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Brief communication

Differential reduction of reactive oxygen species by human tissue-specific mesenchymal stem cells from different donors under oxidative stress

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Clinical trials using human Mesenchymal Stem Cells (MSCs) have shown promising results in the treatment of various diseases. Different tissue sources, such as bone marrow, adipose tissue, dental pulp and umbilical cord, are being routinely used in regenerative medicine. MSCs are known to reduce increased oxidative stress levels in pathophysiological conditions. Differences in the ability of MSCs from different donors and tissues to ameliorate oxidative damage have not been reported yet. In this study, for the first time, we investigated the differences in the reactive oxygen species (ROS) reduction abilities of tissue-specific MSCs to mitigate cellular damage in oxidative stress. Hepatic Stellate cells (LX-2) and cardiomyocytes were treated with Antimycin A (AMA) to induce oxidative stress and tissue specific MSCs were co-cultured to study the reduction in ROS levels. We found that both donor's age and source of tissue affected the ability of MSCs to reduce increased ROS levels in damaged cells. In addition, the abilities of same MSCs differed in LX-2 and cardiomyocytes in terms of magnitude of reduction of ROS, suggesting that the type of recipient cells should be kept in consideration when using MSCs in regenerative medicine for treatment purposes.

Keywords. Oxidative stress; reactive oxygen species; tissue-specific mesenchymal stem cells

Abbreviations: AD, adipose; AMA, Antimycin A; AU, arbitrary units; BM, bone marrow; DP, dental pulp; MFI, mean fluorescence intensity; MSC, mesenchymal stem cell; ROS, reactive oxygen species

1. Introduction

Mesenchymal stem cells have become a popular choice for clinical trials owing to their immuno-modulatory activities, low or no risk of rejection, and their easy availability. They possess the self-renewable capacity and ability to differentiate into multiple lineages. MSCs can be derived from various tissue sources such as bone marrow (BM), adipose (AD), dental pulp (DP) and umbilical cord (UC). Tissue-specific MSCs vary in their proliferative capacity, population doubling time (PDT) and differentiation potential (Kern *et al.* 2006; Chen *et al.* 2015; Li *et al.* 2015). Several mechanisms such as paracrine secretion, mitochondrial transfer, exosome secretion, cell fusion and trans-differentiation contribute towards regeneration of damaged cells (Koyanagi *et al.* 2005; Caplan and Dennis 2006; Bruno *et al.* 2009; Islam *et al.* 2012; Ahmad *et al.* 2014). Recent studies have contributed to the dramatic shift in paradigm suggesting that MSCs regulate repair mechanism primarily by modulating inflammation and immune response by paracrine secretion and via neutralization of increased ROS levels in cells under oxidative stress (Park *et al.* 2009; Ranganath *et al.* 2012; Maumus *et al.* 2013; Raposo and Stoorvogel 2013; Liang *et al.* 2014). Studies have shown that tissue-specific MSCs differ in their immuno-modulatory activities, variation in secretion of cytokines, growth factors and other components of their secretome (Jin *et al.* 2013; Collins *et al.* 2014; Pires *et al.* 2016). Differences in these properties impact their regenerative potential in clinical applications has been observed (Jin *et al.* 2013; Collins *et al.* 2014).

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However, the differences in differential regulation of oxidative stress by tissue-specific MSCs have not been studied yet.

Few reports have suggested that various factors such as tissue-source, donor's age, passage number, conditioned media and health of donor affect the properties of tissue-specific MSCs (Collins *et al.* 2014; Nakamura *et al.* 2015; Khatri *et al.* 2016). However, the effect of these factors on MSCs in a co-culture system with different recipient cells and in variety of pathological conditions has not been explored well. Generation of free radicals causes oxidative stress that leads to diverse pathophysiological conditions (Griendling and FitzGerald 2003). Many diseases such as cardiovascular diseases, neurodegeneration, renal disorder and liver fibrosis are mediated through the excess accumulation of ROS (Poli 2000; Griendling and FitzGerald 2003). Treatment of cells with AMA is known to induce oxidative stress by the generation of superoxide anion, decreased respiration and cellular damage (King and Radicchi-Mastroianni 2002; Dutta *et al.* 2013). Thus, AMA-induced oxidative stress in cells such as cardiomyocytes, hepatocytes and epithelial cells are considered as a model to understand diseases prognosis and treatment modalities (Ahmad *et al.* 2014; Dutta *et al.* 2013; Kawano *et al.* 2014). The antioxidant effect of MSCs to mitigate oxidative damage has been reported in many studies (Ahmad *et al.* 2014; Caicedo *et al.* 2015; Phinney *et al.* 2015). MSCs reduce increased ROS levels by regulating several factors participating in stress pathways, thereby, promoting healing and rejuvenation of injured cells (Cho *et al.* 2012; Liu *et al.* 2012; Ohkouchi *et al.* 2012).

In this study, we have investigated the differences in tissue-specific MSCs to alleviate oxidative stress damage in terms of reduction ROS levels. Considering that oxidative stress is a mediator of liver fibrosis and cardiovascular diseases, hepatic stellate cells (LX-2) and cardiomyocytes were selected as representatives and were treated with AMA to induce oxidative stress. Post AMA treatment, these cells were co-cultured with tissue specific MSCs (BM-MSCs, AD-MSCs and DP-MSCs) separately to study the attenuation of elevated ROS levels in cells under oxidative stress.

