Carvedilol enhances mesenchymal stem cell therapy for myocardial infarction via inhibition of caspase-3 expression

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MSC - Mesenchymal stem cells
ROS - Reactive oxygen species
LAD - Left anterior descending
TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
EPR - Electron paramagnetic resonance spectroscopy
DEPMPO - 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide

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ABSTRACT

Adult stem cells have shown great promise towards repairing infarcted heart and restoring cardiac function. Mesenchymal stem cells (MSC), because of their inherent multipotent nature and their ability to secrete a multitude of growth factors and cytokines, have been utilized for cardiac repair with encouraging results. Preclinical studies showed that MSCs injected into infarcted hearts improve cardiac function and attenuate fibrosis. Although, stem cell transplantation is a promising therapeutic option to repair the infarcted heart, it is faced with a number of challenges including the survival of the transplanted cells in the ischemic region, due to excessive oxidative stress present in the ischemic region. The objective of this study was to determine the effect of Carvedilol, a non-selective β-blocker with antioxidant properties on the survival and engraftment of MSCs in the infarcted heart. MSCs were subjected to a simulated host tissue environment, similar to the one present in the infarcted myocardium, by culturing them in the presence of hydrogen peroxide (H₂O₂) to induce oxidative stress. MSCs were treated with 2.5 µM Carvedilol (Carv) for 1 h in serum-free medium, followed by treatment with H₂O₂ for 2 h. The treated cells exhibited significant protection against H₂O₂-induced cell death versus untreated controls as determined by MTT and TUNEL assays. Similarly, transplantation of MSCs after permanent LAD (left coronary artery) ligation and treatment of animals post myocardial infarction (MI) with Carv (5 mg/kg b.w.) led to significant improvement in cardiac function, decreased fibrosis and caspase-3 expression, when compared to MI or MSC-alone groups.
INTRODUCTION

Cardiovascular disease morbidity and mortality rates have shown a dramatic decrease over the past 50 years due to advances in research and clinical care (Cutler et al., 2006; Yeh et al., 2010; Lauer, 2012). Nevertheless, heart disease remains the leading cause of death worldwide (Mathers and Loncar, 2006). Cellular cardiomyoplasty has emerged as one of the most promising therapeutic approaches for treating ischemic heart disease since the last decade. Cellular transplantation has rapidly evolved from the benchside to clinical trials (Menasche, 2011). In particular, mesenchymal stem cells (MSC) have shown promising results in improving cardiac function and attenuation of fibrosis (Tomita et al., 1999; Fazel et al., 2005). However, the survival of the transplanted stem cells is limited not only due to the hypoxic environment of the myocardium, but also due to the abundance of oxidative stress and reactive oxygen species (ROS) in the infarcted heart (Zhang et al., 2001; Wei et al., 2010). Oxidative stress has been implicated in the prognosis after an acute myocardial infarction particularly during the initiation of reperfusion, due to its direct cytotoxicity (Ferrari et al., 1985; Hill and Singal, 1996), negative inotropism (Hill and Singal, 1996; Flesch et al., 1999) and pro-apoptotic effect (Krown et al., 1996; Ferrari et al., 2004) contributing to ventricular remodeling (Kinugawa et al., 2000).

β-blockers are commonly prescribed for patients with acute MI and they produce their effect by decreasing heart rate, myocardial contractility, myocardial oxygen consumption and the overall work load of the heart, hence improving ischemic heart symptoms (Monteiro et al., 2003; Kopecky, 2006). Carvedilol (Carv) is a non-selective β-blocker with β1, β2 and α1 adrenergic receptor blocking properties (Packer, 1998),
while Atenolol is a selective β1 receptor antagonist. Carv exerts a potential beneficial effect compared to other β-blockers in ventricular remodeling; due to the α-adrenergic blockage properties and its unique antioxidant properties for scavenging superoxide anions and hydroxyl radicals (Yue et al., 1992; Feuerstein et al., 1998). This dual property of Carv makes it substantially unique from drugs with only antioxidant properties. Furthermore, Carv attenuates cardiomyocyte apoptosis and the expression of pro-apoptotic proteins after ischemia reperfusion injury (Zeng et al., 2003). The anti-apoptotic cardioprotective role of Carv is independent of its anti-adrenergic properties (Schwarz et al., 2003). Studies have shown that the antioxidant properties of Carv provide it a unique ability to maintain the viability and inhibit apoptosis in the ischemic myocardium (Cargnoni et al., 2000; Carreira et al., 2006). Additionally, experimental studies have shown that Carv treatment alleviates left-ventricular remodeling, fibrosis and cardiac dysfunction in rats (Barone et al., 2007; Yoshikawa et al., 2010).

