Hypoxic adipose mesenchymal stem cells derived conditioned medium protects myocardial infarct in rat

J. HE¹, Y. CAI², L.-M. LUO³, H.-B. LIU³

¹Integrated Department of South Building of Chinese PLA General Hospital, Beijing, China
²Department of Medical Oncology of South Building of Chinese PLA General Hospital, Beijing, China
³Department of Cardiology of Chinese PLA General Hospital, Beijing, China

Abstract. - OBJECTIVE: The aim of this study was to explore the impact of normoxic and hypoxic cell-culture conditions on the expression and secretion of adipose mesenchymal stem cells (ADMSCs)-derived paracrine molecules, and to evaluate the cardioprotective role of hypoxic condition medium (hypoCM) in vivo.

MATERIALS AND METHODS: Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) analyses of normoxic and hypoxic ADMSCs and their conditioned medium fractions. Then, the effect of hypoCM on cardiomyocytes proliferation and migration was assessed. Moreover, a rat model of myocardial infarct (MI) was established to test the therapeutic effect of hypoCM in vivo.

RESULTS: ADMSCs expressed and secreted significantly higher amounts of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and stromal derived factor-1 (SDF-1 or CXCL12) under hypoxic conditions. Furthermore, compared with the vehicle control, hypoCM significantly enhanced the proliferation and migration of cardiomyocytes. Consistent with the in vitro data, hypoCM decreased the infarct size, apoptosis index and apoptosis related protein in the rat MI model.

CONCLUSIONS: These findings suggest that ADMSCs promote rat MI via hypoxia-enhanced paracrine.

Key Words: Adipose mesenchymal stem cells, Myocardial infarct, Hypoxic, condition medium, Paracrine.

Introduction

Myocardial infarction (MI), also known as heart attack, is the irreversible necrosis of heart muscle secondary to prolonged ischemia. Approximately 1.5 million cases of MI occur annually in the United States and is an increasing cause of death in China. Reasons for this trend include the increasing prevalence of traditional risk factors for atherosclerosis, including hypertension, hyperlipidemia, diabetes mellitus, obesity, and inadequate physical activity in the presence of significant exposure to cigarette smoke, and air pollution. Population aging is further contributing to rising ischemic heart disease incidence. Although reperfusion therapy and several standard oral medications (antiplatelet drugs, β-blockers, statins, and angiotensin-converting enzyme inhibitors) reduce AMI case fatality and are recommended by international and Chinese AMI management guidelines, however, the final results have not changed a lot. Therefore, it is necessary to explore efficient therapeutic measures to prevent myocardial damage induced by MI.

In recent years, many investigators have demonstrated the potential of mesenchymal stem cells (MSCs) to be a beneficial therapy after experimental myocardial infarction (MI) by inhibiting pathological remodeling. These cells have shown benefit when delivered acutely after MI and in dilated cardiomyopathy. Although these results confirm the ability of MSCs to beneficially affect remodeling post-MI, the mechanism(s) underlying these effects is poorly understood. It has been proposed that MSCs are capable of differentiating into cardiac myocytes and regenerating cardiac muscle. However, this occurs infrequently despite the beneficial effects on left ventricular (LV) function. Several groups have suggested that MSCs are capable of secreting angiogenic, antiapoptotic, and mitogenic factors. Specifically, one explanation for the beneficial effects is MSCs may secrete cytokines...
that modulate cardiomyocyte survival and function through an autocrine/paracrine mechanism\textsuperscript{13,15}. Additional support of the paracrine hypothesis is provided by data showing that several cytoprotective cytokines (vascular endothelial growth factorVEGF], fibroblast growth factor-2, hepatocyte growth factorHGF], insulin-like growth factor-IIGF-I], and TB4) are significantly up-regulated in the Akt-MSCs\textsuperscript{16, 17}.

Recently, Das et al\textsuperscript{18} suggested that hypoxia mediated preconditioning of MSCs could reduce hypoxia induced cell death and increase MSCs proliferation and differentiation in vitro. In addition\textsuperscript{19-22}, MSCs could be isolated from a lot tissues other than bone marrow, such as adipose tissue, umbilical cord, placenta.

Here, we investigated the effect of conditioned medium fractions collected from hypoxic adipose MSCs (ADMSCs) in the treatment of rat MI. The results of this study now gave the considerable influence of oxygenation on the biological properties of ADMSCs, and set forward the intriguing possibility that adipose tissue derived products might find utility in cell-free therapies to encourage the treatment of MI.