2. Methods

2.1 *Revival and characterization of cryopreserved human MSCs*

This study was ethically approved by the Institutional Committee for Stem Cell Research (IC-SCR), All India Institute of Medical Sciences (AIIMS), New Delhi. MSCs were isolated from respective tissue samples and cryopreserved after taking prior informed consent from the the MSCs donors. The details of cryopreserved (5 years) human BM-MSCs, AD-MSCs and DP-MSCs are provided in supplementary table 1. All the MSCs used in the experiments were revived in LG-DMEM (Gibco, USA) media containing 10% Fetal Bovine Serum (FBS; Hyclone) supplemented with 1% Glutamax & Penstrep (Invitrogen, USA), incubated at 37°C with 5% CO_2. *In vitro* culture expansion and characterization of MSCs and viability test were done as per previously described lab protocol (Mohanty *et al.* 2013; Nandy *et al.* 2014). Surface marker profiling of CD73 PE, CD90, PECy5, HLA Class I APC, HLA Class II FITC, CD 34 PE (Becton Dickinson, USA) and CD105 APC (eBioscience, USA) and tri-lineage differentiation of MSCs to osteocyte, chondrocyte and adipocytes was performed (Mohanty *et al.* 2013). Controls were made by corresponding isotypes: IgG1 coupled with PE, PECy5, APC and FITC. The cells were acquired on BD LSR II flow cytometer (Becton Dickinson, USA) with at least 10,000 events for each sample and analyzed with Becton Dickinson FACS Diva (ver 6.1.2) for surface marker characterization (Nandy *et al.* 2014; Kakkar *et al.* 2015). Cells at passage 3 were used for all further experiments.

2.2 *Cell cultures*

Human hepatic stellate cells LX-2 cell line were maintained in DMEM media with 2% FBS and supplemented with penicillin (200 µg/mL), streptomycin (200 U/mL) and 1X antimycotic solution, Amphotericin B (Gibco, USA). Human cardiac biopsy obtained from the Department of Cardio Thoracic Vascular Surgery (AIIMS, New Delhi) were cultured for cardiomyocytes and cryopreserved. The cryopreserved cultures were revived as per the standard lab protocol and maintained in DMEM/Ham F12 in ratio 3:1 with 10% FBS. Characterization of cardiomyocytes was performed using flow cytometry and Immunofluorescence for Myosin light chain-2v (Mlc-2v) and Cardiac troponin I (cTnI) as per our established protocol (Kakkar *et al.* 2015).

2.3 *Oxidative stress induction*

AMA (Sigma, USA) at 100 nM was added to culture media of LX-2 and cardiomyocytes (50,000 cells/well in a 12-well culture plate) and incubated at 37°C and 5% CO_2 for 18 h to induce oxidative stress. After 18 h, cells were washed with 1xPBS (pH 7.4) before adding media and MSCs for co-culture experiments.

2.4 *Co-culture*

Three different tissue-specific MSCs (BM-MSCs, AD-MSCs and DP-MSCs) were trypsinized and co-cultured with AMA treated LX-2 or cardiomyocytes, at 1:1 ratio and added equal amounts of respective media and incubated at 37°C with 5% CO_2 for 24 hours.

2.5 *Quantification of ROS*

ROS was measured using MitoSOX red (Life technologies, USA) at a concentration of 4 μM for 20 minutes in respective media and incubated at 37°C with 5% CO_2. The quantification of ROS was done using flow cytometer BD LSR II flow cytometer (Becton Dickinson, USA) with at least 20,000 events for each sample and analyzed with Becton Dickinson FACS Diva (ver 6.1.2). Readings (in duplicates) for mean fluorescence intensity (MFI) in PE region was recorded in arbitrary units (AU). Control comprised of MSCs + LX-2 (or cardiomyocytes) unstained and the MFI which was subtracted from co-culture with MSC + LX-2 (or cardiomyocytes) treated with AMA.

2.6 *ATP detection*

ATP detection was performed using ATP Assay Kit as per manufacturer's instructions (Abcam, USA). Cells were grown in duplicates in 12-well plate for co-culture. Each well contained total 5×10^5cells that were used for ATP assay. After 24 h of co-cultures, cells were lysed in the ATP assay buffer and centrifuged at 15,000*g* for 2 min. The supernatant was added to a 96-well plate and followed by the addition of 50 μL of the reaction mix to each well and detected using colorimetric detection by spectrophotometer (BioTek, USA) at 570 nm. Standard curve was prepared using ATP standard provided in the kit. Relative amount of ATP in samples was calculated using standard curve.

2.7 *Fluorescence imaging*

Tissue-specific MSCs were stained by live imaging by MitoTracker Green FM (Excitation/Emission: 419/560 nm) from Thermo Fisher, Scientific and LX-2 cells were stained with Tetramethylrhodamine ethyl ester (TMRE) from Thermo Fisher, Scientific) using EVOS® FLoid® Cell Imaging Station (Life Technologies, Carlsbad, CA, USA).

2.8 *Statistical analysis*

Statistical analyses were performed using R 2.13.0 and Minitab software (R Development Core Team 2010). One-way and two-way ANOVA analysis was done to determine the effect of tissue- specific MSCs and donors MSCs (significant values, P-value <0.05).