Based upon the widespread use of Carv in hypertensive, post-MI and heart failure patients, the focus of this study was to see the combined effect of Carv in rats receiving MSC transplantation. Since, the morbidity and mortality rates after acute myocardial infarction have increased; it is clear that combination therapy utilizing complementary approaches would act synergistically to decrease further myocardial damage after MI. In the present study we have investigated the in-vitro protective effect of Carv against H₂O₂ induced oxidative stress in MSCs. We have further evaluated the in-vivo effect of treating of MSCs with Carv prior to transplantation and the beneficial effect of further continuing treatment of animals with Carv after MI. The in vitro results demonstrated that Carv pretreatment attenuates apoptosis of MSCs, when subjected to
H$_2$O$_2$-induced oxidative stress. Similarly, in-vivo data showed improved recovery of cardiac function, decreased TUNEL-positive cells and caspase-3 expression in the hearts treated with combined Carv and MSC.

METHODS

Chemicals

Carvedilol (Sigma Chemicals, St. Louis, MO) was freshly prepared in DMSO and diluted with PBS before administration. Xanthine and, xanthine oxidase were obtained from Sigma Chemicals (St. Louis, MO). DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide) was obtained from Radical Vision (Jerome, Marseille, France). All other reagents were analytical grade or higher purchased from Sigma-Aldrich, unless otherwise mentioned.

Measurement of superoxide radicals by EPR spectroscopy

The superoxide radical-scavenging ability of Carv in vitro was determined by EPR spectroscopy (Khan et al., 2007). A mixture of xanthine (0.2 mM) and xanthine oxidase (0.02 U/ml) in PBS (pH 7.4) was used to generate superoxide radicals. EPR measurements were performed in PBS (pH 7.4) containing DEPMPO (1 mM) and in the presence or absence of different concentrations of Carv. The superoxide radicals were detected as DEPMPO-OOH adducts. The attenuation of DEPMPO adduct generation was quantified by double-integration and expressed as percentage of untreated (without Carv) levels.
Cell survival and proliferation assay

MSCs (20,000/well) were cultured in a 96-well plate. After 24 h, the cells were treated with Carv (2.5 µM) or Atenolol (2.5 µM) for 1 h prior to the addition of 800-µM H₂O₂ in a serum-free medium for 2 h. Controls were used in parallel for each treatment group. 0.5 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) in complete media was then applied to the cells for 2 h. The yellow tetrazolium MTT was reduced by metabolically active cells to a purple formazan which was solubilized and quantified by spectrophotometry at 570 nm and referenced higher than 650 nm. Similarly, anti-BrdU proliferation assay was performed. MSCs were incubated with 10-µM BrdU (5-bromo-2'-deoxyuridine) for 2 h. The pyrimidine analogue BrdU gets incorporated in place of thymidine into the DNA of active proliferating cell. The amount of BrdU utilized by the cells was assayed by ELISA technique using anti-BrdU antibody and its substrate.

Preparation of Carv suspension for oral administration

Based on the studies in the literature (Abdulla et al., 2011; Kumar et al., 2011) we have selected 5 mg/kg b.w. as the optimal dose to be administered daily to the rats following MI for 4 weeks. Coreg tablets were crushed to fine powder using a mortar and pestle and mixed with 20 ml of Ora-plus suspending agent to prepare a final concentration of 7.25 mg/ml of Carv suspension. The Carv suspension was prepared freshly every day prior to administration.

