Original Research

Impaired redox environment modulates cardiogenic and ion-channel gene expression in cardiac-resident and non-resident mesenchymal stem cells

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Impact statement

Human mesenchymal stem cells (h-MSCs) are highly promising candidates for tissue repair in cardiovascular diseases. However, the retention of cells in the infarcted area has been a major challenge due to its poor viability and/or low survival rate after transplantation. The regenerative potential of mesenchymal stem cells (MSCs) repudiate and enter into premature senescence via oxidative stress. Thus, various strategies have been attempted to improve the MSC survival in 'toxic' conditions. Similarly, we investigated the response of cardiac resident MSC (hC-MSCs) and non-resident MSCs against the oxidative stress induced by H_2O_2. Supplementation of ascorbic acid (AA) into MSCs culture profoundly rescued the stem cells from oxidative stress induced by H_2O_2. Our data showed that the pre-treatment of AA is able to inhibit the cell death and thus preserving the viability and differentiation potential of MSCs.

Abstract

Redox homeostasis plays a crucial role in the regulation of self-renewal and differentiation of stem cells. However, the behavioral actions of mesenchymal stem cells in redox imbalance state remain elusive. In the present study, the effect of redox imbalance that was induced by either hydrogen peroxide (H_2O_2) or ascorbic acid on human cardiac-resident (hC-MSCs) and non-resident (umbilical cord) mesenchymal stem cells (hUC-MSCs) was evaluated. Both cells were sensitive and responsive when exposed to either H_2O_2 or ascorbic acid at a concentration of 400 μmol/L. Ascorbic acid pre-treated cells remarkably ameliorated the reactive oxygen species level when treated with H_2O_2. The endogenous antioxidative enzyme gene (Sod1, Sod2, TRXR1 and Gpx1) expressions were escalated in both MSCs in response to reactive oxygen species elevation. In contrast, ascorbic acid pre-treated hUC-MSCs attenuated considerable anti-oxidative gene (TRXR1 and Gpx1) expressions, but not the hC-MSCs. Similarly, the cardiogenic gene (Nkx 2.5, Gata4, Mlc2a and β-MHC) and ion-channel gene (I_{KDR}, I_{KCa}, I_{to} *and* $I_{Na.TTX}$) expressions were significantly increased in both MSCs on the oxidative state. On the contrary, reduced environment could not alter the ion-channel gene expression and negatively regulated the cardiogenic gene expressions except for troponin-1 in both cells. In conclusion, redox imbalance potently alters the cardiac-resident and non-resident MSCs stemness, cardiogenic, and ion-channel gene expressions. In comparison with cardiac-resident MSC, non-resident umbilical cord-MSC has great potential to tolerate the redox imbalance and positively respond to cardiac regeneration.

Keywords: Human umbilical cord mesenchymal stem cell, human cardiac mesenchymal stem cell, oxidative stress, redox balance, cardiac gene expression, ion-channel gene expression

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Introduction

Redox imbalance is a major concern of cardiovascular disease including myocardial infarction (MI), ischemia-reperfusion injury, cardiomyopathy, and end-stage heart failure.[1] This redox imbalance is caused either by excess reactive oxygen species (ROS) production or diminished antioxidant system. Numerous studies postulate that, the increased level of ROS is a causal factor for most of the cardiovascular diseases. The primary resource of ROS in the myocardium is the membrane bound NADPH oxidases, the mitochondrial electron transport chain (ETC) and the endoplasmic reticulum of fibroblasts, infiltrating inflammatory cells and

myocytes.[2,3] These intracellular ROS levels are tightly regulated to maintain the redox balance by well-defined antioxidant enzyme systems in myocardium at the steady-state physiological condition. However, at the pathological condition, the ROS levels could be further increased due to an imbalance in the production and recuperation of ROS which potentially leads to irreversible damage of myocardium and ultimately, heart failure.[3–5]

Although the existing treatments improve the cardiac function, the cardiac transplantation (CT) still serves as the best option for heart failure. However, the limitations in CT such as unavailability of donors, post-transplant rejection, and graft-versus-host diseases necessitate revolutionary therapeutic strategies in order to improve the injured heart's function. In line with this, stem cell therapy emerged as one of the alternative approaches to possibly substitute CT. Although cardiomyocytes propagation is an issue since these are matured end-differentiated cells, transplantation of stem or progenitor cells showed a potential therapeutic outcome in patients with cardiovascular diseases.[6–9]

To date, various types of stem cells have been experimentally tested for cardiac repair and regeneration including cardiac-resident stem/progenitor cells and non-resident stem cell such as embryonic stem cells (ESCs), bone marrow-derived stem cells (BMSCs), umbilical cord or cord blood and, adipose-derived stem cells (ADSCs), skeletal myoblasts and recently, induced pluripotent stem cells.[10–12] Among all, MSCs which are the adult stem cells found in almost all tissues hold great potential for cardiac regeneration. Despite being the stem cells of mesodermal tissues, MSCs as well transdifferentiate into non-mesodermal lineage tissues which expand the utilization of MSCs in various tissue injuries regardless of anatomical and physiological barriers.[13] The current research and clinical trial data advocate the successful use of MSC in repair, restore, regenerate, and reduce inflammation in the cardiac vicinity. Nevertheless, MSC therapy for heart function is still witnessed as a partial or incomplete functional recovery due to the poor survival or retention rate.

Redox balance also plays a pivotal role in the regulation of self-renewal and differentiation of stem cell. The origin of adult stem cell niches is characterized by low level of ROS, which preserve the stemness and self-renewal of stem cells. On the contrary, self-renewal ability of stem cells is skewed towards differentiation when the ROS level exceeds than the basal level.[14,15] However, stem cells have remarkable ability to balance the redox state by engaging an array of endogenous antioxidant enzyme system to scavenge the excess intracellular ROS. Several factors including redox imbalance have been identified to be involved in the inefficient/compromised cardiac regenerative ability.[16] Although the effect of redox imbalance on the limited sources of MSC is fairly established, the consequence of such imbalance on cardiogenic differentiation and ion-channel gene expression pattern of cardiac-resident and non-resident (umbilical cord) MSCs remain elusive. Therefore, a better understanding of the behavioral nature of MSC in a harsh environment such as elevated ROS is deemed necessary. We therefore sought to determine whether redox imbalance modulates the cardiogenic differentiation and ion-channel gene expression on cardiac and umbilical cord-derived MSC.

Methodology

Human umbilical cord-derived MSCs

The present study was conducted after obtaining the written informed consent from respective parents. Fresh human umbilical cords ($n = 6$) were collected after the childbirth and transported to the central facility in cold phosphate-buffered saline (PBS) incorporated with antibiotics and antimycotics. hUC-MSCs were generated and identified as suggested by our previous protocol.[17] In brief, Wharton's jelly of umbilical cord tissue was separated and chopped into minute pieces and enzymatically digested with 0.4% type II collagenase (Worthington, Lakewood, NJ) and 0.01% DNAse (Worthington) followed by mechanical dissociation using a handheld cell homogenizer (Hassen Wagger) at 9000 rpm/min for 10–15 min. The cell suspension was filtered using 70 μm cell strainer, washed, and finally seeded into 75 cm^2 tissue culture flasks at a density of 1 million cells/mL. Third to fifth passages of expanded hUC-MSCs were used for the experiments. Immunophenotyping was performed to characterize the hUC-MSCs by using standard MSC surface markers.