3. Results

3.1 *Differential reduction of ROS by tissue-specific MSCs in LX-2 cells under oxidative stress*

Cryopreserved MSCs were successfully revived and characterized based on the presence of all surface markers including CD105, CD73 PE, CD90 PECy5, HLA Class I APC and absence of HLA Class II FITC, CD 34/45 PE/FITC (Becton Dickinson, USA) (figure 1A). Viability and trilineage differentiation potential of MSCs were also confirmed (figure 1B). Florescence images of tissue-specific MSCs labeled with Mitotracker Green FM and LX-2 cells with TMRE demonstrated healthy cells (figure 2A). The shift in ROS levels of LX-2 before and after treatment of AMA and post co-culture with tissue-specific MSCs in PE region by flow cytometry data depicted lowering of ROS levels by MSCs (figure 2B). The base level ROS of LX-2 increased significantly from average MFI 2592 AU to 4092 AU after oxidative stress induced by treatment of AMA (P<0.05) (figure 2C). Overall, average ROS reduction by all tissue-specific MSCs was 72.88% in LX-2 cells. Differential ROS reduction abilities of ten donor tissue-specific MSCs, numbered from 1 through 10, was observed in their co-culture with LX-2 (AMA) (figure 2C). Average percentage reduction by BM-MSCs (n=3), was found to be 64.09%, AD-MSCs (n=4) was 68.61% and by DP-MSCs was 87.4% (figure 2C). Lower values of ROS in the bar graphs demonstrate higher reduction ability in co-cultures with LX-2 (AMA). The reduction in ROS by all three tissue-specific MSCs was found to be significantly different and the order of reduction abilities were noted as DP-MSCs > AD-MSCs > BM-MSCs.

3.2 *Rescue abilities of MSCs are affected both by donor's age and tissue source*

Donor MSCs were divided into two groups, younger age group (10–30 years, n=6) and older age groups (31–60 years, n=4), details are given in supplementary table 1. It was also found that ROS values of young MSCs donors were lower than the old donors indicating their better ability to reduce ROS (figure 3A). The average amount of ROS reduction in young donor group was found to be higher in DP-MSCs and in the following order: DP-MSCs > AD-MSCSs > BM-MSCs. Older age BM-MSCs showed the lowest reduction capacity. Also, older AD-MSCs were found to reduce more ROS than older BM-MSCs (figure 3A). One-way ANOVA clearly demonstrated that donor source significantly impacts ROS reduction capacity (P<0.01). Interestingly, Two-way