Induction of myocardial infarction in vivo

Fisher-344 rats (200-250 g) were used as an in vivo acute model for myocardial infarction (permanent LAD ligation). Rats were randomly divided into 5 groups of 6 animals each: (1) Sham (Untreated); (2) MI (LAD ligation only); (3) Carv (MI + Carvedilol
(5 mg/kg b.w.); (4) MSC (MI+MSC-treated); and (5) MSC+Carv (MI+MSC+Carv treated). Rats were anaesthetized with ketamine (50 mg/kg, i.p) and xylazine (5 mg/kg, i.p) and maintained under anesthesia using isoflurane (1.5-2.0%) mixed with air. Myocardial infarction (MI) was created by permanently ligating the left-anterior-descending coronary artery (LAD), as described previously (Khan et al., 2009). After permanent ligation of LAD, successful infarction was confirmed by an ST elevation on electrocardiograms that were recognized in all surgical groups of animals. MSCs or MSCs treated with Carv (2.5 µM) were transplanted in the ischemic heart at 30 min after permanent LAD ligation and the chest was closed. Shams (untreated animals) were anaesthetized as described previously and the chest cavity was opened and closed without LAD ligation. After re-initiation of spontaneous respiration, animals were extubated and allowed to recover from the anesthesia. The following day after surgery, rats were administered orally with Carv suspension (5 mg/kg b.w.) daily for 4 weeks using a clean oral gavage. All the procedures were performed with the approval of the Institutional Animal Care and Use Committee of the Ohio State University and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23).

**Echocardiography (M-mode) for cardiac functional analysis**

Assessment of cardiac function was performed at the baseline and at 4 weeks after MI or stem cell transplantation. Rats were anaesthetized using 1.5-2% isoflurane and M-mode ultrasound images were acquired using a Vevo 2100 (VisualSonics; Toronto, Canada) ultrasonic rodent imaging system as described previously (Khan et al., 2012).
Measurement of fibrosis

Rats were anaesthetized after 4 weeks of MI/stem cell transplantation, and their hearts were excised and washed with ice-cold PBS. The hearts were then frozen for 10-min at -20°C and sliced into 2 mm sections using a heart matrix. The sections were then incubated in formalin overnight for Masson’s trichrome staining for collagen/fibrosis. To determine the fibrosis, images were acquired by a dissecting microscope (Nikon; Tokyo, Japan). The fibrosis area was quantified by computerized planimetry using MetaMorph image analysis software (Molecular Devices; Sunnyvale, CA) as described previously (Khan et al., 2009).

Assessment of apoptosis by TUNEL assay

To assess cell death or apoptosis, cultured MSCs/cryopreserved heart sections (5-μm thickness) were fixed in freshly prepared 4% paraformaldehyde for 20-min at room temperature. The cells/slides were incubated for 30-min in PBS and washed twice with fresh PBS. The samples were then incubated with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate, freshly prepared) for 2 min on ice. DNA strand breaks were detected using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay kit procured from Roche Diagnostics (Indianapolis, IN). Briefly, sections were covered with 50 μL of TUNEL reaction mixture and incubated in this solution for 60 min at 37°C in a humidified chamber. After rinsing with PBS, the sections were mounted with Vectasheild HardSet Mounting Medium with DAPI (Vector Labs; Burlingame, CA) and visualized under a fluorescence microscope. The apoptotic index (the percentage of TUNEL-positive cardiomyocytes relative to total nuclei) was then calculated from at least 3 slides, 5 fields each in the peri-infarct area.
using MetaMorph (Molecular Devices; Sunnyvale, CA) image analysis software as published earlier (Khan et al., 2010).

**Immunostaining for α-SMA and vWF**

Immunostaining was performed in formalin fixed paraffin-embedded heart sections (5-micron). The fixed tissue sections were serially rehydrated as described previously (Khan et al., 2012). The tissue sections were then incubated with 2% goat serum and 5% bovine-serum albumin in PBS to reduce nonspecific binding. The sections were then incubated for 2 h with mouse, anti-α-smooth muscle actin (α-SMA) and anti-von Willebrand factor (vWF) monoclonal antibodies (Cell Signaling, MA). The sections were then incubated with the appropriate anti-mouse secondary antibodies (1:200) conjugated to Texas red (α-SMA) and FITC (vWF). The nuclei were counterstained with hardest DAPI (Vector Labs). The tissue sections were visualized by inverted Nikon fluorescence microscope (TE 2000). Blood vessels and capillaries stained positive for α-SMA and vWF in peri-infarct regions of the heart were acquired. Separate sections were also stained without primary antibodies to indentify nonspecific binding.