Human cardiac-derived MSCs

Myocardial biopsies were collected from the patients aged between 40 and 60 years ($n = 3$) who underwent heart surgery after obtaining a written informed consent in accordance with the ethical committee requirements of National Heart Institute, Malaysia. The generation and culture of hC-MSCs were carried out as per our established methodology.[17] In brief, myocardial biopsies were diced into minute pieces, washed, and finally seeded into a 75 cm^2 tissue culture flasks. Third to fifth passages of hC-MSCs were used for the following experiments. Immunophenotyping was performed to characterize hC-MSCs by using standard MSC surface markers.

Immunofluorescence staining

The hUC-MSCs and hC-MSCs were cultured on coverslips and were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.5% Triton X-100 and blocked with 5–10% goat serum in PBS. The cells were then probed with REX1 and Notch1 (ab50828 and ab52627, Abcam, Cambridge, MA) for overnight incubation under room temperature, followed by probing with an anti-rabbit antibody with Alexa Fluor 488 for 1 h. The slides were then mounted with mounting medium containing nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) (ab188804, Abcam). Immunofluorescence images were captured using a fluorescent microscope (Olympus ix51, USA).

Cytotoxicity assay

The hUC-MSCs and hC-MSCs (2×10^4 cells/well) were plated in 96-well plates in triplicates and were allowed to adhere for 24 h before treatment. The cells were then treated with different concentrations of AA (50 μmol/L to 500 μmol/L) (Sigma Chemical Co., St. Louis, MO) and H_2O_2 (100 μmol/L to 1200 μmol/L) (Merck, USA) for 24 h and 4 h, respectively. The cytotoxicity of AA and H_2O_2 was determined using MTT assay as previously published.[17] Similarly, hUC-MSCs and hC-MSCs (2×10^4 cell/well) were plated in 96-well plates in triplicates and were pre-treated with or without 400 μmol/L of AA for 24 h. Cells were then washed with PBS twice and treated with or without 400 μmol/L of H_2O_2 for 4 h. The viability of the cells was detected using MTT method at a wavelength of 570 nm by a microplate reader.

Measurement of ROS

ROS levels were measured in hUC-MSCs and hC-MSCs that were pre-treated with AA (24 h) and post-treated with H_2O_2 (4 h) by fluorescent probe 2′7′-dichlorodihydrofluorescein diacetate. Cells were grown on coverslips and were grouped into three arms. One group was treated with 400 μmol/L of H_2O_2 for 4 h; the second group was treated with 400 μmol/L of AA for 24 h; the third group was pre-treated with 400 μmol/L of AA for 24 h followed by 400 μmol/L of H_2O_2 for 4 h. After treatment, the cells were washed with PBS and incubated with the media containing 10 μmol/L 2′7′-dichlorodihydrofluorescein diacetate for 30 min. Fluorescence images were acquired using fluorescent microscopy (Olympus ix51). Fluorescence intensity of the images was analyzed using ImageJ software, NIH.

Differentiation assay

Cells (hUC-MSCs and hC-MSCs) were divided into four groups upon achieving 80% of cell confluency. The group one was treated with 400 μmol/L of H_2O_2 for 4 h; the second group was treated with 400 μmol/L of AA for 24 h; the third group was pre-treated with 400 μmol/L of AA for 24 h followed by 400 μmol/L of H_2O_2 for 4 h. The fourth group served as a control without treatment. After the respective treatments, cells from all four groups were introduced into osteocytes and chondrocytes using respective differentiation medium (STEMPRO®), USA. Differentiated cells were fixed and stained with Alizarin Red and Alcian blue to visualize the osteogenic and chondrogenic differentiation, respectively.

Semi-quantitative RT-PCR

Total RNA was extracted from three independent hC-MSCs and hC-MSCs treated with or without AA and/or H_2O_2 using RNeasy Mini Kit (Qiagen, Germantown, MD) as per manufacturer's instructions. The preparation of the first-strand cDNA was conducted using the QuantiTect Reverse Transcription Kit (Qiagen). Quantification of mRNA levels was performed using gene-specific primers and the reaction was performed in 20 μL containing 1 μL cDNA, 2 μL primers (1 pmol final concentration), 10 μL OneTaq DNA polymerase mixers (BioLabs, USA), and 10 μL of DNase-free water. After incubation for 2 min, amplification was carried out for 35 cycles of 45 s at 95°C, 30 s at 50–65°C, and 45 s at 72°C. The gene expression was normalized to housekeeping gene, GAPDH/β-actin and gene expression level was quantified by ImageJ software. Details of the primers are provided in Table 1.

Western blot

Cells were homogenized with radioimmunoprecipitation assay (RIPA) buffer (Merck Millipore, Germany) containing protease-phosphatase inhibitor cocktail (Roche and Sigma-Aldrich) and clarified by centrifugation at 10,000 r/min for 10 min. Bradford method was followed to quantify the extracted protein. Protein lysates were mixed with 3x loading dye containing 5% β-mercaptoethanol followed by boiling for 3 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation. The separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk in 1x TBST-T for 1 h. After washing, the membranes were then probed with respective primary antibodies (REX1 and Notch1 (ab50828 and ab52627 Abcam) at dilution from 1:1000 to 1:2500 in 1x TBST buffer containing 2–3% bovine serum albumin for overnight at 4°C. Following washes, membranes were incubated with horseradish peroxidase conjugated anti-mouse/anti-rabbit/anti-human (1:10,000) antibodies for 1 h at room temperature. Finally, membranes were subjected to chemiluminescence detection using ECL kit (Western Bright, USA). The intensity of the signals was visualized by bio-illuminator (FluorChem 5500, CA) and the quantification was performed using ImageJ software.

Statistical analysis

Data from each group were expressed as mean and standard error (SE) of at least three separate experiments performed. Statistical comparison between groups was analyzed using Student's *t* test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Stemness and differentiation potency of hUC-MSC and hC-MSC

We recently reported that human umbilical cord and cardiac-derived MSCs had similar expression level of cell surface markers (CD44, CD73, CD90 and CD105) and ion-channels, and tri-lineage differentiation pattern.[17] In the present investigation, we analyzed the stemness and potential of differentiation between the sources of MSCs using established markers, REX1 and Notch1, respectively. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed a significant ($P < 0.05$) increase of REX1 and Notch1 expressions in cardiac-derived MSCs when compared to hUC-MSCs (Figure 1(b)). In contrary, the immunofluorescence and Western blot analysis revealed that there were no significant variances noted in the protein expression of REX1 and Notch1 in both sources of MSCs. These results indicated that although mRNA