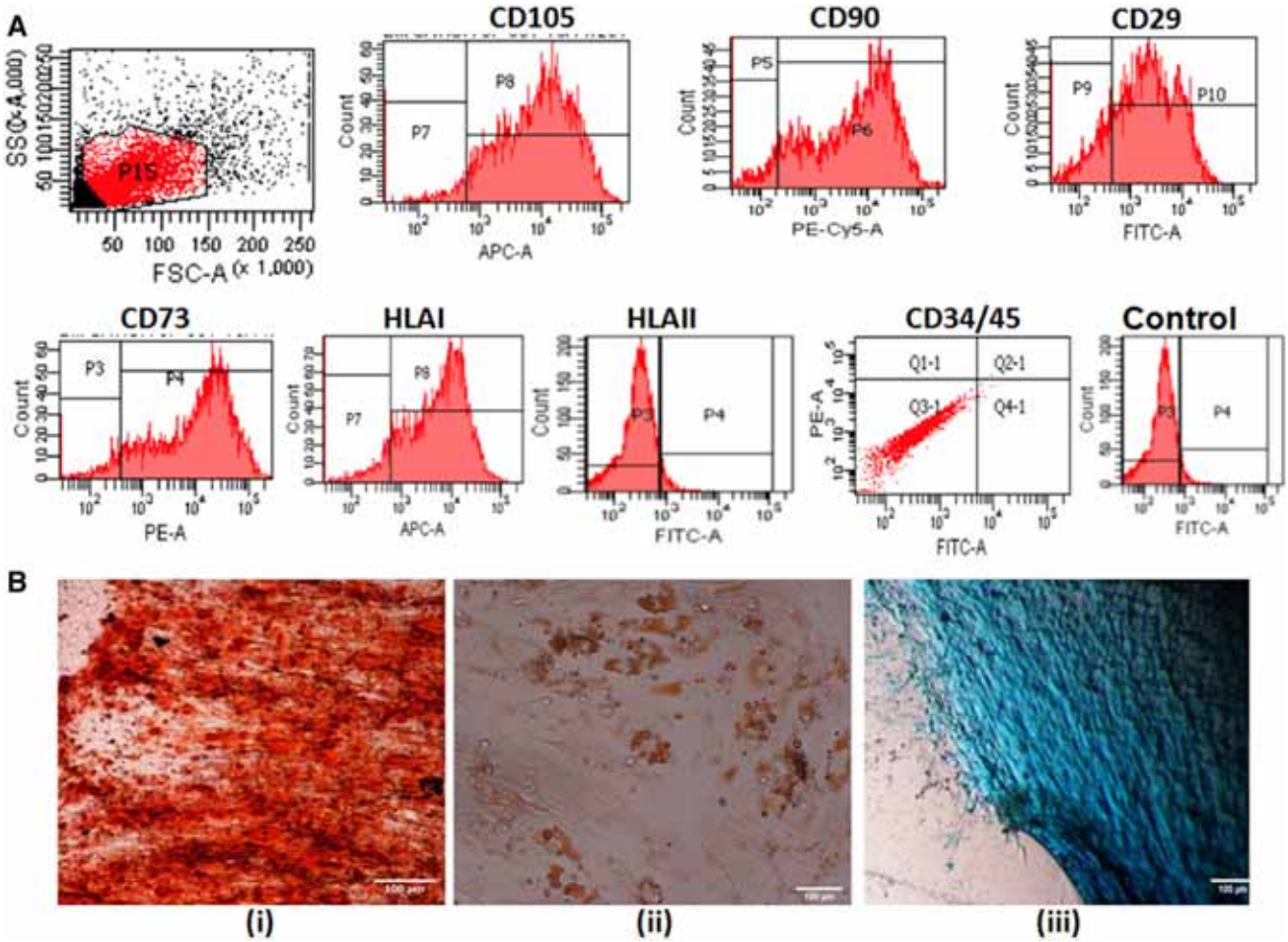

Figure 1. Characterization of MSCs by (**A**) flow cytometry: representative surface marker Profiling of BM-MSCs by flow cytometry, CD 105 (90%), CD 90 (96.9%), CD 29 (80.8%), CD 73 (84.5%), HLA I (86.5%), HLAII (0%), CD 34/45 (0%), control (autoflurescence). (**B**) Tri-lineage differentiation: (i) Alizarin Red S staining of the extracellular mineralized matrix of the osteogenic differentiation. (ii) Oil red O stained fat globules post Adipogenic differentiation. (iii) Alcian Blue staining post Chondrogenic differentiation (20× magnification).

ANOVA analysis demonstrated the equal contribution of both donor and tissue source in reducing ROS levels ($P<0.05$) (figure 3B). Interaction plots showed the effect of donor type and each tissue-specific MSC and their combined contributions in affecting ROS levels (figure 3B).

3.3 *Rescue effect of MSCs on cardiomyocytes*

Cardiomyocytes were characterized for Mlc-2v (78.5%) and cTnI (77.5%) using flow cytometry and fluorescent microscope imaging with cardiomyocytes specific-markers (figure 4). We observed that all three MSCs type were able to differentially reduce ROS levels, also in cardiomyocytes under oxidative stress (figure 5A). The shift in the mean fluorescence intensity under oxidative stress and post-culture with tissue-specific MSCs is shown by flow cytometry data (figure 5A). All the tissue-specific MSCs were able to reduce elevated levels of ROS, and total average reduction of ROS in cardiomyocytes was noted as 61.29%. It was observed that overall co-cultures of stressed cardiomyocytes with DPMSCs (71.27%), demonstrated lowest absolute ROS levels followed by AD-MSCs (67.3%) and BM-MSCs (37.29%) (figure 5B). ANOVA analysis revealed significant differences in the reduction capacities of tissue-specific MSCs ($P<0.05$). Thus, although average ROS reduction abilities of DP-MSCs > AD-MSCs > BM-MSCs remained similar but, if considered donor-wise comparisons of MSC donors (8 of the 10 donors depicted by numbers) in both LX-2 and cardiomyocytes a different pattern in their reduction abilities was observed along with noted changes in magnitude difference in ROS reduction abilities (figure 6).

3.4 *No correlation between reductions in ROS and ATP production*

We expected that lowering in ROS would be accompanied by a subsequent increase in ATP levels and tested it in two