**Western-blot analysis for molecular signaling**

Western blots for pAkt, pERK1/2, Bcl-2 and caspase-3 signaling were performed with the tissue homogenates as described previously (Khan et al., 2012). The anterior walls of the left ventricles were collected from Sham, MI, Carv, MSC and MSC+Carv groups. After the treatment periods in all the groups, rats were anesthetized using pentobarbital sodium and the hearts were harvested and immediately rinsed in ice-cold PBS (pH 7.4). The protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride
(PVDF) membrane, and probed with primary antibodies for pERK1/2, p-Akt (ser-473), Bcl-2 and caspase-3 (Cell Signaling; Beverly, MA). The membranes were incubated overnight at 4°C with the primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) for 1 h. The membranes were then washed and developed using an enhanced chemiluminescence detection system (ECL Advanced Kit, GE healthcare). The same membranes were then reprobed for GAPDH. The protein intensities were quantified by an image-scanning densitometer (Un-Scan It, UT). To quantify the phospho-specific signal in activated proteins, we first subtracted the background and then normalized the signal to the amount of GAPDH or total target protein in the tissue homogenate (Khan et al., 2012).

DATA ANALYSIS

The statistical significance of the results was evaluated by one-way analysis of variance (ANOVA) and all pairwise multiple comparison procedures were done by Tukey’s Post Hoc Test. The values were expressed as mean±S.D. A p value of <0.05 was considered significant.

RESULTS

Carv attenuates superoxide generation in vitro

To evaluate the superoxide scavenging property of Carv in vitro, we performed EPR spectroscopy. Detection of superoxide was performed by DEPMPO spin trap (1 mM) as DEPMPO-OO adduct. Carv significantly (p<0.05) reduced superoxide radical generation in vitro. Figure 1A shows representative spectra obtained by EPR spectroscopy. Figure 1B shows the scavenging effect of Carv against superoxide radicals. Carv (up to 1 mM),
challenged against DEPMPO (1-mM), showed a decrease in the intensity of the DEPMPO-OOH spectrum by more than 35% (Figure 1A & B). On the other hand, Atenolol (1 mM) which is also a β-blocker, did not have any scavenging effect on superoxide radicals, when compared to Carv (1 mM). The *in vitro* data clearly established that Carv is capable of scavenging superoxide radicals in a dose-dependent manner when compared to atenolol.

**Carv pretreatment improves cell survival and decreases apoptosis of MSCs under ischemic stress**

Treatment of MSCs with H$_2$O$_2$ led to significant reduction in cell survival and proliferation. On the other hand, pretreatment of MSCs with Carv showed significant (p<0.05) increase in cell survival and proliferation when compared with H$_2$O$_2$-treated group (Figure 2A and 2B). Correspondingly, apoptosis of MSCs assessed by TUNEL assay showed decreased cell death in Carv+H$_2$O$_2$ group, when compared with the group treated with H$_2$O$_2$ alone (Figure 2C).

**Carv adjuvant treatment with MSC improves cardiac function**

Cardiac function was measured at baseline and four weeks after MI by M-mode ultrasound echocardiography. Figure 3A shows representative images of M-mode echocardiography. The recovery of cardiac function was significantly (p<0.05) improved in all treatment groups when compared to MI group. However, the functional recovery (ejection fraction and fractional shortening) was further improved in rats treated with MSC+Carv, when compared to MSC-alone group (p<0.05; Figure 3B & 3C). In addition, the LV inner diameters at end-systole (LVIDs) and end-diastole (LVIDd) were
significantly (p<0.05) restored in MSC+Carv group, when compared to MI, Carv and MSC groups (Figure 3D and 3E).

**Carv and/or stem cells treatment ameliorates cardiac fibrosis**

To further understand whether improved cardiac function correlates to cardiac fibrosis, masson-trichrome staining was performed for the assessment of fibrosis four weeks after MI. Data showed a significant (p<0.05) decrease in fibrosis in Carv, MSC and MSC+Carv treated groups, when compared to MI group (Figure 4A). Moreover, the fibrosis was significantly lower in Carv and MSC+Carv groups, when compared to MSC-alone group. The fibrosis data supports our hypothesis that adjuvant treatment of MSCs with Carv attenuates cardiac fibrosis.