Table 1 List of primers for RT-PCR

Gene	Forward primer sequence	Reverse primer sequence	Length (bp)
Kv2.1[32]	TACTGGGGCATCGACGAGA	GACTGGCCGAACTCATCGA	308
Kv4.3[32]	GATGAGCAGATGTTTGAGCAG	AGCAGGTGGTAGTGAGGCC	106
Kv7.3[32]	GGAGAGGAGATGAAAGAGGAG	TGAAGAAAGGAAAAGAGACGAC	358
KCNN3[32]	GCCATCCTCCACCCTTCCTCCA	CGGGAGGAGATGACGATCTC	320
KCNN4[32]	CCTTTCAGACACACTTTGGCTGATCC	CAGTGCTAAGCAGCTCAGTCAGG	529
hNE-Na[32]	GCTCCGAGTCTTCAAGTTGG	GGTTGTTTGCATCAGGGTCT	446
GAPDH[33]	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT	217
α-MHC[34]	CACAGAAGAGGCCCGAGTAG	CCACCCAAGTTCGACAAGAT	127
β-MHC[34]	CAGGGTGTTGACCTTGTCCT	TGATCTGGAGCTGACACTGG	196
MLC-2a[34]	ATTGAGCTTCTCCCCAAAGAG	GCAGACCTGAGGGAGACCTAC	135
NKx 2.5 [34]	AGATCTTGACCTGCGTGGAC	GAGAAGACAGAGGCGGACAA	187
GATA-4[34]	GTGGACATAGCCCCACAGTT	CTCCTACTCCAGCCCCTACC	207
Tropnin-1[34]	CGTCTCTCGATCCTGTCTTTG	CATGGAGAAGGACCTGAATGA	108
SOD1[35]	TTACACCACAAGCCAAACGAC	TGTTGAGCCGGGCAGTGT	359
SOD2[35]	TGTTGAGCCGGGCAGTGT	CTCCCAGTTGATTACATTC	631
CAT[35]	TTTGGCTACTTTGAGGTCAC	TCCCCATTTGCATTAACCAG	439
TrxR1[35]	TCGCTTTGGAGTGCGCTGGA	GATTGCAACTGGGGTGAGCT	439
GI-GPx[35]	TCACTCTGCGCTTCACCATG	AGCAGTTCACATCTATATGGC	601
NOTCH1[36]	GACATCACGGATCATATGGA	CTCGCATTGACCATTCAAAC	666
REX1[37]	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA	306
β-Actin[38]	CACGAAACTACCTTCAACTCC	CATACTCCTGCTTGCTGATC	265

expression of REX1 and Notch1 were remarkably high in cardiac-derived MSCs, their translation protein expression was comparable between the two different sources of MSCs (Figure 1(a) and (c)).

Effects of H_2O_2 and AA on viability and proliferation of hUC-MSC and hC-MSC

When the proliferation rate and percentage of cell viability were compared between these groups upon H_2O_2 treatment, a similar trend of cell damage was noticed in dose- and time-dependent manners (Figure 2(a)). It was noted that the median effective dose (ED_{50}) was approximately 400 μmol/L for 4 h. The effects of AA on the proliferation and viability of hUC-MSCs and hC-MSCs were tested at concentration ranging from 50 to 500 μmol/L for 24 h (Figure 2(b)). Unlike H_2O_2, cell viability was profoundly increased in a dose-dependent manner (~1.5 fold of hCU-MSC; ~1.3 fold of hC-MSC) when treated with 400 μmol/L of AA. Since, the AA treatment delivered a positive influence on cell (hUC-MSC and hC-MSC) proliferation we have hypothesized that whether preconditioned AA can rescue the MSCs from H_2O_2-mediated cellular damages. To test our hypothesis, MSCs were preconditioned with 400 μmol/L AA for 24 h followed by an immediate exposure of 400 μmol/L H_2O_2 for 4 h. As we expected, the result shows that there was no significant effects up on cellular (hUC-MSC and hC-MSC) viability (Figure 2(c)). However, subsequent exposures (without AA preconditioning) of H_2O_2 significantly affect the cellular viability. These results revealed that preconditioning of MSCs with AA had drastically reduced the detrimental effect inflicted by H_2O_2 treatment, whereby only 20% of cells were dead. Overall our results clearly show that AA preconditioning certainly enhances the protective response in both resident and non-resident MSCs when challenged by an oxidative stress environment.

AA precondition preserves the osteogenic and chondrogenic differentiation from oxidative stress-mediated damage

In order to examine the effect of H_2O_2 and preconditioning of AA on MSCs, cells were cultured in the respectice osteogenic and chonddrogenic inducive media during the differentiation process or prior to the induction. The short-term exposure of 400 μmol/L H_2O_2 has negatively impacted the differentiations of MSCs into osteogenic and chondrogenic lineages; however, AA promotes the differentiations of MSCs towards both lineages as compared to untreated controls (Figure 3). When MSCs were preconditioned with AA and treated subsequently with H_2O_2, the differentiation ability of MSCs towards osteogenic and chondrogenic lineages is not affected, indicating a protective nature of AA on induced oxidative stress.

ROS production attenuated in AA preconditioned MSCs

The intracellular redox homeostasis changes in MSCs as a result of H_2O_2 and/or AA exposures were measured by detecting ROS level using fluorescent probe (2′7′-dichlorodihydrofluorescein diacetate). A significant increase in ROS levels was observed in H_2O_2-treated hUC-MSC and hC-MSC. Although both hC-MSC and hUC-MSC responded to the oxidative insult by releasing ROS, it was noted that the magnitude of ROS production is greater in hUC-MSC.