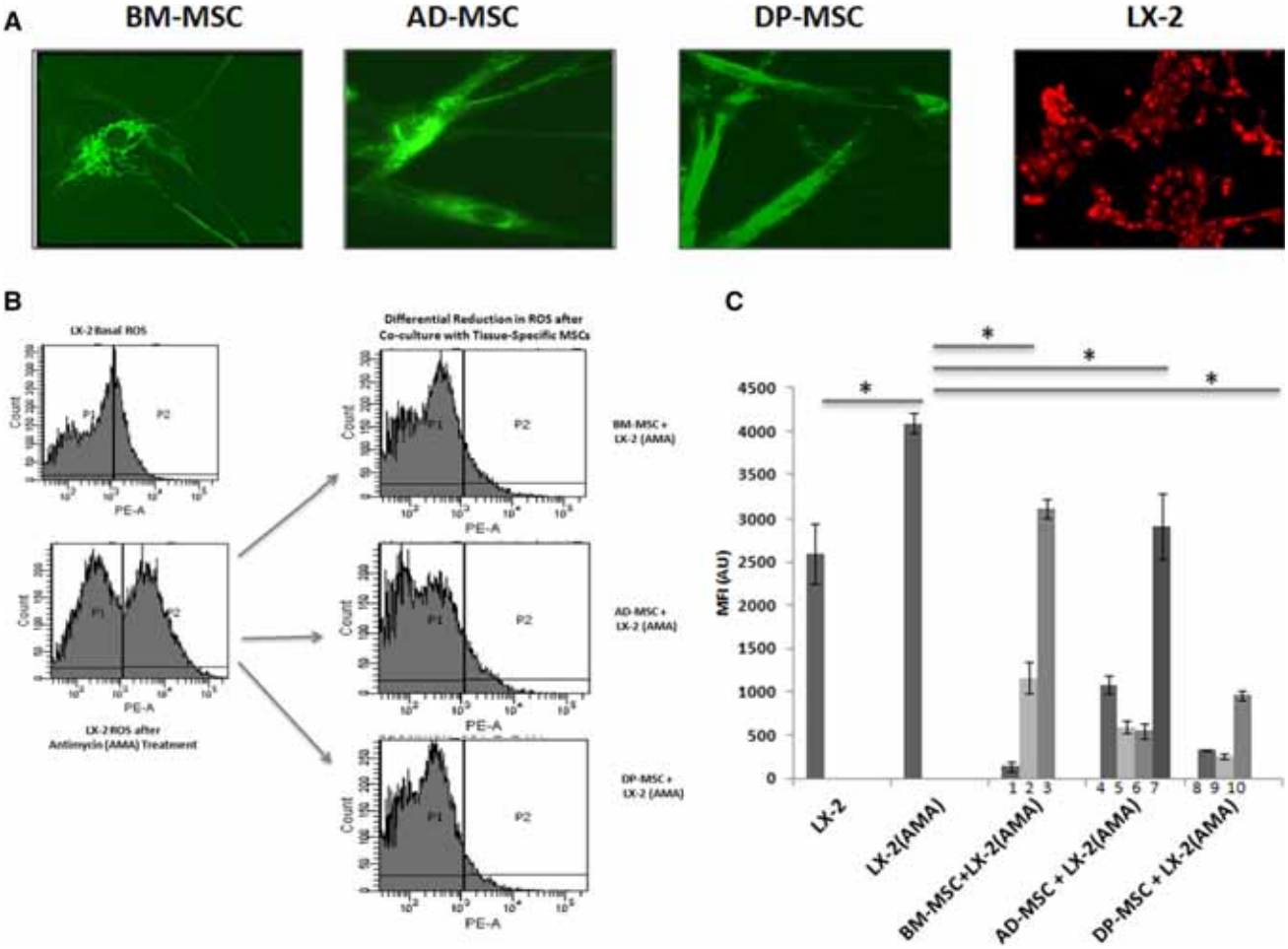

Figure 2. Differential Reduction of ROS by Tissue Specific MSCs co-cultured with LX-2 cells under oxidative stress. (**A**) Fluorescent Images to show healthy MSCs, labelled with MitoTracker Green FM and LX-2 stained with tetramethylrhodamine ethyl ester (TMRE) (20× magnification). (**B**) Flow cytometry data depicting Mean Florescence intensity of ROS (MFI) values by Arbitrary Units (AU) in LX-2 alone, treated with AMA and co-cultured with tissue-specific MSCs. Assessment of ROS fluorescence in PE region for cells stained with MitoSOX deep red was performed. (b) Bar graphs depicting reduction in ROS for donor MSCs in BM-MSCs, AD-MSCs and DP-MSCs categories (*P<0.001).

young donors chosen from each tissue-specific MSCs (total n=6). Although we found that when MSCs were co-cultured with cardiomyocytes (AMA treated), there was subsequent increase in ATP along with the decrease in ROS (figure 7), but did not find any significant correlation between reduction in ROS and increase in ATP values.

4. Discussion

Effective clinical outcomes demand the use of optimal source of MSCs that can lead to consistent and better clinical outcomes. Although MSCs are popularly being used for clinical trials, the availability of many options of tissue source makes it difficult to determine the optimal source that can be used in regenerative medicine. It has been challenging to identify a suitable source of stem cells due to several confounding factors including the heterogeneous population of stem cells, different isolation procedures, variation in expansion media along with other confounding factors including donor's age and tissue source of MSCs. Although all the tissue-specific MSCs share a few similar characteristic properties such as surface marker profiling, they vary in many other characteristics such as cellular repair capacity and secretion of immunomodulatory factors (Jin *et al.* 2013). This further suggests that these MSCs also differ in their treatment efficacy on the basis of their origin and mechanism involved in rejuvenation of damaged cells (Collins *et al.* 2014). One of the mechanisms through which MSCs alleviate cell damage under oxidative stress is by neutralization of enhanced ROS. Liver fibrosis and cardiovascular diseases are caused by oxidative damage and thus, reduction in ROS levels in stressed cells by tissue-