**Carv and/or MSC treatment increases angiogenesis**

To detect neovascularization in the infarcted heart, the cardiac tissue sections were stained with smooth muscle cell marker (α-SMA) and endothelial cell surface marker (vWF). The results demonstrated an increased number of α-SMA and vWF positive vessels/capillaries respectively in the peri-infarct regions of the Carv+ MSC group compared to MSC-alone group.

**Carv and/or MSC treatment decreases myocardial apoptosis**

One of the key events after acute MI; the apoptosis of cardiomyocytes is due to the activation of caspases that cleave DNA and other components. Apoptosis data in heart sections was evaluated at four weeks after MI by TUNEL assay. The data showed decreased TUNEL-positive cells in Carv and MSC+Carv treated groups when compared
to MSC-alone group (Figure 5A), indicating that adjuvant treatment of MSCs along with Carv further decreases apoptosis of cardiomyocytes in the infarcted heart.

**Carv adjuvant treatment with MSC attenuates caspase-3 expression in vivo**

Caspase-3 expression was found to be significantly (p<0.05) decreased in rat hearts treated with Carv and MSC+Carv groups when compared with MI or MSC-alone groups (Figure 5C). The results indicate that Carv as an adjuvant treatment plays a crucial role in attenuating apoptosis of surviving myocytes in the infarcted area of the damaged heart. Since MSCs were pretreated with Carv prior to transplantation, and consecutively animals were treated with Carv daily for four weeks, this combined treatment might be responsible for significant decrease in caspase-3 expression in MSC and Carv combined group compared to MSC alone group.

**Carv and MSC treatment modulates the phosphorylation of Akt, ERK1/2**

PI3K-Akt signaling and ERK1/2 signaling cascades are activated due to a wide range of receptors and are involved in regulating cell survival, proliferation and differentiation (Widmann et al., 1999; Cross et al., 2000). The results of our study showed a significant increase in phosphorylation of Akt and ERK1/2 in MI hearts when compared to all the treated groups. Interestingly, the levels of phospho-Akt and ERK1/2 were significantly downregulated (p<0.05) in Carv, MSC and MSC+Carv groups, when compared to MI group (Figure 6).

**DISCUSSION**

Overall, the present study showed that the pretreatment of stem cells with Carv decreases H₂O₂-induced cell death and caspase-3 expression. Furthermore, *in vivo*
studies showed that transplantation of Carv pretreated stem cells along with daily treatment of Carv, (i) enhanced the functional recovery of the heart; (ii) decreased cardiac fibrosis; (iii) increased angiogenesis and (iv) decreased caspase-3 expression; thereby suppressing apoptosis of surviving myocytes in the infarcted heart. The beneficial effects of Carv in protecting cardiomyocyte apoptosis and survival may seem to be due to its antioxidant properties.

Although, numerous pre-clinical studies have demonstrated the benefits of MSC therapy for treating the damaged heart after MI (Tomita et al., 1999; Kudo et al., 2003; Fazel et al., 2005), one of the key factors that determine the clinical outcomes of transplanted stem cells is their survival in the ischemic heart. Previous studies from our group have shown that the important determinants that might affect the survival of stem cells in the ischemic heart are the apoptosis of transplanted cells due to low oxygen and oxidative stress (Khan et al., 2007; Wisel et al., 2009). Therefore, the main goal of this study was to understand whether pretreatment and/or post-conditioning of animals with Carv a non-selective β-blocker could improve the outcome of stem cell therapy.

**Antioxidant effect of Carv on MSC in vitro**

The potential sources of ROS in cardiomyocytes include mitochondrial electron transport chain, xanthine oxidase, non-phagocytic NADPH oxidases, dysfunctional NO synthase, lipoxygenase, heme-oxygenase and the cytochrome P450 monooxygenases. During cardiac disease the major sources of ROS in cardiac disease are the mitochondria, the xanthine oxidase and the NADPH oxidase (Grieve et al., 2004; Arozal et al., 2010). One of the important observations of our study is the dose-dependent increase in the superoxide-scavenging property of Carv compared to
Atenolol. Similarly, MSCs subjected to oxidative stress by H$_2$O$_2$ treatment were protected when they were pretreated with Carv prior to H$_2$O$_2$ treatment. The decrease in number of TUNEL positive cells further indicated the anti-apoptotic effect of Carv pretreatment on stem cells. This data showed the potential benefit of Carv pretreatment to decrease the apoptosis of transplanted cells. We speculate that through Carv’s dual properties as a β-blocker and antioxidant, it maintains a unique ability to restore cardiac function and scavenge superoxide radicals (Hayashi et al., 2010). These dual properties of Carv distinguish it from drugs with only antioxidant properties.