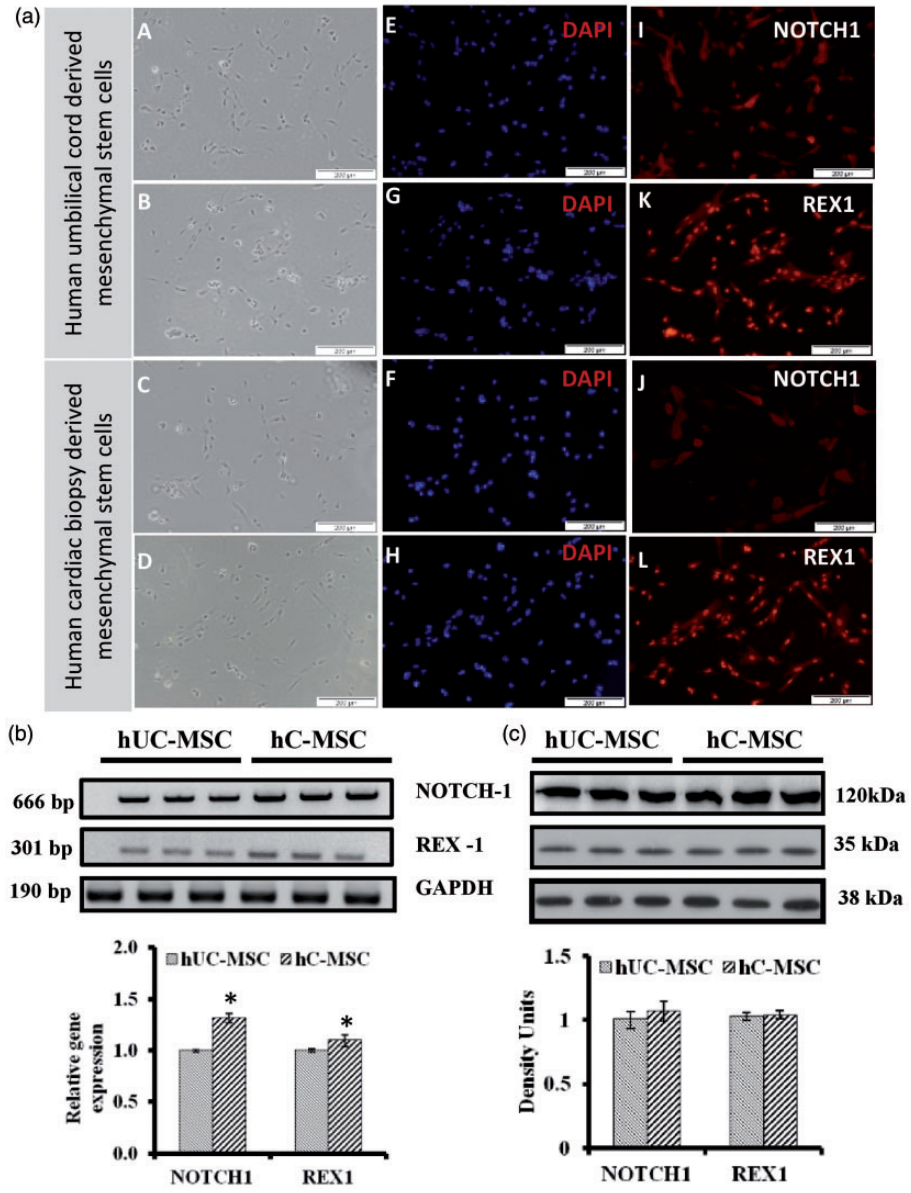

Figure 1 Immunofluorescence analyses of NOTCH1 and REX1 protein expression in two distinct MSC sources (a). In order to examine the distribution of the NOTCH1 receptor (a single-pass transmembrane receptor protein important in cell–cell communication), while REX1 is important in maintaining proliferative state and to prevent differentiation in MSCs. (A–D) Images aliened in first column are captured using phase contrast microscopy for unstained hUC-MSCs and hC-MSCs. The cells are monomorphic with a spindle-shaped morphology and contain scant cytoplasm. (E–H) DAPI, a nucleus staining, was employed to image the distribution of nucleus in both *in vitro* expanded hUC-MSC and hC-MSC which were derived from passage 4. (I and K) Cells were positive for NOTCH1 and REX1 in hUC-MSCs. (J and L) Cells were positive for NOTCH1 and REX1 in hC-MSCs. Scale bar: 200 μm. Gene (NOTCH1 and REX1) expression level between hC-MSCs and hUC-MSC (b) mRNA expression, Western blot analysis of NOTCH1 NAD REX1 expression (c). GAPDH/β-actin was used as an internal control gene. The variation within each set of triplicates is shown with mean of SD ±: *(between hUC-MSCs and hC-MSC) where $P < 0.05$ ($n = 3$). (A color version of this figure is available in the online journal.)

When the same experiment was repeated but with AA pre-conditioned MSCs, the production of ROS was remarkably reduced in all cells (Figure 4). These results demonstrate that both MSCs are highly sensitive to H_2O_2 and pre-treatment with AA serve as an efficient cyto-protective tool.

Redox imbalance and primary antioxidant gene expressions in MSCs

Since H_2O_2 treatment has elevated the ROS production in MSCs the expressions of primary antioxidant enzymes genes such as SOD1, SOD2, Catalase, GPX1, and TXRX1were evaluated as these genes are crucial in eliminating the intracellular ROS. The mRNA expressions of primary antioxidant enzymes (SOD1, SOD2, GPX1 and TXRX1) were down regulated in both hUC-MSCs and hC-MSCs. On the other hand, preconditioning with AA considerably strengthened the hUC-MSCs endogenous antioxidant system (TXRX1 and GPX1), but not in hC-MSCs. Moreover, AA pre-treated hUC-MSCs show more resistance towards oxidative stress by stabilizing or increasing the expression of antioxidantgenes (SOD1,