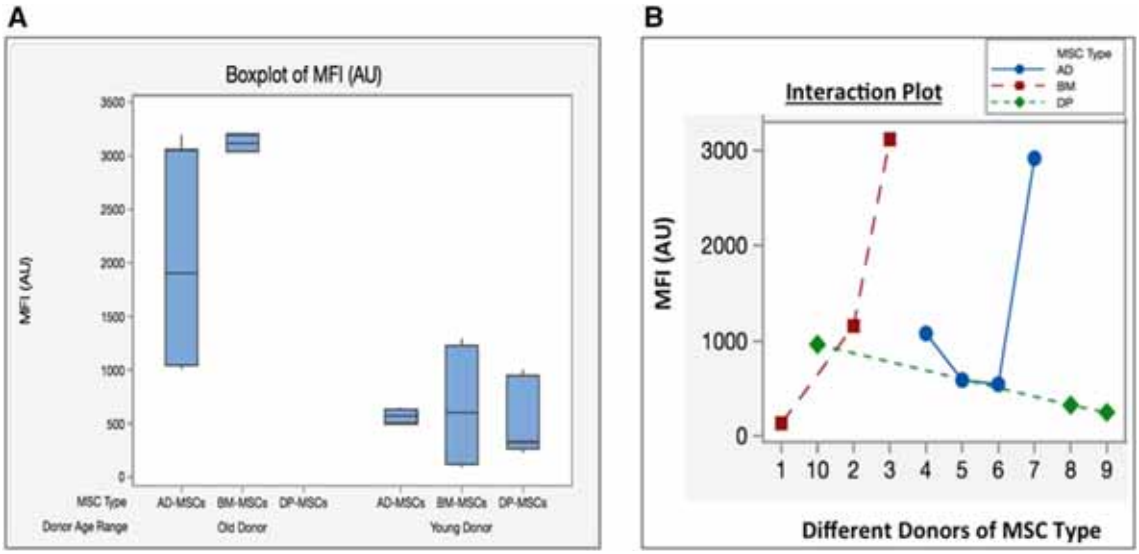

Figure 3. Tissue-specific MSCs along with donor source together affect ros reduction under oxidative stress. (**A**) Box-plots for Tissue-specific MSCs show variation in young and old donor MSCs, younger donor (10–30 years) and older donor (31–60 years). As dental samples were obtained from deciduous shed teeth so only pediatric group was available and the data for older group is missing. (**B**) Interaction plot depicting two-way interaction between donor and tissue-specific MSCs. Two-way ANOVA revealed significant contributions of both donor age and origin matters ($P<0.05$), Confidence Interval 95%. Young donors (10–30 years): n=6; old donors (31–60 years): n=4.

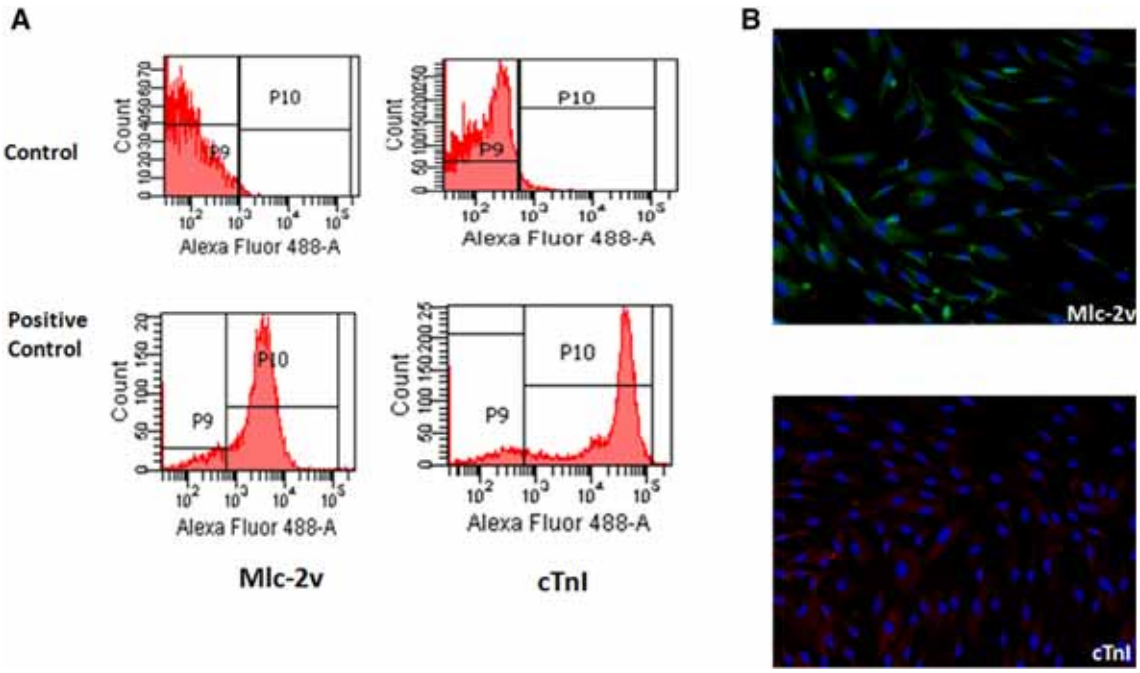

Figure 4. Characterization of cardiomyocytes using cardiac-specific markers Mlc-2v and cTnI by (**A**) flow cytometry analysis and (**B**) immunofluorescence images (20× magnification).