**Effect of Carv and MSC on cardiac function and fibrosis**

Several pre-clinical studies have shown an improvement in cardiac function after MSC transplantation (Tomita et al., 1999; Kudo et al., 2003; Fazel et al., 2005). However, this is the first study to show that the combination of Carv along with MSCs treatment enhances the functional recovery of the heart when compared to MSC cardiomyoplasty alone. This data demonstrated the clinical significance of Carv in patients who are on Carv treatment and undergoing stem-cell transplantation. Patients might have a better clinical outcome in terms of improvement of cardiac function than patients receiving Carv only. On the other hand, cardiac fibrosis which is another key determinant in the process of left-ventricular remodeling was attenuated in both Carv and MSC groups. To an even greater extent, the anti-fibrotic effect was further enhanced in Carv+MSC group. The results suggest the crucial role of Carv, either alone as a β-blocker or in conjunction with stem cells showing phenomenal benefits in attenuation of myocardial fibrosis.
Effect of Carv and MSC on angiogenesis

We have previously reported that MSCs transplanted in the ischemic heart induce angiogenesis (Khan et al., 2009; Khan et al., 2012). The immunohistological findings from our current study showed an increased vessel and capillary density in the combined Carv and MSC group (Figure 4B & 4C). The results strongly suggest that the combinatorial treatment enhanced angiogenesis. However, the exact mechanism by which the combined treatment affects angiogenesis needs further investigation.

Effect of Carv and MSC on cardiomyocyte apoptosis and caspase-3 expression

One of the potential pathophysiological mechanisms in the progression of heart failure is cardiomyocyte apoptosis (Sabbah, 2000; Abbate et al., 2002), which is responsible for cardiac cell loss after acute MI (AMI) (Olivetti et al., 1996). This leads to changes in the shape, size and contractility of the left ventricle. Cardiac remodeling is not limited to the first 7 days after AMI; it is a progressive process which continues for weeks and up to several months after AMI (Abbate et al., 2002; Jessup and Brozena, 2003). Studies in human post-mortem and animal tissues have demonstrated an increased rate of apoptosis in the peri-infarct and remote myocardium for several months after AMI (Palojoki et al., 2001; Baldi et al., 2002). Apoptosis seemed to be higher in the peri-infarct regions, particularly in cases of recurrent ischemia (Abbate et al., 2002). A recent study by Abbate et al (Abbate et al., 2007) showed that permanent occlusion of infarct-related artery (IRA) is associated with increased apoptosis of cardiomyocytes in the peri-infarct regions of the heart and persisted for up to 8 weeks after MI in an animal model of permanent occlusion of the left coronary artery. In the present study, we have shown that in animals treated with Carv and MSCs, there was a significant reduction in
apoptosis as indicated by decreased TUNEL positive cells (Li et al., 2010). Additionally, caspase-3 which is an important mediator in regulating apoptosis was down-regulated in the combined treatment group (Baldi et al., 2002). The results demonstrated that the decrease in apoptosis might be due to the synergism between the antioxidant properties of Carv and the paracrine signaling effect of MSCs ultimately leading to a decrease in cardiomyocytes apoptosis in the peri-infarct regions of the heart. Overall, the data demonstrates the significance of combined therapy with β-blocker (Carv) and stem cells for ameliorating myocardial apoptosis after AMI.