Materials and Methods

Isolation of Adipose MSCs

Fat tissue was obtained from a healthy male human donor and clinical-grade ADMSCs were isolated according to previous descriptions\textsuperscript{23}. Briefly, fat was digested with type IV collagenase (Life Technologies, Carlsbad, CA, USA) at 37°C for 45 min, filtered by a 200-mesh strainer and centrifuged at 2000 \(g\) for 10 min. The cell sediment was suspended in complete medium containing low glucose Dulbecco’s modified Eagle’s medium (DMEM) and 20% fetal bovine serum (FBS) (Both were from Life Technologies), seeded at a density of 4000 cells/cm\(^2\) and grown at 37°C with 5% CO\(_2\) in a humidified atmosphere. After reaching 80% confluency, the cells were trypsinized and passaged. Cells used for experiments were between passages three to five.

ADMSCs Hypoxia Induction

Hypoxia was induced in ADMSCs by the use of a sealed chamber that was flushed with a humidified gas mixture composed of 2% O\(_2\), 5% carbon dioxide (CO\(_2\)) and 93% atmospheric nitrogen (N\(_2\)). The oxygen partial pressure value (pO\(_2\)) in the chamber was controlled and validated with a Compact Oxygen Controller (Biospherix, Lacona, NY, USA). The hypoxic cell culture chamber was kept closed throughout the entire experimental procedure.

Preparation of Conditioned Medium Fractions Derived from Normoxic and Hypoxic ADMSCs

ADMSCs were cultured and expanded under normoxic conditions in serum-containing complete medium. At passage 3-4, the ADMSCs were seeded into 100 mm tissue culture dishes and allowed to reach 80% confluence. The medium was then changed to serum-free RPMI-1640 (10 ml) (Life Technologies, Carlsbad, CA, USA), and the cells were cultured under normoxic (20% O\(_2\), 5% CO\(_2\) and 95% air) or hypoxic conditions for another 48 h. Next, conditioned medium fractions were collected from the normoxic and hypoxic ADMSCs to yield nCM and hypoCM, respectively, centrifuged at 3,000 \(\times g\) at 4°C for 15 min, collected cell-free supernant and stored at -80°C prior to further experiments.

For the in vivo analysis, aliquots of conditioned media were concentrated by 10 kD Millipore filter (Millipore, Billerica, MA, USA) by centrifugation at 5,000 \(\times g\) at 4°C for 25 min.

Proliferation Evaluation

ADMSCs ADMSCs (1×10\(^5\) cells) were seeded into 100 mm tissue culture dishes. The cells were incubated in serum-containing complete culture medium or serum-free Roswell Park Memorial Institute (RPMI)-1640 under normoxic or hypoxic conditions for 24, 48 and 72 h.

H9c2 cardiomyocytes Cardiomyocytes (5×10\(^4\) cells) were seeded into 12-well tissue culture plates in vehicle control medium or ADMSC hypoCM, and incubated for various periods of time. The media were changed once on day 4 of culture.

The cells were detached from the plates or dishes and evaluated at the indicated times by use of MTT assay. Cells were treated with 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and proliferation was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were reported as ratio of the optical density (OD) value between hypoCM treated group and control group.

Transwell Migration Assay

A transwell migration assay was performed to assess the chemotactic and migration proper-
ties of ADMSC hypoCM in vitro. Cell suspensions (5×10^4 H9c2 cardiomyocytes in 100 µl of medium) were added to the upper chambers of a 24-well Transwell plate (pore size: 8.0 mm; Corning Costar, USA). Aliquots of vehicle control medium, or hypoCM (600 µl) were then added to the lower chambers. Cells were maintained at 37°C at 24 h. The cells that had not migrated were removed from the upper face of the filters using cotton swabs, cells that migrated from the upper chambers to the lower chambers were then fixed with 4% formaldehyde and stained by 0.1% crystal violet. 3 random × 10 fields were photografted and counted as described previously.24

Realtime Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA extraction of ADMSCs and the reverse transcription reaction were performed according to the manufacturer's instruction. VEGF, HGF, CXCL12, IL-6, IL-10, IL-1β and TNF-α specific sequences were amplified during 32 cycles of 30 s denaturing at 95°C, 60 s annealing at 59°C, and 60 s extension at 72°C, with the primers listed in Table I.

ELISA Analysis

ADMSC nCM and hypoCM samples were subjected to ELISA analysis for their content of specific cytokines, growth factors and chemokines relevant to wound healing. ELISA kits from R&D Systems (Minneapolis, MN, USA) were used according to the manufacturer’s instructions.

