Pharmacological Preconditioning of Mesenchymal Stem Cells with Trimetazidine (1-[2,3,4-Trimethoxybenzyl]piperazine) Protects Hypoxic Cells against Oxidative Stress and Enhances Recovery of Myocardial Function in Infarcted Heart through Bcl-2 Expression

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ABSTRACT

Stem cell transplantation is a possible therapeutic option to repair ischemic damage to the heart. However, it is faced with a number of challenges including the survival of the transplanted cells in the ischemic region. The present study was designed to use stem cells preconditioned with trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine; TMZ), a widely used anti-ischemic drug for treating angina in cardiac patients, to increase the rate of their survival after transplantation. Bone marrow-derived rat mesenchymal stem cells (MSCs) were subjected to a simulated host tissue environment by culturing them under hypoxia (2% O2) and using hydrogen peroxide (H2O2) to induce oxidative stress. MSCs were preconditioned with 10 μM TMZ for 6 h followed by treatment with 100 μM H2O2 for 1 h and characterized for their cellular viability and metabolic activity. The preconditioned cells showed a significant protection against H2O2-induced loss of cellular viability, membrane damage, and oxygen metabolism accompanied by a significant increase in HIF-1α, survivin, phosphorylated Akt (pAkt), and Bcl-2 protein levels and Bcl-2 gene expression. The therapeutic efficacy of the TMZ-preconditioned MSCs was evaluated in an in vivo rat model of myocardial infarction induced by permanent ligation of left anterior descending coronary artery. A significant increase in the recovery of myocardial function and up-regulation of pAkt and Bcl-2 levels were observed in hearts transplanted with TMZ-preconditioned cells. This study clearly demonstrated the potential benefits of pharmacological preconditioning of MSCs with TMZ for stem cell therapy for repairing myocardial ischemic damage.

Myocardial infarction (MI) is a major contributor to chronic heart disease leading to mortality in humans. Transplantation of stem cells (cellular cardiomyoplasty or cell therapy) in the infarcted myocardium has been considered a possible therapeutic option to repair the infarcted myocardium and restore the function of the damaged heart (Dimmeler et al., 2008). A variety of cells including embryonic stem cells, fetal cardiomycocytes, cardiac stem cells, skeletal myoblasts, smooth muscle cells, hematopoietic stem cells, or mesenchymal stem cells are being explored as potential choices for myocardial cell therapy (Dimmeler et al., 2008). Mesenchymal stem cells (MSCs), which are self-renewing precursor cells of nonhematopoietic stromal tissues, are currently under intense investigation for cardiac repair (Nesselmann et al., 2008). MSCs are adult pluripotent cells, which can be isolated from bone marrow and other adult tissues and easily propagated in vitro (Pittenger et al., 1999). These cells can be directed to differentiate into osteoblasts (Heino and Hennunen, 2008), chondrocytes (Pereira et al., 1995), vascular endothelial cells (Yue et al., 2008), or cardiomycocytes (Toma

ABBREVIATIONS: MI, myocardial infarction; MSC, mesenchymal stem cell; TMZ, trimetazidine, 1-[2,3,4-trimethoxybenzyl]piperazine; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; LDH, lactate dehydrogenase; H2O2, hydrogen peroxide; PI, propidium iodide; OCR, oxygen consumption rate; EPR, electron paramagnetic resonance; pAkt, phosphorylated Akt; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAD, left anterior descending (coronary artery); LV, left ventricle; EF, ejection fraction; FS, fractional shortening.
et al., 2002) using specific growth factors and conditions. Furthermore, MSCs can suppress local inflammation (Djouad et al., 2003) and trigger local production of growth factors and cytokines favoring endogenous cardiac repair. Thus, MSCs appear to be an ideal cell choice for myocardial tissue repair.

Stem cell transplantation to the infarcted myocardium is faced with additional challenges beyond finding the ideal cell type for use. The infarct region is usually ischemic, with the development of a scar tissue that may not facilitate the transport of essential nutrients and oxygen to support the engraftment and survival of the transplanted stem cells. Most of the cells die within hours of transplantation in the infarcted heart because of interplay of ischemia, inflammation, and apoptosis (Menasché, 2008). Several strategies have been proposed to improve revascularization of the ischemic tissue or to enhance the longevity of the transplanted cells in the hostile ischemic environment. For example, preconditioning the stem cells using chemokines, growth factors, or pharmacological agents has been shown to improve their survival at the site of transplantation (Shmelkov et al., 2005; Niagara et al., 2007; Pasha et al., 2008).