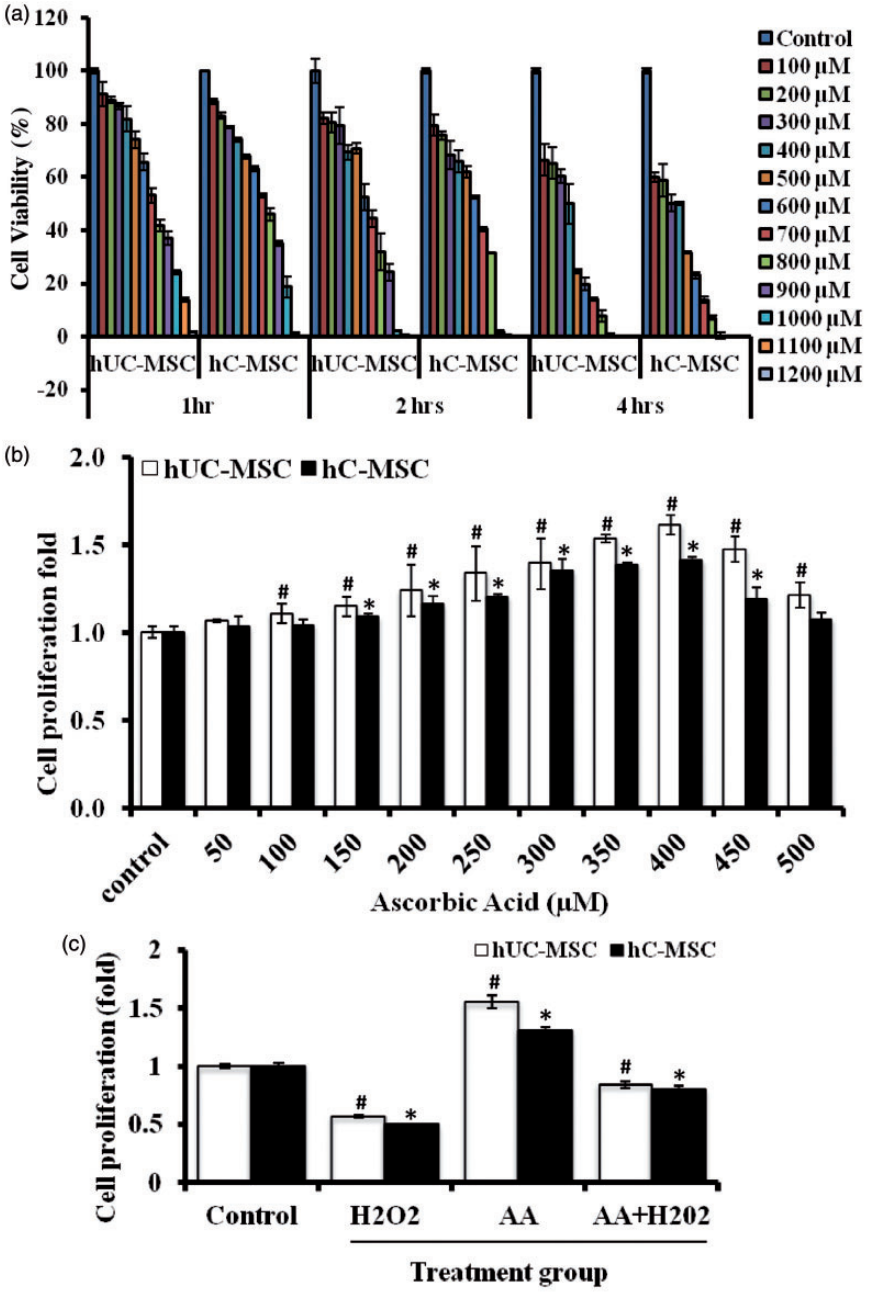

Figure 2 The cell growth profile after the treatment with different concentrations of hydrogen peroxide and ascorbic acid in two distinct MSC sources. H_2O_2 was employed to validate the growth inhibition while inducing oxidative stress to MSCs. Ascorbic acid was used to stimulate MSCs proliferation and to reduce oxidant induced by H_2O_2. The cell viability assay was performed by MTT method. (a) MSCs exposed to various concentration of H_2O_2 at different time interval. H_2O_2 has significantly reduced the cell number in dose- and time-dependent manner. (b) Cells were supplemented with different concentrations of AA for 24 h in complete media. AA significantly induces the cell growth until the concentration 400 µmol/L, after that cell growth starts to decrease (c). Cells were pre-treated with 400 µmol/L for 24 h followed by exposure to 400 µmol/L of H_2O_2 for 4 h. Pre-treatment with AA cells significantly tolerate the oxidative stress than untreated cells. The variation within each set of triplicates is shown with mean of SD ±: # (between hC-MSCs, compared with control) and *(between hUC-MSCs, compared with control) where $P < 0.05$ ($n = 3$). (A color version of this figure is available in the online journal.)

SOD2, GPX1 and TXRX1). Whereas, AA pre-treated hUC-MSCs failed to restore the expression of antioxidant genes (Figure 5). These findings demonstrate that both hUC-MSCs and hC-MSCs are sensitive to oxidative stress when stimulated with low levels of antioxidant system. Nevertheless, hUC-MSCs have the ability to withstand oxidative stress by stabilizing their antioxidant enzyme genes' expressions upon pre-exposure to AA.

Redox imbalance regulates the cardiac specific gene expression in MSCs

To verify whether the H_2O_2/AA-induced redox imbalance regulates cardiac gene expression, we have estimated the expression levels of cardiac-specific markers, such as Nkx2.5, Gata4, α-MHC, β-MHC, Mlc2a, and Troponin I. The RT-PCR analysis revealed that the mRNA expression of Gata4, Mlc2a, β-MHC, and Troponin I was significantly

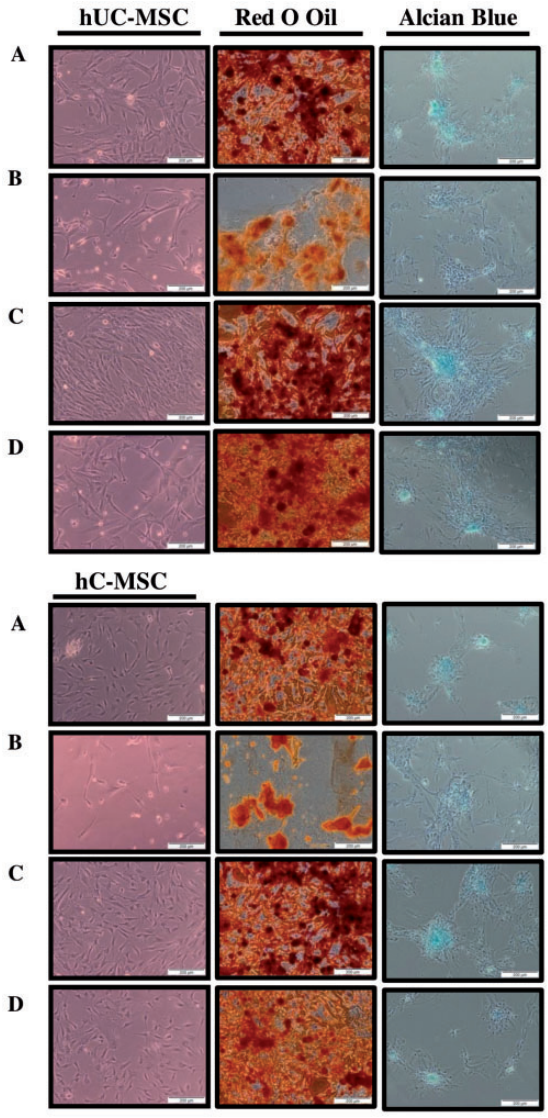

Figure 3 Comparative analysis of osteogenic and chondrogenic differentiation ability of MSCs exposure to an oxidant and reducing agent. (a) Control group (untreated). (b) Cells exposed to H_2O_2. (c) Cells exposed to AA. (d) Cells pre-exposed to AA followed by H_2O_2. The osteogenic lineage was stained with red O oil and chondrogenic lineage was stained with Alcian blue stain. Picture images are taken using phase contrast microscope at 100× magnification. Scale bar = 200 μm. (A color version of this figure is available in the online journal.)

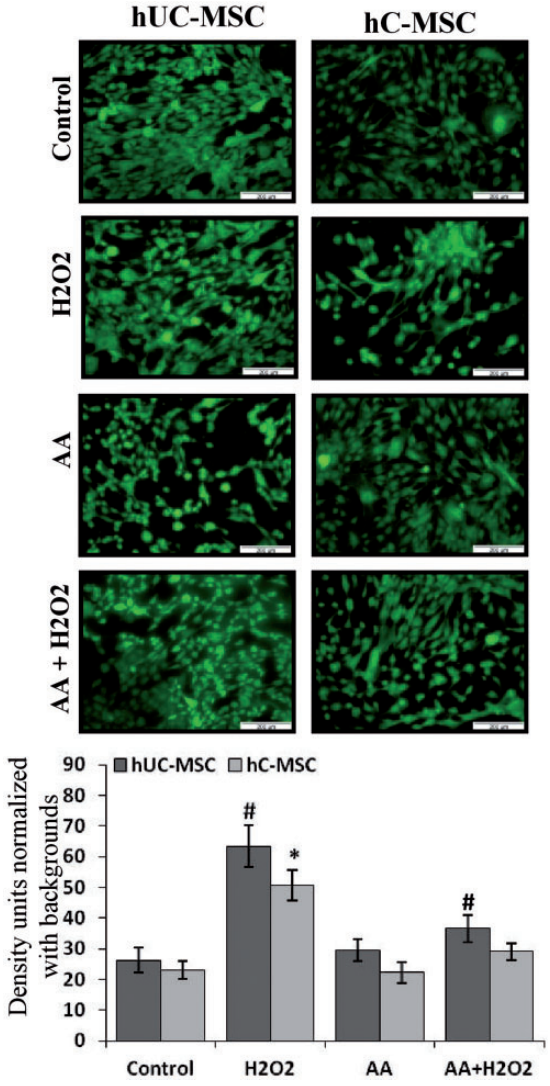

Figure 4 Measurement of intracellular reactive oxygen species (ROS) in hUC-MSC and hC-MSC after induction with and without 400 μmol/L AA and H_2O_2. (a) Cells were incubated with 2′,7′-dichlorofluorescein diacetate (DCFDA) fluorescent probe and imaged under fluorescent microscope, 100× magnification. Scale bar = 200 μm. (b) Bar graph demonstrates the levels of ROS at different groups. The variation within each set of triplicates is shown with mean of SD ±: # (between hC-MSCs, compared with control) and *(between hUC-MSCs, compared with control) where $P < 0.05$ ($n = 3$). (A color version of this figure is available in the online journal.)