Rat Grouping and Myocardial Infarction Model

All the animal stuff and related experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Male Wistar rats weighted 220-260 g (SLAC Laboratory Animal, China) were randomly divided into myocardial infarction group (n = 25) and sham group (n = 25). These two groups were further divided into 1, 3, 6, 12 and 24 h time points (5 rats at each time point). In hypoCM treated experiment, 15 rats were equally and randomly divided into sham group, MI+vehicle group and hypoCM treated group (n = 5).

Rats were anaesthetized with intraperitoneal (IP) injection of 2% pentobarbital natrium (40 mg/kg) (Sinopharm Chemical Reagent Co., Ltd, China). Endotracheal intubation was used for breathing. The rat was placed in supine position. Myocardial infarction (MI) model was induced by the left anterior descending coronary artery (LAD) ligation. A left thoracotomy was performed and the LAD was ligated at a level immediately below the bottom of the left atrium by irreversible tightening of a suture loop. A successful performance of coronary occlusion was confirmed by a typical ST segment elevation on the electrocardiogram (ECG) and regional cyanosis of the myocardial surface distal to the suture. No LAD ligation was performed in sham operation group.

Meanwhile, in hypoCM treated experiment, vehicle control or hypoCM was applied 10 min after LAD ligation by 5 injection sites into anterior and lateral aspects of the viable myocardium bordering the infarction with a 31-gauge needle (BD Bio-sciences, San Jose, CA, USA). LAD ligated rats with blank medium injection served as controls.

Haematoxylin-Eosin Staining

The heart tissues from each group were fixed in 4% formalin and then stained with haematoxylin-eosin. Pictures were taken using microscope at different magnification.

Table I. Primer sequence of related genes.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>GGCTTTACTGCTGTACCTCC</td>
<td>CAAATGTTTCTCGCTCT</td>
</tr>
<tr>
<td>HGF</td>
<td>ACCCTACAAGGTGAAAGCTACA</td>
<td>AATTGTGCGGTTGTTGTTCT</td>
</tr>
<tr>
<td>CXCL12</td>
<td>TGCCAGACAAAGACACCTCC</td>
<td>CTCGAAACAAACAGAATACAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGCCAGTTGCCTTCTTGG</td>
<td>CCGTCCTGGACATGTGAAAG</td>
</tr>
<tr>
<td>IL-10</td>
<td>CACGTGTCATGGTGCTGCTC</td>
<td>TGGCCAGGCTGCTCCCTCTTT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GGACCCAAAGCAGCTTCTTT</td>
<td>AGACAGCAGAGCCTTTTTT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TCAGTCTCATGGCCACAGA</td>
<td>GTGGCTTTGGAGATCTCCATT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCACCACCAACTGCTTA</td>
<td>GGATGCAGGGATGATGTC</td>
</tr>
</tbody>
</table>
**TUNEL Staining**

TUNEL staining was used for the pretreated heart tissue slices processing according to the manufacture’s instruction (Roche, Basel, Switzerland). The samples were analyzed under light microscope with a magnification of 400×. Five fields were randomly picked and the cells with brown particles presented in the nucleus were recognized as positive. The following equation was used for apoptotic index (AI) calculation: AI = positive cell numbers in one field/total cell numbers in one field × 100%.

**Triphenyltetrazolium Chloride (TTC) Staining**

The heart was rapidly removed and cooled in ice-cold saline for 10 min. Coronal sections (2 mm) were cut and immersed in 1% TTC at 37°C for 30 min, and then transferred to 4% paraformaldehyde in 0.01 M PBS (pH 7.4) for 24 h fixation. Normal hearts were red while MI hearts were white. The heart slices were photographed and analyzed with Image J software.

**Lactate Dehydrogenase (LDH), Creatine Kinase (CK) and Superoxide Dismutase (SOD) Activity Measure**

LDH, CK and SOD activities in myocardial tissue were measured using Assay kits (Beijing ZSGB-BIO, China) according to the manufacturer’s instructions.

**Statistical Analysis**

All the statistical analyses were performed by SPSS18 software (SPSS Inc., Chicago, IL, USA). The data was presented as Mean ± SD. Student t-test or one way-ANOVA was used to examine the difference in two or multiple groups. *p < 0.05 was recognized as significant difference.