Oxygen is an essential metabolic substrate required for the production of energy to support the survival, proliferation, and differentiation of the transplanted cells in the infarct myocardium. Under aerobic conditions, cellular energy production (ATP) involves predominantly fatty acid oxidation pathway, which is oxygen intensive. However, under hypoxic conditions, which occur in the infarct myocardium, it would be advantageous for cells to switch to alternate pathways, such as anaerobic glycolysis, for energy production, thereby reducing dependence on tissue oxygenation. In the clinical setting, this is usually achieved through the application of anti-ischemic drugs, such as trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine; TMZ; also known as Vastarel in the United States), which is used to reduce ischemia-induced metabolic damage by lowering the tissue demand for oxygen (Lopaschuk et al., 2003). TMZ reduces the rate of free fatty acid oxidation, with a concomitant increase in anaerobic glucose oxidation rates during low-flow ischemia (Kantor et al., 2000). The likely mechanism of TMZ action is through the inhibition of 3-ketoacyl CoA thiolase enzyme, which is crucial to the β-oxidation of fatty acids. Thus, inhibition of the fatty acid oxidation pathway by TMZ appears to be a clinically relevant solution to compromise the reduced supply of oxygen to the ischemic heart tissue. However, the pharmacological efficacy of anti-ischemic agents, such as TMZ, in augmenting myocardial stem cell therapy has not yet been reported.

Therefore, the goal of the present study was to investigate whether pharmacological preconditioning of MSCs with TMZ could enable them to survive in the hypoxic environment in the infarct tissue upon transplantation. Bone marrow-derived rat MSCs were subjected to a simulated host tissue environment by culturing them under hypoxic conditions and inducing oxidative stress using hydrogen peroxide. These cells were further treated with TMZ and characterized for their cellular viability and metabolic activity. The therapeutic efficacy of the TMZ-preconditioned cells was studied in an in vivo rat model of myocardial infarction. The results clearly demonstrated the potential benefits of TMZ in preconditioning MSCs before implantation to offer a significant enhance-

### Materials and Methods

**Reagents.** Dulbecco’s modified Eagle’s medium with GlutaMax 1 (4500 mg/l glucose) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Aerobic cultures of cells were maintained using 5% CO₂ in air (20% O₂) at 37°C in a humidified incubator. Hypoxic cultures were performed using cells of third or fourth passages at ~80% confluence. The cells were incubated in serum-free media under hypoxic conditions (2% O₂, 5% CO₂, balance N₂) using a BioSpherix (Redfield, NY) growth chamber for 12 h before treatments. The 2% O₂ was chosen to provide a sublethal dose of hypoxia corresponding to the levels in the ischemic heart, which ranges from 0.2 to 2.6% O₂ (Khan et al., 2007). The cells, under hypoxic conditions, were treated with TMZ (10 μM) for 6 h followed by H₂O₂ (100 μM) for 1 h to induce oxidative stress. The optimal dose of TMZ and H₂O₂ and treatment periods were determined from a preliminary dose/time study.

**Cell Viability Using Propidium Iodide Binding Assay.** The nuclear viability of the cells was measured using an automated cell counter (NucleoCounter; New Brunswick, Edison, NJ). This technique uses propidium iodide (PI), which binds to cellular nuclei. Depending upon sample preparation, the counts provide the total number of cells and number of nonviable cells, from which the number of viable cells is calculated.

**Cell Viability (Mitochondrial Activity) by MTT Assay.** The effect of TMZ on the mitochondrial activity of MSCs was determined by MTT assay using MTT colorimetric assay kit. Cells were seeded in 96-well plates at a concentration of 5 × 10⁴ cells/well in a 200-μl volume of growth medium. After treatment period, the supernatant was removed, washed three times with 1× PBS, and 200 μl of MTT reagent in plain RPMI-1640 medium was added. The plates were incubated for 4 h at 37°C in a humidified incubator. The MTT reagent solution was removed from each well, and acidified-methanol was added to dissolve the formazan salt. The plates were then loaded into an automated plate reader (Beckman Coulter, Fullerton, CA; AD340) and analyzed at λ = 490 nm to determine the quantity of formazan product present in each well. All assays were run in at least three parallels and repeated three times.