increased in hUC-MSC when compared to hC-MSC at the basal state. Upon treatment with short-term H_2O_2, hUC-MSCs showed an increased mRNA expression of Nkx2.5, Gata4, MLC2a, and β-MHC as compared to the untreated controls. Although, hC-MSC responded to the H_2O_2 and showed an increased expression of Nkx2.5, and had no effects on Gata4, α-MHC, β-MHC, MLC2a, and Troponin I expressions. On the other hand, AA treatment revealed a significant alteration in cardiac genes expressions. The mRNA expressions of Gata4, Mlc2a, and β-MHC were remarkably increased in hUC-MSCs and AA significantly down regulated the cardiac markers except Gata4 and Troponin I. However, the AA pre-treatment was not significantly affecting the oxidative stress-induced cardiac gene expression in both MSCs compared to the time-matched H_2O_2-treated MSCs (Figure 6).

Redox imbalance regulates the ion-channel gene expression in MSCs

Ion-channels play a crucial role in cellular electrophysiological activities by regulating the potential of membranes. It is indispensable to study the nature of ion-channels properties of stem cell in cardiac regenerative medicine. Recently, we have also reported the presence of well-defined ion-channels in both MSCs. Here, we have evaluated the impact of induced redox imbalance in modulating the ion-channel expression in both MSCs. In this context, three types of K^+ channels and one type of Na^+ channel

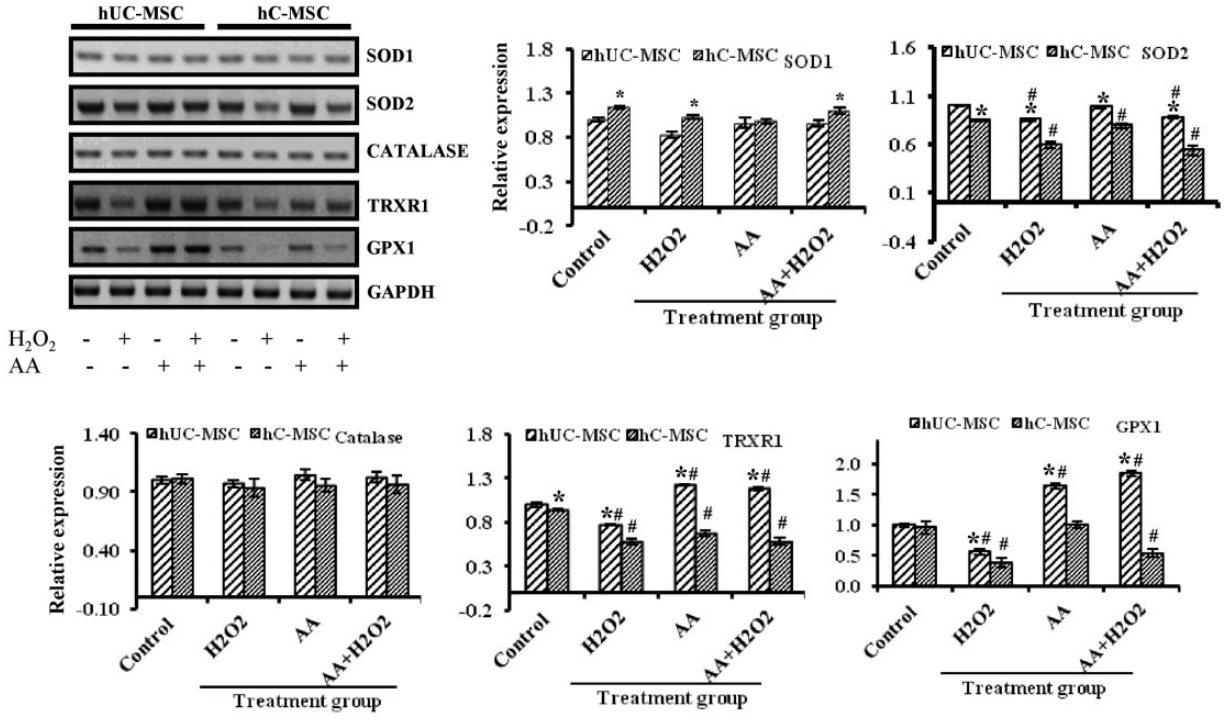

Figure 5 Antioxidants gene expression in hUC-MSC and hC-MSCs. Relative mRNA expression of antooxidant enzymes of SOD1, SOD2, catalase, TrxR1, and glutathione peroxides (GPx). The relative gene expression levels were normalized with GAPDH/β-actin (housekeeping gene). The variation within each set is shown with mean of SD ±: *(between hUC-MSCs and hC-MSC); # (between control and experiment) where $P < 0.05$ ($n = 3$)

expression was quantified at mRNA level. The RT-PCR analysis revealed that the oxidative stress promotes all their subunits (delayed rectifier K^+ current [IKDR], Ca^{2+}-activated K^+ current [IKCa] and transient outward K^+ current [Ito]) of K channels (INa.TTX), and one Na channel gene expression in hUC-MSC compared to the basal control. Unlike hUC-MSC, almost all types of ion-channels were significantly increased in hC-MSC, except K channel subunits of Kv7.3 (IKDR) and Kv4.3 (Ito). Whereas the treatment of MSCs with AA (400 μm) had no substantial effect on ion-channel expressions, except Kv2.1 and Kv4.3 in hUC-MSC. In the meantime, AA pre-incubation negatively promoted the H_2O_2-induced ion-channels gene expression in both MSCs. Overall results demonstrate that oxidative stress potentially induced the ion channel gene expressions in both cardiac residential and non-residential MSCs, whereas reduced environment did not affect the ion-channel expression (Figure 7).

Discussion

The global research data from preclinical studies and clinical trials have indicated MSCs as an excellent therapeutic tool for various ischemic heart diseases.[18] However, the poor survival of transplanted stem cell at the affected or injured site often hampers the potential for cardiac regeneration. The unfavorable or harsh environment (inflammation, oxidative stress, etc.) at ischemic area jeopardies engraftment and execution of the repair mechanisms the transplanted stem cells and eventually causes cell death.[16,19,20] This emphasizes the importance of conducive micro-environment in delivering the expected therapeutical effects of stem cells. However, it is also equivalently crucial to elucidate the response and homeostasis behavior of stem cells when transplanted or homed at non-conducive micro-environments. Since the source and the anatomical regions where the stem cells were harvested determine their responses towards a new micro-environment through a reciprocal manner, thus, this present study evaluated the behavior of hC-MSC and hUC-MSC against redox imbalance induced either by H_2O_2 and/or AA.

