TMZ (2.08 mg·kg-1·day-1) for 3 days before AMI to day 28 after AMI. Other animals were fed daily on equal volume of saline.

1.8 Inflammatory Cytokines Measurements

CRP was measured by particle-enhanced immuno-nopelometry by using a 1:400 sample dilution. A TNF-α cytoscreen ELISA kit was used and the resulting yellow to blue color intensity was recorded at 450 nm by a Sorin-Biomedica microplate reader.

1.9 Structure and Function Measurements Using Echocardiography

Echocardiogram was performed on rats using a sequia 512 equipped with a 3-7 MHz linear transducer (7v3), before operation and at 28th day after AMI. The anterior chest area was shaved, and 2D images and M-mode tracings were recorded.

1.10 Tissue Collection and Infarct Size Characterization

After echocardiogram measurements, 10 mL cardioplegic solution was injected to arrest the heart in diastole. The heart was removed from the chest, and left ventricle was separated and sectioned from apex to base, then cryoembedded after protection with 20% sucrose in PBS. One-mm thick sections were mounted on a set of gelatin-coated glass slides. These slices were stained with hematoxylin and eosin, and Masson’s trichrome stain, and photographed. Infarct size (%) was calculated from the ratio of surface area of infarct wall to the entire surface area of the left ventricle, by the use of image analysis software.