**Results**

**Hypoxia Increases the Proliferation of ADMSCs**

As shown in Figure 1, with or without serum, proliferation of cultured ADMSCs was significant-

![Figure 1. ADMSCs Proliferation in serum-containing complete medium (A) and serum-free medium (B). Cell proliferation was examined under hypoxia or normoxia conditions, respectively. Data are given as the means ± SD; *p < 0.05 compared with thenormoxia group at 24, 48 and 72 h. †p < 0.05 compared with indicated earlier time points.](image-url)
ly increased by hypoxia at 48 h (for ADMSCs grown in complete culture medium, 1.72 ± 0.09 vs. 1.37 ± 0.15, *p < 0.05; for ADMSCs grown in serum-free LG-DMEM, 1.69 ± 0.05 vs. 1.48 ± 0.05, *p < 0.05) and 72 h (for ADMSCs grown in complete culture medium, 2.45 ± 0.08 vs. 1.91 ± 0.07, *p < 0.05; for ADMSCs grown in serum-free LG-DMEM, 1.78 ± 0.08 vs. 1.35 ± 0.09, *p < 0.05). Proliferation of hypoxic ADMSCs grown in complete culture medium increased in a time-dependent manner (Figure 1A, *p < 0.05). Similar results were observed when cell proliferation was evaluated in serum-free LG-DMEM, with the exception of a slight decrease in the proliferative capacity of the 72 h hypoxic incubation group (Figure 1B, 24 h vs. 48 h, 0.65 ± 0.02 vs. 1.68 ± 0.05, *p < 0.05; 48 h vs. 72 h, 1.68 ± 0.05 vs. 1.77 ± 0.09, *p < 0.05).

**Hypoxia up-regulates the mRNA Expression and Protein Secretion of ADMSC-Derived Paracrine Factors**

The selected growth factors, cytokines and chemokines associated with the MI in normoxic and hypoxic ADMSCs were quantified by Real-time RT-PCR. As shown in Figure 2A, hypoxia differentially up-regulated the mRNA expression levels of VEGF (2.61-fold), HGF (2.3 fold), C-X-C motif ligand 12 (CXCL12, 1.98-fold), interleukin 1 beta (IL-1β, 1.8-fold) and interleukin 6 (IL-6, 2.21-fold) (*p < 0.05). No difference was found on interleukin 10 (IL-10, 1.03-fold) and tumor necrosis factor-alpha (TNF-α, 0.98-fold) (*p > 0.05).

Then, ELISA was applied to measure the protein level of VEGF, HGF and CXCL12. We found the concentration of the VEGF (1.3-fold) (Figure 2B), HGF (3.02-fold) (Figure 2C) and CXCL12 (Figure 2D) (1.57-fold) were significantly up-regulated in the hypoCM than that in norCM (*p < 0.05).

**Hypoxia Condition Medium Enhances Proliferation and Migration of H9c2 Cardiomyocytes in vitro**

The hypoCM was then used to evaluate the effect on the behavior of H9c2 cardiomyocytes. Compared with vehicle control, we
found a significant increase on cell proliferation at Day 2, Day 4 and Day 6 ($p < 0.05$) (Figure 3A). Furthermore, hypoCM exhibited a significantly greater chemo-attractive effect (38-fold higher) than vehicle control medium on the migration of cardiomyocytes ($p < 0.05$) (Figure 3B).

**Hypoxia Condition Medium Improve the MI in vivo**

As shown in Figure 4, the successful establishment of the MI was confirmed by HE staining (Figure 4A) and TUNEL staining (Figure 4B). The apoptosis index was also calculated and a time dependent manner was observed (Figure 4B). After application with the hypoCM treatment, we assessed the therapeutic effect in the model. According to the HE staining, the myocardial damage in hypoCM treated group was alleviated compared with the vehicle control group (Figure 5 A). TUNEL staining showed the similar result and apoptosis index decreased significant in hypoCM treated group (Figure 5 B). The MI size assessed by TTC staining was decreased significantly in the hypoCM treated group compared with the vehicle control group ($p < 0.05$) (Figure 5 C). The examination of LDH, CK and SOD activities showed that the activities of CK and LDH were decreased and SOD was increased in the hypoCM treated group, compared with the vehicle control group (Figure 5 D).

**Discussion**

In present study, we focused on the impact of hypoxia on the paracrine functions of ADMSCs in regard to MSC stimulated cardiomyocytes repair. And demonstrated that ADMSCs expressed and secreted VEGF, HGF, CXCL12 and other factors, which was consistent with previous study. These growth factors, cytokines and chemokines function together to modulate the local environment, affecting the proliferation, migration, differentiation and functional recovery of resident cells.