Various research studies have confirmed that MSCs are being sensitive to redox stress.[21] Our data showed that both MSCs were ceased in proliferation and committed in the rapid cell death upon treatment with ≥400 μmol/L of H_2O_2 / AA. It was previously demonstrated that stem cells were susceptible for transient cell cycle arrest and apoptosis when exposed to the short-term and sub-lethal doses of H_2O_2.[22] Nevertheless AA preconditioning improved the viability of MSCs after the oxidative stress challenge. It could be possible that AA preconditioning may directly or indirectly influence the endogenous antioxidant system of MSCs and protect them from short-term oxidative stress. Others as well reported a similar observation when various antioxidants such as *N*-acetylcysteine, edaravone, and AA were consumed.[23–25] Furthermore H_2O_2-induced oxidative stress had significantly affected the osteogenic and chondrogenic differentiation ability of MSCs from both cardiac and non-cardiac origins. In line with this, other scientific reports too had documented inhibition of osteogenic and chondrogenic differentiation capability of MSCs after being treated with H_2O_2.[26,27]

It has been recently reported that MSCs also have developed defence system against wide range of stresses, which

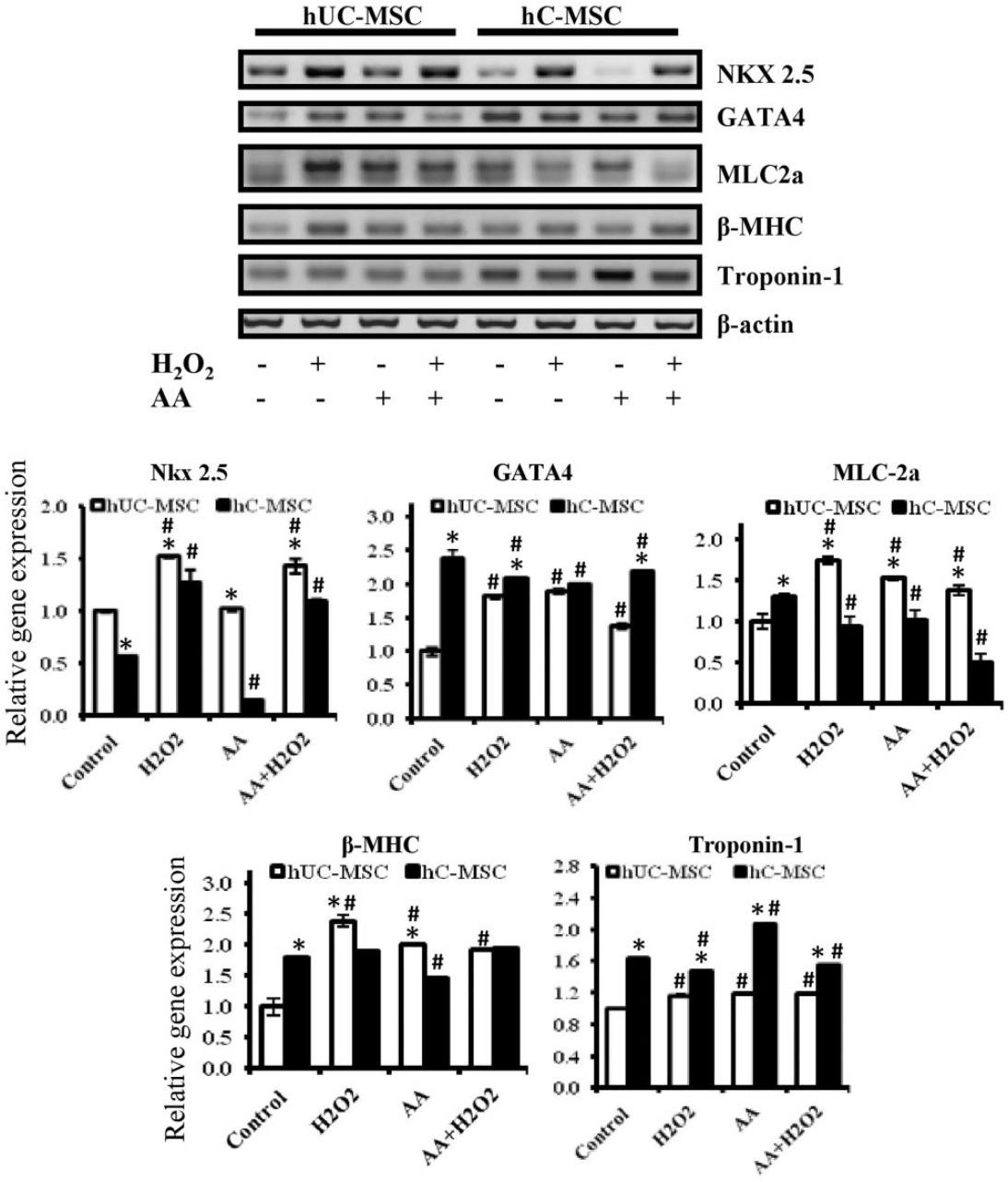

Figure 6 mRNA expression of cardiogenic gene in distinct MSCs. mRNA and cDNA were prepared from control and treatment groups and were subjected to PCR for quantifying the relative mRNA expression. GAPDH/β-actin was used as an internal control gene. The experiment was conducted in triplicates using three different samples. The variation within each set is shown with mean of SD ±: *(between hUC-MSCs and hC-MSC); # (between control and experiment) where $P < 0.05$ ($n = 3$)

are more or less equal to differentiated cells.[27] In general, MSCs generate low levels of intracellular ROS and constitutively express high levels of endogenous antioxidant enzymes, which is critically important to maintain the hypoxic or redox status of MSC niches. However, an adequate level of ROS is essential to sustain the physiological process of stem cells such as proliferation, differentiation, and apoptosis. Since MSCs from cardiac and non-cardiac origins exposed to the short-term and sub-lethal concentration of H_2O_2 a remarkable level of oxidative stress was created by producing an abundant amount of intracellular ROS. On the other hand, AA pre-treated MSCs showed a relatively lesser ROS accumulation after being challenged by the oxidative stress. Our result support the notion that MSCs are highly sensitive to the sub-lethal concentration of H_2O_2 and are incapable to maintain the redox status by up-regulating the endogenous antioxidant enzymes.[21] We presumed that the short and sub-lethal treatment of H_2O_2 may affect the expression of endogenous antioxidant enzymes. We indeed found that the expression of primary antioxidant enzymes SOD1, SOD2, TRXR1, and Gpx1 were down-regulated, but the expression of catalase was not altered. These findings are in agreement with a previous study which postulated that the umbilical cord blood derived MSCs are highly