**Effect of Carv and MSCs on phosphorylation of AKT and ERK**

The increase in phosphatidylinositol-3-kinase/Akt (PI-3K/Akt) activation and extracellular signal-regulated kinase (ERK) pathways play an important role in the regulation of cardiomyocyte apoptosis and left-ventricular function (Li et al., 2009). It has been reported that activation of ERK is associated with inhibition of cardiomyocyte apoptosis both *in vitro* and *in vivo* models of ischemia-reperfusion (Bueno et al., 2000; Foncea et al., 2000). A study by Li et al (Li et al., 2009) showed an increase in ERK phosphorylation in saline-injected control hearts at 1 week after MI and this increase was considerably less pronounced in MSC transplanted hearts. Additionally, increased p-ERK1/2 activation was reported in infarcted and remote regions in the post-MI hearts (Yeh et al., 2010). The study also suggested that the increase in MEK-1/ERK signaling may be predominantly localized in the non-myocytes in the scar and fibrotic regions. Although, cardiomyocytes contribute to the majority of the adult myocardial mass, they comprise of only 30% of the total cells in the heart and the remaining being poised of non-myocyte cells (Zak, 1973), these non-myocyte cells might be responsible for
alterations in Akt and ERK1/2 in post-MI hearts. Interestingly, the results from our study indicated an increased phosphorylation of Akt and ERK1/2 in post-MI hearts at 4 weeks. In contrary, there was a significant decrease in phosphorylation of Akt and ERK1/2 in Carv, MSC and MSC and Carv treatment groups. The decreased activation of AKT and ERK1/2 in the treated groups might be due to decreased cardiomyocytes apoptosis. Furthermore, increased activation of Akt and ERK1/2 in cardiac fibroblasts (as seen in MI hearts) might lead to increased fibrosis. Further studies are needed to understand the mechanism by which Carv and MSC treatment modulates the activation of Akt and ERK1/2.

CONCLUSIONS

Our study suggests that Carv treatment along with MSC transplantation improved cardiac function, decreased fibrosis, enhanced cardiomyocyte survival and decreased caspase-3 expression in the infarcted heart. The combined treatment strategy might be very useful in clinical settings, where infarcted patients are frequently treated with Carv, which could be supplemented with allogenic stem-cell transplantation, thereby producing a more promising clinical outcome in recovery of cardiac function.
Authorship contributions

*Participated in research design:* Fatemat Hassan and Mahmood Khan

*Conducted experiments:* Fatemat Hassan, Sarath Meduru, Mahmoud Mostafa, Kazuki Taguchi and M. L. Kuppusamy

*Contributed new reagents or analytic tools:* Mahmood Khan and Periannan Kuppusamy

*Performed data analysis:* Fatemat Hassan, Kazuki Taguchi, Sarath Meduru and Mahmood Khan

*Wrote or contributed to the writing of the manuscript:* Fatemat Hassan and Mahmood Khan
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1: **Carvedilol attenuates superoxide radicals:** Superoxide-scavenging ability of Carv was determined by EPR spectroscopy using DEPMPO spin trapping (A) Representative EPR spectra of DEPMPO spin trap (1 mM) was used to detect of superoxide radicals as DEPMPO-OOH adduct. (B) 1 mM of Carv, challenged against 1-mM DEPMPO, decreased the intensity of the DEPMPO-OOH spectrum by more than 35%, which was not seen with Atenolol (B). Overall, the EPR data clearly established that the Carvedilol is capable of scavenging superoxide in a dose-dependent manner. (NS represents not significant). Values are expressed as Mean ± SD; n=3.

Figure 2: **Effect of Carvedilol on the survival and proliferation of MSC:** MSCs (2 x 10^4/well) were cultured in a 96-well plate. After 24 hours, the cells were pre-treated with Carv (2.5 µM) or Atenolol (2.5 µM) for 2 hour prior to the addition of 800-µM H_2O_2 in a serum-free medium for 2 hours. Controls were used in parallel for each treatment group. Carv-treated MSCs showed increased protection against H_2O_2 induced cell death than Atenolol-treated group. (A) MTT and (B) BrdU assays were used to assess cell viability and proliferation respectively. (C) The effect of Carv treatment on apoptosis was assessed by TUNEL assay. Treatment of MSCs with 800-µM H_2O_2 increased the number TUNEL-positive cells. However, preconditioning with Carv protected against H_2O_2-induced cell death and oxidative stress. Values are expressed as Mean ± SD; n=3.