Controversies were existed on the ability of ADMSCs to transdifferentiate into cell lineages of all three germ layers, the employment of ADMSCs derived paracrine molecules as a possible therapeutic approach to MI is of marked interest and great clinical significance. Such an approach could hypothetically minimize the biological variability of cell-based therapy, conquer the problem about cell origin and immunocompatibility, and allow precise dosage with identified paracrine components; thus, leading to the development of safe and effective cell-free regenerative strategies with predictable and controllable outcomes. However, the identified stem-cell phenotype of ADMSCs was still controversial, which result in the failure to fully achieve their therapeutic potential. According to the previous study, oxygen tension, which helps the maintenance of ADMSCs proliferative capacity and paracrine mechanisms of regenera-

---

**Figure 3.** Effects of ADMSC-derived conditioned medium samples on paracrine cell proliferation and migration. Cardiomyocytes ($5 \times 10^4$ cells) were incubated with vehicle control medium, nCM or hypoCM. Cell proliferation was evaluated at 2, 4, 6 days. (A, Data are given as the means ± SD; $^\ast p < 0.05$ compared with the vehicle control or the nCM group. Equal numbers of cardiomyocytes were added to the upper chambers of 24-well transwell plates, with the indicated medium added to the lower chambers ($n = 4$ wells per treatment). Cells that migrated to the bottom of the filter were stained and evaluated (B). Data are given as the means ± SD; $^\ast p < 0.05$ compared with the vehicle control or the nCM group.
ADMSCs mediated paracrine improve cardiomyocytes repair in MI through several pathway by the action of ADMSCs derived conditioned medium and secreted molecules: (1) ADMSCs derived secreted mitogens may stimulate the proliferation of cardiomyocytes in vitro; (2) ADMSCs derived conditioned medium may regulate cell migration in response to MI; (3) ADMSCs derived conditioned medium may act as a chemoattractant to recruit specific cell type to the MI region. We here used to hypoxia to enhance the ADMSCs elicited paracrine responses in cardiomyocytes in vitro and in vivo.

Significantly increased proliferation of ADMSCs under hypoxic conditions was observed in our setting here, which was consistent with previous study. In addition, real time RT-PCR and ELISA data showed that the paracrine function of MSCs toward target MI was enhanced by hypoxia, genes VEGF, HGF, CXCL12, IL-1β, IL-10 and IL-6 were significantly upregulated in response to a low oxygen concentration environment. Meanwhile, the secretion of VEGF, HGF and CXCL12 was also amplified. Of these factors, VEGF was suggested to exert pro-angiogenesis role and cell differentiation. HGF is cardioprotective and it attenuates ischemia/reperfusion injury by directly protecting cardiomyocytes. CXCL12 is also involved in cardiogenesis, migration of primordial germ cells and recruitment of endothelial progenitor cells to sites of ischemic tissue. These factors together may exert the best effect in MI treatment.

For future application of ADMSCs conditioned medium or purified factors from conditioned medium in MI, pathways involved in enhanced se-

**Figure 4.** Establishment of the rat myocardial infarction model. The morphological changes were observed by Haematoxylin-Eosin staining and apoptosis of cardiomyocytes was examined by using TUNEL after myocardial infarction. **A**, Morphological changes were determined after myocardial infarction 24h and sham operation by HE staining. **B**, Cardiomyocytes apoptosis was measured by TUNEL at 1, 3, 6, 12, 24 h after myocardial infarction. The graph shows the apoptotic index of cardiomyocytes in different groups (mean ± SD). (**A-B 40x**).
cretion of growth factors and cytokines by hypoxic ADMSCs still requires more information and further investigation.

We also found that conditioned medium derived from hypoxic ADMSCs (hypoCM) significantly enhanced the migration and proliferation of H9c2 cardiomyocytes \textit{in vitro}. Furthermore, hypoCM significantly improve the cardiac damage caused by MI compared with vehicle control. The possible reason is that hypoCM contains elevated levels of proteins involved in cardioprotection.

**Conclusions**

The culture of BM-MSC under hypoxic conditions increased their proliferation and paracrine effects to cardiomyocytes. These observations indicate that hypoxic ADMSCs and their secreted products might be employed in regenerative medicine strategies to decrease cardiac damage after MI.

**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

**References**

Hypoxic ADMSCs derived conditioned medium protects myocardial infarct in rat


16) Gneecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Nisieux N, Zhang L, Pratte RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005; 11: 387-398.

17) Gneecchi M, He H, Nisieux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratte RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006; 20: 661-669.


22) Zhao YF, Luo YM, Xiong W, Ding W, Li YR, Zhao W, Zeng HZ, Gao HC, Wu XL. Mesenchymal