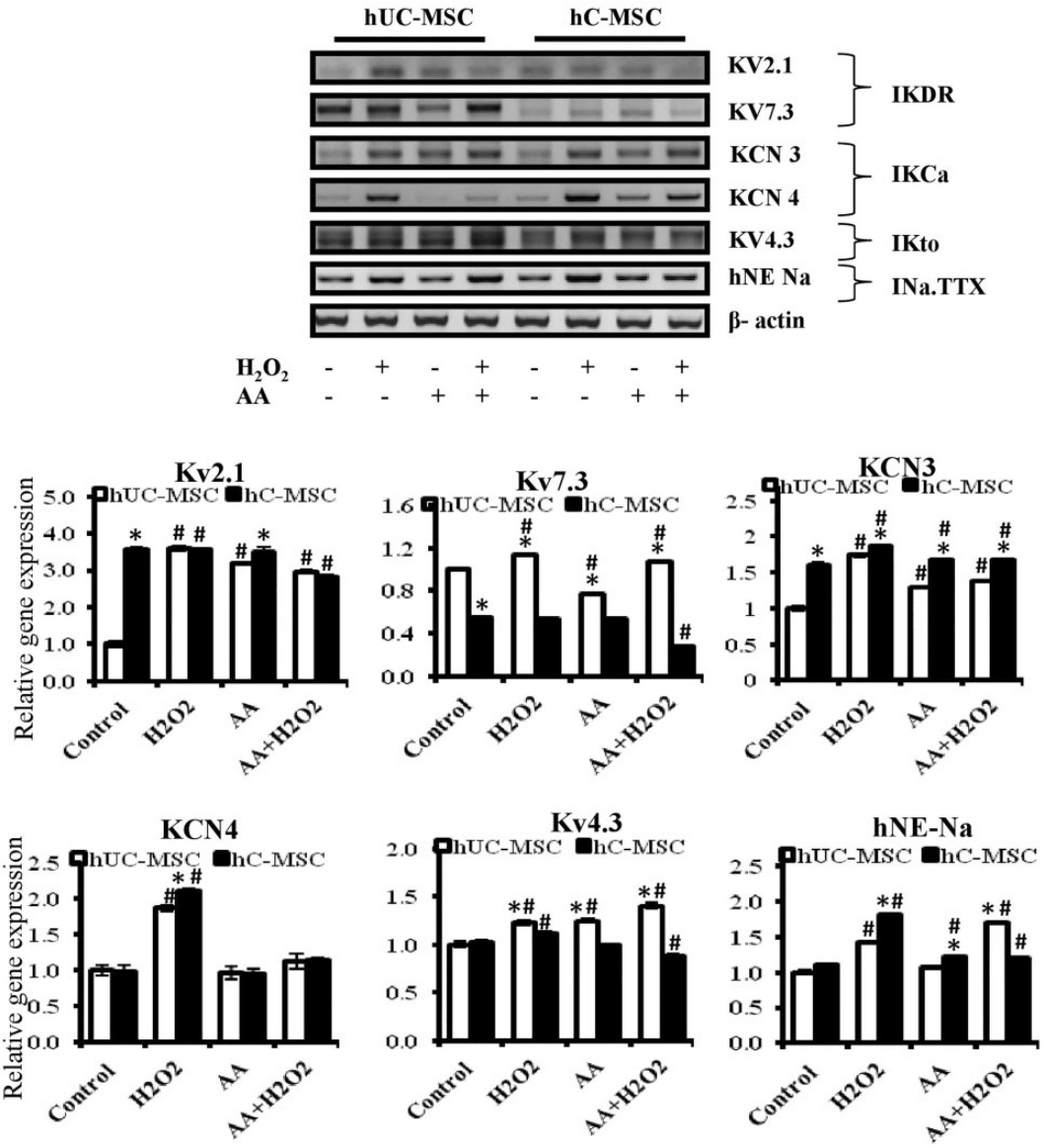

Figure 7 mRNA expression of multiple functional ion channel currents subtypes in different sources of MSCs. mRNA and cDNA were prepared from control and treatment groups and were subjected to PCR for quantifying the relative mRNA expression. GAPDH/β-actin was used as an internal control gene. The experiment was conducted in triplicates using three different samples. The variation within each set is shown with mean of SD ±: *(between hUC-MSCs and hC-MSC); #(between control and experiment) where $P < 0.05$ ($n = 3$)

sensitive to H_2O_2 in correlation with low levels of antioxidant enzyme activity.[28] However, MSCs from different sources might possess a variable capacity of endogenous antioxidant system as bone marrow-derived MSCs shown to have a higher resistance towards oxidative stress.[29,30] Although redox homeostasis is an important modulator of stem cell self-renewal and differentiation, the increase in oxidative stress did not influence the MSCs self-renewal and differentiation as measured by Rex1 and Notch1. Guo et al.[22] demonstrated a similar observation where the self-renewal and differentiation potency of ESC were not affected by the short-term exposure of H_2O_2.

Next, we investigated whether the redox imbalance has any influence on cardiac specific gene expression in both cardiac-resident and non-resident MSCs. To test this hypothesis, both MSCs were subjected to the pathological levels of H_2O_2/AA for 4 h and 24 h, respectively. The result shows that the short-term and sub-lethal concentration of H_2O_2 significantly induced the early cardiogenic genes expressions in both MSCs. However, in comparison with hC-MSCs, the cardiac gene expressions (Nkx2.5, Gata4, Mlc2a and β-MHC) were markedly increased in hUC-MSCs. On other hand, AA considerably induced the cardiac gene expression in hUC-MSCs than that of hC-MSCs. This result support that the redox imbalance crucially influences the cardiac gene expression in MSCs. This finding confirms that the oxidative stress promotes cardiac genes expressions in MSCs.[4]

We also investigated whether the oxidative stress induced by H_2O_2 upregulates the electrophysiology gene expression in MSCs. It was found that the gene expression level of delayed rectifier-like K^+ current (Kv2.1, Kv7.3),

Ca^{2+}-activated K^+ current channel subunits (KCNN3 and KCNN4), transient outward K^+ current (Ito) encoding gene KV4.3, and TTX-sensitive transient inward sodium current (INa.TTX) encoding genes hNE-Na were significantly high in both MSCs when exposed to the oxidative stress. To the best of our knowledge, this is the first evidence that reports H_2O_2-mediated oxidative stress in regulating the level of channels at the cell surface. Moreover, it has been reported that several Kv channels could be regulated by oxidative stress.[31]

In summary, this study has demonstrated that the cardiac-resident and non-resident MSCs are highly sensitive and respond to the oxidative stress due to poor antioxidant enzyme activity. However, redox imbalance plays a major role to promote cardiac-specific gene expressions and ion-channel specific gene expressions in both sources of MSCs. Although the redox imbalance induces the cardiac and ion-channel specific gene expression in MSCs, it also potentially affects the cell proliferation and viability. Hence, an appropriate cyto-protective pre-treatment with right dosage and period is vitally important to warrant a successful stem cell transplantation and stable engraftment.

Author contributions: In this study SB acted as the project worker; SS as co worker; CR as co worker; SP as supervisor; RR as co-supervisor and lab in-charge.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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