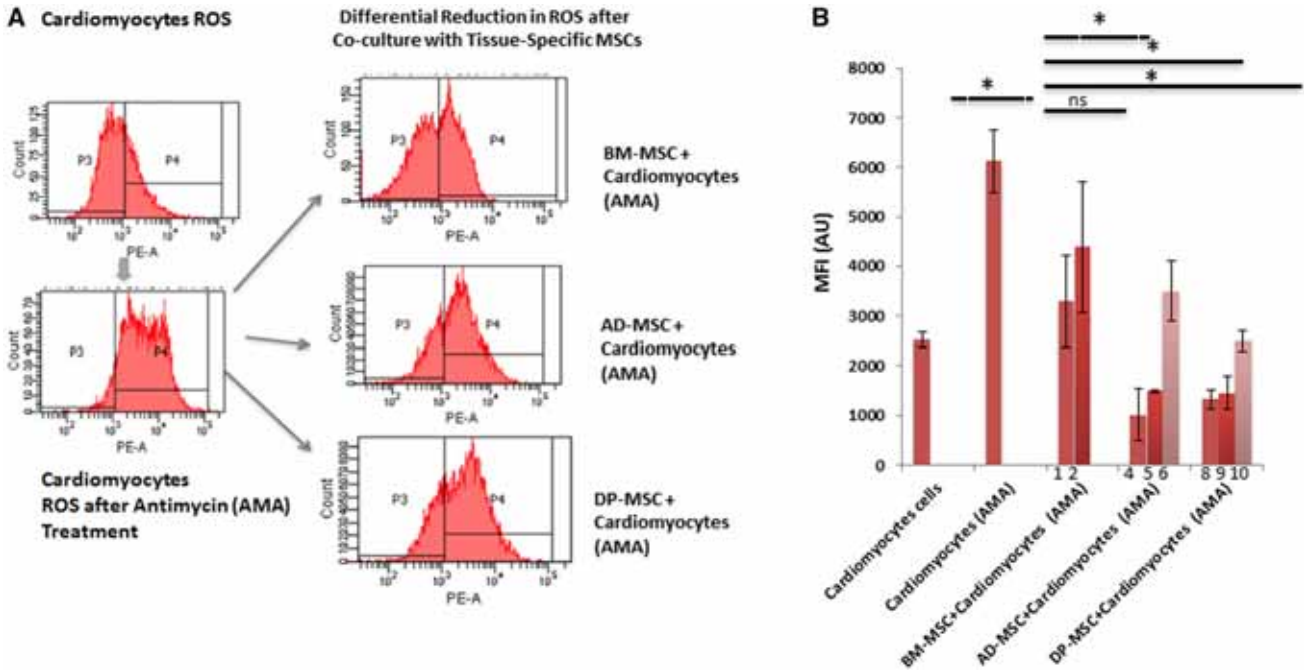

Figure 5. Differential reduction in ROS levels by tissue specific mscs, co-cultured with cardiomyocytes under oxidative stress. (**A**) Flow cytometry data depicting mean florescence intensity of ROS (MFI) values by arbitrary units (AU) in cardiomyocytes alone, treated with AMA and co-cultured with tissue-specific MSCs. Assessment of ROS fluorescence in PE region for cells stained with MitoSOX deep red was performed. (**B**) Bar graphs depicting reduction in ROS for eight patient samples of BM-MSCs, AD-MSCs and DP-MSCs (*P<0.001).

specific MSCs may indicate their ability to ameliorate cellular damage. Several mechanisms of MSC action are known including anti-inflammatory activity, secretion of paracrine factors and reduction in enhanced ROS levels of damaged cells that contribute in repair and rejuvenation (Liu *et al.* 2012; Ohkouchi *et al.* 2012). In case of myocardial infarction, decrease in ROS levels is found to correlate with reduction in infarct size (McCully *et al.* 2009). Ahmad *et al.* (2014) have demonstrated that MSCs have ability to reduce ROS in case of acute lung injury along with increase in ATP production. Despite these evidences, the differential ability of tissue-specific MSCs to reduce excess ROS, in case of oxidative damage still remains unexplored. Till date, no study has compared the ROS reduction abilities of tissue-specific MSCs from multiple donors. In this study, we investigated differential ability of tissue-specific MSCs to reduce accumulation of ROS under oxidative stress condition in LX-2 and cardiomyocytes induced by AMA. Hepatic stellate cells are activated under oxidative stress that leads to liver fibrosis, thus AMA that inhibits complex III, leading to accumulation of free radicals was used to generate oxidative stress condition (Poli 2000; Proell *et al.* 2007; Bataller and Lemon 2012). In accordance, with findings of other research group showing reduction in ROS in other cells, we found that similar results when MSCs were co-culture with stressed cells (Ahmad *et al.* 2014). We also found differential lowering of ROS by tissue-specific MSCs obtained from multiple donors in co-cultures of LX-2 and cardiomyocytes under oxidative stress. Interestingly, we found that DP-MSCs were able to demonstrate more reduction in ROS as compared to BM-MSC and AD-MSCs (figure 2). It should be noted that we did not include any older age group for DP-MSCs due to lack of donor availability and this adds to limitation of our study along with non-inclusion of other favored tissue-specific MSCs such as UC-MSCs (Li *et al.* 2012; Jin *et al.* 2013). Our data demonstrates that younger donor were more efficient in reducing ROS than older MSCs donors (figure 2A). This finding is in line with other studies that have shown that better proliferative and immunomodulatory activities of younger MSCs as compared to MSCs from older age donors (Beane *et al.* 2014; Bruna *et al.* 2016; Khatri *et al.* 2016).

In addition, two-way ANOVA reveals that both donors and tissue source significantly impacts ROS reduction capabilities of MSCs under oxidative stress (figure 2B). This suggests that both donor source and tissue are critical factors that affect use of MSCs for therapeutic application. Differential reduction in ROS was also observed in cardiomyocytes treated with AMA (figure 5). Interestingly the pattern of reduction of ROS was not same in co-cultures of stressed LX-2 and cardiomyocytes (figure 6). This suggests that the interaction between tissue-specific MSCs and the injured cell also matters and the origin or type of recipient should also be