**Cytotoxicity Using LDH Assay.** After treatment, MSCs were washed, trypsinized, centrifuged, and resuspended in 15 ml of growth medium without serum. Cytotoxicity was determined by measuring the quantity of LDH found in the culture medium using a standardized LDH assay. The culture supernatants from each experiment were collected and stored at ~80°C until the assays were performed. Samples were thawed, and LDH assays were performed at 25°C. Readings were taken at 340 nm using a Varian (model Cary

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MSCs (a total of 5 × 10^5 cells/ml) were given in the infarct regions of the hearts 30 min after LAD ligation. The chest cavity was closed by bringing together the third and fourth ribs with one 4-0 polypropylene silk suture. The layers of muscle and skin were closed with a 4-0 polypropylene suture, and the rats were allowed to recover under a warm light.

The animals were divided into three groups, each consisting of six animals. The MI group received a sham treatment and serum-free growth medium without MSCs; the MSC group received transplanted MSCs alone, cultured under hypoxic conditions; and the MSC + TMZ group received transplanted MSCs preconditioned with TMZ under hypoxic conditions. The animals were sacrificed 4 weeks after cell transplantation. The hearts were explanted and immediately snap-frozen for Western blot studies. Hearts were also fixed in formalin for histological studies for evaluation of tissue fibrosis and key biochemical markers.

**Echocardiography.** Transthoracic M-mode echocardiography measurements were conducted at baseline and at 4 weeks after MSC transplantation using a GE Vivid 7 ultrasound imaging system equipped with a 15-MHz linear array transducer. Rats were anesthetized with 2% isoflurane in air for the duration of the procedure. Heart size and shape were calculated using the M-mode and two-dimensional short-axis image plane of the LV. Measurements were averaged from three cardiac cycles. The data were used to estimate percentage LV ejection fraction (EF) and fractional shortening (FS).

**Measurement of Fibrosis.** After measurement of hemodynamic function, the animals were euthanized, and the hearts immediately recovered and washed two to three times in cold PBS. The excised hearts were then cut into three transverse slices. Each slice was fixed in 4% paraformaldehyde and embedded in paraffin. The middle transverse section was stained with Masson-Trichrome for infarct size (fibrosis) determination. Fibrosis was defined as the sum of the epicardial and endocardial infarct circumference divided by the sum of the total LV epicardial and endocardial circumferences using computer-based planimetry. Quantitative assessment of each parameter was performed using MetaMorph software (Molecular Devices, Sunnyvale, CA).

**Data Analysis.** The statistical significance of the results was evaluated using analysis of variance and a Student’s t test. Values were expressed as mean ± S.D. A p value of <0.05 was considered significant.

### Results

**Effect of Hypoxia and H₂O₂ on the Viability of MSCs.** The effect of hypoxic culture on the nuclear and mitochondrial viability of MSCs was studied. Cells cultured using 2% O₂ for 24 h did not show any significant change in their viability compared with normoxic (20% O₂) culture (Fig. 1, A and B). MSCs treated with H₂O₂ (100 μM) for 1 h under hypoxic conditions caused significant reductions in the nuclear (40%) and mitochondrial (44%) viabilities compared with hypoxic culture without H₂O₂ treatment (Fig. 1, C and D). The results established that culture of MSCs under hypoxic conditions had no effect on cellular viability, but subsequent exposure to H₂O₂ was able to significantly reduce cellular viability.

**Preconditioning of MSCs with TMZ Protects Cells against H₂O₂-Induced Toxicity.** To study the effect of TMZ pretreatment on H₂O₂-induced cytotoxicity, MSCs grown under hypoxic conditions were treated with 10 μM TMZ in culture for 6 h, followed by exposure to H₂O₂ for 1 h. At the end of treatment period, the cells were analyzed for nuclear and mitochondrial viability. As anticipated, H₂O₂ exposure resulted in a significant reduction in cell viability compared with untreated control (Fig. 2, A and B). TMZ alone had no effect on cellular viability, and cells pretreated with TMZ
Viability (M/H9262 control. Both PI and MTT assays demonstrated that 100% inhibition of H2O2-induced damage in MSCs by TMZ exposed under hypoxic conditions, were treated with TMZ for 6 h, followed by incubation with 100 μM H2O2 for 2 h. The expression of HIF-1α was determined by RT-PCR analysis. The data (Fig. 3C) showed a substantial overexpression of Bcl-2 in preconditioned MSCs, suggesting that TMZ increased the Bcl-2 protein at the expression level.