Figure 3: **Carvedilol adjuvant treatment with stem cells improves cardiac function:** (A) Representative images of ultrasound echocardiography (M-mode) performed on rats to assess the cardiac function. The functional parameters (B) ejection
fraction, (C) fractional shortening, (D) LV internal dimensions at end-systole (LVIDs) and (E) LV internal dimensions at end-diastole (LVIDd) were analyzed at the baseline and at 4 weeks after MI or stem cell transplantation. MSC+Carv treated group showed enhanced functional recovery after MI in comparison to MSC or Carv alone treated groups. Values are expressed as Mean ± SD; n=6 animals/group.

**Figure 4:** Effect of Carv and/or stem cells treatment on myocardial fibrosis and angiogenesis. (A) Masson-trichrome staining for cardiac fibrosis showed a significant decrease in fibrosis in MSC+Carv treated groups, when compared to MI or MSC groups (p<0.05). (B) α-SMA, a marker for vasculogenesis (white arrows) was increased in MSC+Carv group, when compared to MSC group. (C) v-WF, a marker for neovascularization or capillary density was also increased in MSC+Carv group, when compared to MSC group. Values are expressed as Mean ± SD; n=6 animals/group.

**Figure 5:** Carv treatment decreases myocardial apoptosis post-MI. Evaluation of cellular apoptosis in heart tissue was performed by TUNEL assay. (A) TUNEL (positive) cells (green) and total nuclei (DAPI/Blue) were imaged in the peri-infarct regions of the heart tissues at four weeks after MI. (B) Increased TUNEL-positive nuclei (green) were seen in the MI group, whereas Carv-treated hearts showed a significant decrease (p<0.05) in TUNEL-positive nuclei, when compared with MSC group. (C) Western Blot analysis of Caspase-3 expression in heart tissues showed significant (p<0.05) reduction in caspase-3 expression in the combined MSC+Carv treated groups in comparison to the single treated groups (MI, Carv and MSC groups). Values are expressed as Mean ± SD; n=4.
Figure 6: Effect of Carv and/or stem cell treatment on modulation of AKT, ERK1/2 and Bcl-2 after four weeks of MI. (A) Representative western blots for different groups were normalized with GAPDH. (B) Western blot analysis data showed a significant (p<0.05) increase in phosphorylation of Akt, and (C) ERK1/2 in MI group. However, the phosphorylation of AKT and ERK1/2 were significantly (p<0.05) attenuated in all the treated groups i.e. Carv, MSC and MSC+Carv. On the other hand, there was no change in the expression of pro-survival Bcl-2 in any of the groups. Values are expressed as Mean ± SD; n=3.
Figure 1
Figure 3

Comparison of Ejection Fraction, Fractional Shortening, LVIDs, and LVIDd among Sham, MI, Carv, MSC, and MSC+Carv groups.

A: Ultrasound images of Sham, MI, Carv, MSC, and MSC+Carv groups.

B: Bar graph showing Ejection Fraction with significant differences between groups (p<0.05).

C: Bar graph showing Fractional Shortening with significant differences between groups (p<0.05).

D: Bar graph showing LVIDs with significant differences between groups (p<0.05).

E: Bar graph showing LVIDd with significant differences between groups (p<0.05).
**Figure 6**

(A) Table showing protein expression levels:

- **PO**: + + + + +
- **CARV**: - - + - +
- **MSC**: - - + + +

Protein expressions and their molecular weights:

- **p-ERK**: 42/44 KDa
- **Total ERK**: 42/44 KDa
- **GAPDH**: 37 KDa

(B) Graph showing phosphorylated-ERK (p-ERK) levels:

- **Sham**
- **MI**
- **Carv**
- **MSC**
- **MSC + Carv**

Phosphorylated-ERK (p-ERK) levels are significantly higher in MI compared to Sham. The levels decrease with MSC and MSC + Carv treatments.

(C) Graph showing phosphorylated-Akt (p-Akt) levels:

- **Sham**
- **MI**
- **Carv**
- **MSC**
- **MSC + Carv**

Phosphorylated-Akt (p-Akt) levels are significantly higher in MI compared to Sham. The levels decrease with MSC and MSC + Carv treatments.

**Note:** The images and graphs above illustrate the changes in protein expression and phosphorylation levels in response to different treatments.