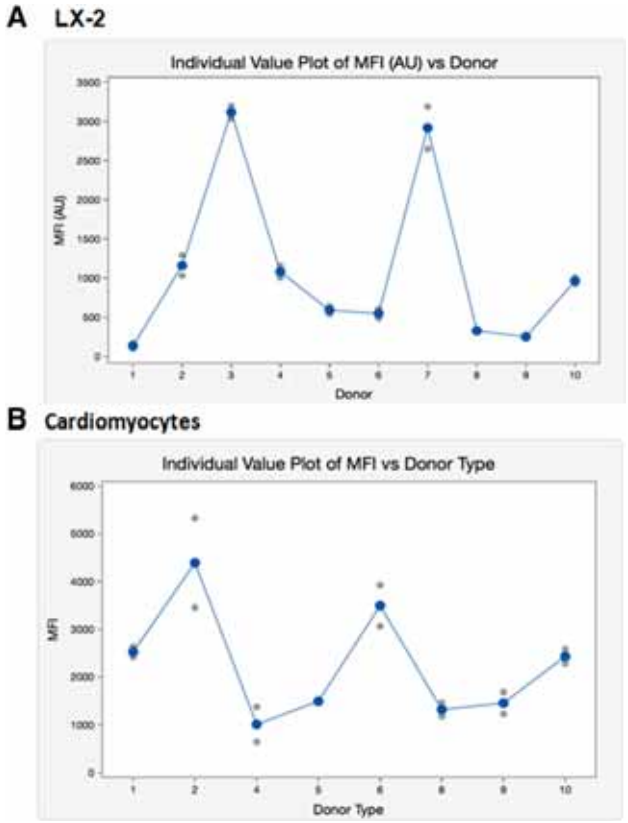

Figure 6. The effect of donor MSCs differs in LX-2 and cardiomyocytes. (**A**) MFI plot for different donors when co-cultured with LX-2. (**B**) MFI plot for different donors when co-cultured with cardiac cells. The donor number is kept same in both the plot for same donor source.

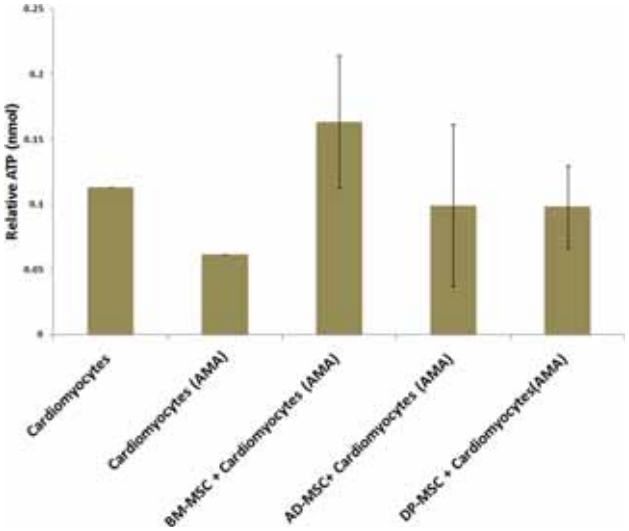

Figure 7. Relative ATP Levels: Assessment of ATP generation in tissue-specific MSCs were co-cultured with cardiomyocytes using ATP Colorimetric Assay Kit (Abcam) and readings were taken at 570 nm.

considered when using MSCs in regenerative medicine. Also, it indicates that a particular tissue specific MSCs may be able to ameliorate a disease condition better than other MSCs in specific injured tissues. It was found that all MSC have the capacity to reduce ROS under stress levels, but each has different capacity depending upon a particular given condition. It is important to note that, small sample size of another limitation of this study and a larger data set would have provided robust statistical analysis. Nonetheless, these are first observation of this kind and can serve as proof of concept for future research along this direction.

During oxidative stress induced by AMA, there is blockage of complex III of electron transport chain in mitochondria affecting the mitochondrial bioenergetics and ATP production (King and Radicchi-Mastroianni 2002). We expected that reduction would be accompanied by an increase in ATP indicating restoration of cellular health and mitochondrial bioenergetics. Although, we did find increase in ATP, after stressed cardiomyocytes were co-cultured with tissue-specific MSCs but the differences were not significant (figure 7). Also, no significant correlation was observed between reduction in ROS and increase in ATP. As DP-MSCs showed maximum reduction in ROS, we expected that they would lead to maximum ATP production. However, BM-MSCs were found to show most elevated levels of ATP as compared to AD-MSCs and DP-MSCs, which demonstrated comparable levels of ATP in co-culture (figure 7). It is plausible that other factors such as genetic components, mitochondrial health, basal ROS and ATP levels of MSCs, mitochondrial biogenesis, anti-oxidant mechanisms and paracrine factors might also be responsible for contributing ability to reduction in ROS levels.

In conclusion, we found that tissue-specific MSCs differ in their capacity to reduce ROS in injured cell under oxidative stress. It was observed that younger donors were more efficient in reducing ROS as compared to MSCs obtained from older age donors. Thus, the source of donor MSCs and their age greatly impacts the ability to alleviate ROS in stress conditions. The rescue abilities of same MSCs also differed in magnitude and patterns of reduction for donor-specific MSCs in LX-2 and cardiomyocytes. This demonstrates that the interaction between donor MSCs and recipient cells should also be considered when using MSCs for clinical and therapeutic applications. This study suggests that these different parameters cannot be ignored when selecting the best-suited MSC candidate prior to using MSCs as off-the-shelf therapy in regenerative medicine. Further investigations in this direction are warranted to increase our insight of these mechanisms and to identify an optimum source of MSCs in treatment of diseases manifested by oxidative stress.

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