Functional Improvement in the Infarct Hearts Transplanted with Preconditioned MSCs. Four weeks after stem cell transplantation in MI heart, cardiac functions were evaluated by M-mode echocardiography (Fig. 4). LVEF and left-ventricular FS were significantly decreased in the MI group compared with the noninjured control (baseline). LVEF and FS were significantly improved in the MI group treated with MSCs cultured under hypoxic conditions (MSC). An even greater recovery of cardiac function was observed in the group treated with MSCs grown under hypoxic conditions and pretreated with TMZ (M + TMZ).

Overexpression of Bcl-2 Protein in the Infarct Hearts Transplanted with Preconditioned MSCs. Western blot analysis of the explanted heart tissue showed significant increases in pAkt and Bcl-2 expression in the hearts treated with preconditioned MSCs compared with non-preconditioned MSC or MI alone hearts (Fig. 6).

Discussion

Cell survival is crucial for the success of transplantation therapy (Haider and Ashraf, 2008). It requires the adaptation of the transplanted cells to endure the hostile environment of the ischemic myocardium. We evaluated a pharmacological strategy that included preconditioning of MSCs with TMZ to make them resistant to subsequent exposure to lethal conditions upon transplantation. The major findings of our study are: 1) TMZ preconditioning significantly attenuated the H2O2-induced impairment of cellular viability and membrane damage in MSCs under hypoxic conditions; 2) TMZ preconditioning markedly increased the levels of cell survival proteins survivin, pAkt, Akt, and Bcl-2, by Western blotting. MSCs, cultured under hypoxic conditions, were treated with TMZ for 6 h, followed by incubation with 100 μM H2O2 for 2 h. The expression of HIF-1α increased significantly in cells cultured under hypoxic conditions compared with the cells cultured under normoxic conditions (Fig. 3). HIF-1α expression was markedly reduced in cells not exposed to TMZ but exposed to H2O2. Pretreatment with TMZ resulted in a significant increase in HIF-1α expression compared with H2O2-treated hypoxic cells. Likewise, in cells exposed to TMZ before H2O2 challenge, there was an enhancement of survivin and Bcl-2 protein expression and increased phosphorylation (activation) of Akt. Overall, the Western blot studies indicated a marked increase in the expression of HIF-1α, survivin, Bcl-2, and pAkt in MSCs pretreated with TMZ. To further confirm whether the increased level of Bcl-2 was due to overexpression of Bcl-2, we performed RT-PCR analysis. The data (Fig. 3C) showed a substantial overexpression of Bcl-2 in preconditioned MSCs, suggesting that TMZ increased the Bcl-2 protein at the expression level.

TMZ Preconditioning up-Regulates the Expression of Survival Proteins in MSCs. To understand the molecular mechanisms and biochemical pathways that lead to the inhibition of H2O2-induced damage in MSCs by TMZ exposure, we investigated the expression levels of some key hypoxic and survival marker proteins, including HIF-1α, survivin, pAkt, Akt, and Bcl-2, by Western blotting. MSCs, cultured under hypoxic conditions, were treated with TMZ for 6 h, followed by incubation with 100 μM H2O2 for 2 h. The expression of HIF-1α increased significantly in cells cultured under hypoxic conditions compared with the cells cultured under normoxic conditions (Fig. 3). HIF-1α expression was markedly reduced in cells not exposed to TMZ but exposed to H2O2. Pretreatment with TMZ resulted in a significant increase in HIF-1α expression compared with H2O2-treated hypoxic cells. Likewise, in cells exposed to TMZ before H2O2 challenge, there was an enhancement of survivin and Bcl-2 protein expression and increased phosphorylation (activation) of Akt. Overall, the Western blot studies indicated a marked increase in the expression of HIF-1α, survivin, Bcl-2, and pAkt in MSCs pretreated with TMZ. To further confirm whether the increased level of Bcl-2 was due to overexpression of Bcl-2, we performed RT-PCR analysis. The data (Fig. 3C) showed a substantial overexpression of Bcl-2 in preconditioned MSCs, suggesting that TMZ increased the Bcl-2 protein at the expression level.

Fig. 1. Effect of hypoxia and hydrogen peroxide on the nuclear and mitochondrial viability of MSCs. Cells were cultured under normoxic (20% O2) or hypoxic (2% O2) conditions for 24 h. After 24 h in hypoxic culture and when the cells reached 80% confluence, H2O2 (100 μM) was included in the culture medium for an additional 1 h. Cellular viability was determined by MTT and PI-binding assays. The data were expressed as mean ± S.D. from three independent experiments. A, viability of MSCs determined by PI staining. There was no significant change in the viability of MSCs cultured under hypoxic conditions compared with normoxic controls. B, mitochondrial viability as determined by MTT assay. Hypoxic (2% O2) had no significant effect on the mitochondrial viability of MSCs. C, effect of H2O2 on the nuclear viability of MSCs cultured under hypoxic conditions. D, effect of H2O2 on the mitochondrial viability of MSCs cultured under hypoxic conditions. * p < 0.05 versus untreated control. Both PI and MTT assays demonstrated that 100 μM H2O2 was cytotoxic to MSCs cultured under hypoxic conditions.
**Fig. 2.** Effect of TMZ on H2O2-induced cytotoxicity in MSCs cultured under hypoxic conditions. MSCs, kept in hypoxic culture for 24 h, were treated with 10 μM TMZ for 6 h, followed by 100 μM H2O2 for 2 h. The data were expressed as mean ± S.D. from three independent experiments. A, cell viability assay as determined by PI staining. H2O2 induced a significant reduction in the cell viability (*, p < 0.05 versus control). Preconditioning of cells with TMZ significantly reversed the H2O2-induced cytotoxicity (#, p < 0.05 versus H2O2). B, cell viability as determined by MTT assay. H2O2 exposure caused a significant reduction in cell viability (*, p < 0.05 versus control). Preconditioning of cells with TMZ significantly reversed the H2O2-induced cytotoxicity (#, p < 0.05 versus H2O2). C, membrane damage as determined by LDH assay. H2O2 exposure induced a significant increase in LDH level (*, p < 0.05 versus control), which was attenuated by preconditioning of cells with TMZ (#, p < 0.05 versus H2O2). D, oxygen consumption in MSCs pretreated with TMZ for 6 h followed by treatment with H2O2 for 2 h under hypoxic conditions. Data are expressed as mean ± S.D. of OCR using five independent experiments. *, p < 0.01 versus control. #, p < 0.05 versus H2O2. Preconditioning of cells with TMZ shows a significant decrease in OCR. A significant decrease in OCR is also observed in cells treated with H2O2, which may be due to H2O2-induced impairment of mitochondrial respiration.

**Fig. 3.** Analysis of hypoxia and survival proteins expressed by the MSCs treated with TMZ and H2O2. Western blots were obtained from MSCs cultured under normoxia (20% O2), hypoxia (2% O2, control), and treatment with H2O2 (2 h) without or with TMZ preconditioning (6 h). A, representative blots of HIF-1α, survivin, pAkt, Akt, and Bcl-2 are shown. B, quantitative analysis of HIF-1α, survivin, pAkt, and Bcl-2 using data obtained from three different blots and expressed as mean ± S.D. #, p < 0.05 versus normoxia; *, p < 0.05 versus H2O2. TMZ preconditioning significantly increased the levels of HIF-1α, survivin, pAkt, and Bcl-2 proteins. C, RT-PCR analysis of Bcl-2 gene expression in MSCs preconditioned with TMZ followed by treatment with H2O2. Bcl-2 gene expression was substantially enhanced by TMZ preconditioning.
metabolism by reducing fatty acid oxidation through selective inhibition of 3-ketoacyl CoA thiolase enzyme in the mitochondria of cardiomyocyte. As a result, TMZ attenuates the adverse effects of free fatty acid-associated oxidative stress (Gambert et al., 2006), lessens oxygen demand by decreasing oxygen consumption (Monteiro et al., 2004), and improves

**Fig. 4.** Recovery of cardiac function at 4 weeks after MSC transplantation in the infarct heart. Transthoracic echocardiography was performed in noninfarct (baseline), infarct (MI), infarct hearts treated with MSCs (MSC), and infarct hearts treated with MSCs preconditioned with TMZ (MSC + TMZ). Representative recordings of M-mode echocardiogram (A), LV ejection fraction (B), and fraction shortening (C) are shown. Results are expressed as mean ± S.D. using six animals/group. #, *p < 0.05 versus baseline; *, **p < 0.05 versus MI; ***, p < 0.05 versus MSC. The MI hearts treated with TMZ-preconditioned cells had greater functional recovery than those with non-preconditioned cells.

**Fig. 5.** Effects of preconditioning on tissue fibrosis 4 weeks after MSC transplantation. Masson-Trichrome staining of heart sections was performed in infarcted hearts (MI) and infarcted hearts treated with non-preconditioned MSCs (MSC) and MSCs preconditioned with TMZ (MSC + TMZ) before transplantation. A to C, representative images of heart sections stained with Masson-Trichrome. D, percentage of fibrosis in hearts 4 weeks after transplantation, as determined by computer planimetry. Data are expressed as mean ± S.D. using six hearts per group. Hearts treated with MSCs preconditioned with TMZ exhibit a significant reduction in fibrosis compared with the MSC group.
mitochondrial metabolism and cardiac performance during ischemia (Kantor et al., 2000). At the cellular level, TMZ preserves ATP production, reduces the generation of oxygen free radicals (Maupoil et al., 1990; Gambert et al., 2006; Kutala et al., 2006), and reduces intracellular acidosis and calcium overload (Kantor et al., 2000). TMZ has been shown to protect hearts from ischemia-induced electrical dysfunc-
tion leading to ventricular fibrillation (Vaillant et al., 2008), ischemia-reperfusion-induced damage to mitochondrial res-
piration (Guarnieri and Muscari, 1993), and ischemia-reper-
fusion injury by decreasing myocardial lactate content early
at reperfusion (Pantos et al., 2005). Trittio et al. (2005) dem-
onstrated that TMZ attenuated tissue injury in postischemic
hearts by inhibiting the activation of neutrophils. We re-
ported recently that pretreatment of hearts with TMZ signif-
icantly enhanced the functional recovery by combined effects
of antioxidant and anti-ischemic activities and enhanced pro-
survival Akt activity (Kutala et al., 2006).

MSCs, such as used in this study, are derived from hypoxic
niches in bone marrow, where the pO2 is quiet low. Usually,
in vitro expansion and incubation of MSCs are carried out
under normoxic (20% O2) conditions to achieve high cellular
vitality and proliferation rates. However, when these cells are
transplanted in the infarct tissue, they encounter severe
hypoxic conditions, typically less than 0.5% O2 (Khan et al.,
2007, 2008; Mohan et al., 2009), which can induce apoptosis
and cell death. This hypoxia-induced apoptosis and cell loss
can be prevented by hypoxic preconditioning, that is, by
exposure of MSCs to less severe hypoxic conditions (1–3% O2)
for a period of time before transplantation into the ischemic
heart (Hu et al., 2008; Rosová et al., 2008; Wang et al., 2008).
Since the focus of this work was to study the effect of TMZ on
the survival of transplanted MSCs under the hypoxic and
oxidative environments in the ischemic heart, we used cells
that were cultured and treated under hypoxic (2% O2) condi-
tions. We did not find significant change in the viability of
MSCs cultured under hypoxic conditions, compared with nor-
moxic culture. However, hypoxia exposure significantly
increased the HIF-1 level and induced Akt activation, which
is known to promote cell survival by inhibiting apoptosis.
Rosová et al. (2008) have demonstrated that human bone

Bcl-2 is an antiapoptotic protein originally found to be
overexpressed in B-cell lymphoma. It is a critical inhibitor
of apoptotic cell death in ventricular myocytes. In the ischemic
heart, Bcl-2 contributes to cardiac protection by regulating
the metabolic functions of mitochondria (Kirshenbaum and
de Moissac, 1997). The role of Bcl-2 protein in myocardial
stem cell therapy has been reported. Li et al. (2007) geneti-
cally modified adult rat bone marrow-derived MSCs to over-
express Bcl-2 and demonstrated substantial resistance of the
transplanted cells to apoptosis and remarkable functional
recovery in an acute model of myocardial infarction in rats.
A recent report by Hu et al. (2008) demonstrated increased
expression of prosurvival and proangiogenic factors, includ-
ing HIF-1α and Bcl-2 by hypoxic preconditioning of mouse
MSCs. They further showed that transplantation of the
hypoxic-preconditioned MSCs in the infarcted mouse hearts
resulted in increased angiogenesis and enhanced morpho-
logic and functional benefits of stem cell therapy. Our in vivo
results showed a significantly increased expression of Bcl-2
in TMZ-preconditioned MSCs subjected to H2O2-induced ox-
idant stress, suggesting that TMZ increased the Bcl-2 protein
at the expression level. A significantly higher level of Bcl-2
was also observed in the hearts treated with the TMZ-pre-
conditioned MSCs. However, the precise role and mecha-
nism by which TMZ regulates Bcl-2 expression is yet to be
understood.

This is the first report on the use of TMZ for precondition-
ing of cells. However, there is abundant literature on the use
of TMZ for myocardial preconditioning (Opie, 2003; Argaud
et al., 2005; Kara et al., 2006). TMZ is reported to inhibit
mitochondrial permeability transition pore opening and pro-
tect the rabbit heart from prolonged ischemia-reperfusion
injury (Argaud et al., 2005). Preconditioning of myocardium
with TMZ has been shown to protect the heart against isch-
emia-induced arrhythmias, reduce myocardial infarct size,
preserve the effects of ischemic preconditioning and pharma-
cological preconditioning, and mimic ischemic precondition-
ing in anesthetized rats (Kara et al., 2004). In the present
study, we transplanted MSCs preconditioned with TMZ, but
we did not treat the animals with TMZ, either pre- or post-
transplantation. Thus, the present study does not establish
whether the beneficial effects of transplantation of TMZ-
preconditioned MSCs is entirely due to TMZ. Further studies
using treatment of animals with TMZ are required to delin-
eate the mechanism of protection by TMZ in vivo. In addition,
it remains to be established whether the preconditioning
effect observed in the present study is actually due to the
shift toward glucose metabolism or to other effects of TMZ.
Because the MSCs are physiologically exposed to low oxygen
tension in bone marrow, a prevalence of glycolysis over mi-
 tochondrial respiration could actually be the normal condi-
tion for these cells. Hence, it is possible that TMZ is exerting
its beneficial effect, not so much because it induces a new

Fig. 6. Protein expression in the explanted tissue of hearts treated with
MSCs preconditioned with TMZ under hypoxic conditions. A, representa-
tive Western blot images showing pAkt, Akt, and Bcl-2 proteins in the explanted
tissue. Values are expressed as mean ± S.D. obtained from six blots. * p < 0.05
versus MI or MSC groups. Hearts treated with TMZ-preconditioned
MSCs show a significantly enhanced Akt and Bcl-2 expressions.
phenotype, but rather because it may help preserving/restoring the glucose-avid phenotype congenial to these cells. Furthermore, it has long been known that glycolytically generated ATP is preferentially employed in driving a number of chemical processes occurring at mitochondrial level (Nakamura et al., 1993; Cappelli-Bigazzi et al., 1997). Thus, favoring glycolysis may have a favorable impact on a variety of functions of stem cells.

In summary, MSCs preconditioned with TMZ showed a significant protection against H2O2-induced loss of cellular viability, membrane damage, and oxygen metabolism accompanied by a significant increase in HIF-1α, survivin, pAkt, and Bcl-2 protein levels and gene expression. A significant improvement in the recovery of myocardial function, decrease of tissue fibrosis, and up-regulation of pAkt and Bcl-2 were observed in infarcted heart tissues treated with TMZ-preconditioned cells. This study clearly demonstrated the beneficial effects of pharmacological preconditioning of MSCs with TMZ for stem cell therapy for repairing myocardial ischemic damage.

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Cappelli-Bigazzi M, Battaglia C, Pannain S, Chiariello M, and Ambrosio G (1997) TMZ-preconditioned cells. This study clearly demonstrated a reduction of tissue fibrosis, and up-regulation of pAkt and Bcl-2. Improvement in the recovery of myocardial function, viability, membrane damage, and oxygen metabolism accompanied by a significant increase in HIF-1α, survivin, pAkt, and Bcl-2.


Guarrinieri C and Muscari C (1993) Tissue fibrosis, and up-regulation of pAkt and Bcl-2. Improvement in the recovery of myocardial function, viability, membrane damage, and oxygen metabolism accompanied by a significant increase in HIF-1α, survivin, pAkt, and Bcl-2. This study clearly demonstrated the beneficial effects of pharmacological preconditioning of MSCs with TMZ for stem cell therapy for repairing myocardial ischemic damage.

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