Differentiation of Mesenchymal Stem Cells Towards Nephrogenic Lineage and Their Enhanced Resistance to Oxygen Peroxide-induced Oxidative Stress

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Introduction. Mesenchymal stem cells (MSCs) have been publicized to ameliorate kidney injury both in vitro and in vivo. However, very less is known if MSCs can be differentiated towards renal lineages and their further application potential in kidney injuries.
Materials and Methods. The present study developed a conditioning system of growth factors fibroblast growth factor 2, transforming growth factor-β2, and leukemia inhibitory factor for in vitro differentiation of MSCs isolated from different sources towards nephrogenic lineage. Less invasively isolated adipose-derived MSCs were also compared to bone marrow-derived MSCs for their differentiation potential to induce renal cell. Differentiated MSCs were further evaluated for their resistance to oxidative stress induced by oxygen peroxide.
Results. A combination of growth factors successfully induced differentiation of MSCs. Both types of differentiated cells showed significant expression of pronephrogenic markers (Wnt4, Wt1, and Pax2) and renal epithelial markers (Ecad and ZO1). In contrast, expression of mesenchymal stem cells marker Oct4 and Vim were downregulated. Furthermore, differentiated adipose-derived MSCs and bone marrow-derived MSCs showed enhanced and comparable resistance to oxygen peroxide-induced oxidative stress.
Conclusions. Adipose-derived MSC provides a promising alternative to bone marrow-derived MSC as a source of autologous stem cells in human kidney injuries. In addition, differentiated MSCs with further in vivo investigations may serve as a cell source for tissue engineering or cell therapy in different renal ailments.

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INTRODUCTION

Kidney injuries, either acute or chronic, are posing a serious global health problem with elevated rates of morbidity and mortality.[1-3] Because of the complicated anatomical structure of the kidney, restoration of injury is much more difficult than the regeneration of any other organs.[4] Therefore, effective strategies to restore kidney function, as well as to prevent the damage progression are urgently needed. Unfortunately, no operational therapies are available to date to prevent or treat kidney injuries except organ transplantation, which is a very invasive method.

In the past decade, stem cells-based regenerative therapy has raised the hope for a new and potent therapeutic approach for the treatment of kidney injuries and many other diseases.[5-12] Mesenchymal stem cells (MSCs) provide an ideal population of stem cells and endow a promising potential in clinical therapy against kidney injuries due to their

low toxicity, as well as autologous transplantation eliminating the chances of immune rejection and avoid ethical ambiguities of using embryonic stem cells.[13,14] Primarily, most of the studies have been carried out using bone marrow-derived MSCs (BMDMSCs).[15,16] However, derivation of BMDMSCs in human not only is painful but also gives less yield as they constitute only approximately 0.01% of the total nucleated bone marrow cells. Recently, MSCs population derived from adipose tissue has provided an attractive alternative cell therapy due to their less invasive means of derivation and high yield. It has been reported that adipose-derived MSCs (ADMSCs) are 500 times more prevalent than BMDMSCs within an equivalent volume of adipose tissue.[17]

Work done on different organs also revealed that differentiated MSCs towards a specific lineage showed better transplantation and cytoprotective efficacy compared to MSCs. To improve efficiency of MSCs transplantation, interventions that promote differentiation of MSCs towards a specific lineage can be a rational approach. Previously, a study performed on the developing metanephric kidney showed that fibroblast growth factor 2 (FGF2), transforming growth factor-β2 (TGF-β2), and leukemia inhibitory factor (LIF) were secreted by inductive rat bud cells and cooperated to induce nephrogenesis especially tubule formation.[18] Later on, in vitro treatment of multipotent renal progenitor cells with cocktail of TGF-β2, LIF, and FGF2 showed that multipotent renal progenitor cells were successfully differentiated towards a renal cell lineage.[19]

The present animal study was planned to use conditioning system of growth factors to differentiate MSCs isolated from different sources towards nephrogenic lineage in vitro. Differentiation potential of ADMSCs was compared to BMDMSCs to produce renal cell-like cells. Moreover, differentiated cells were further evaluated for their resistance to ischemic injury compared to control MSCs.

MATERIALS AND METHODS

Animals

Sprague Dawley rats (5 to 6 months old) were used in the study. Animals were kept and maintained in the animal house facility in 12 hours' light-and-dark cycle with free access to water and food. All animals were treated in accordance with the standard procedures approved by the Committee of Animal Care, National Centre of Excellence in Molecular Biology, University of the Punjab Lahore.

Isolation and Culturing of Mesenchymal Stem Cells

Male Sprague Dawley rats of average age of 3 months were used to isolate MSCs from 2 different sources of bone marrow and adipose tissue. Briefly, BMDMSCs were isolated from the tibias and femora of the rats (n = 5) according to their ability to adhere to plastic surface of a culture flask and were cultured as described previously,[20] and ADMSCs were cultured from the Sprague Dawley rats as described previously.[21] Cells were plated in 25-cm^2 vented culture flasks at 37°C with 5% carbohydrate. The ADMSCs were grown up to a 2nd passage in Dullbecco modified Eagle medium-low glucose with 15% fetal bovine serum and the medium was changed every 3rd day.

Differentiation of Stem Cells Towards Nephrogenic Lineage

Both ADMSCs and BMDMSCs were grown up to 70% confluency on fibronectin-coated 24 and 6 well plates and incubated with a cocktail of growth factors that contained FGF2 (50 ng/mL), TGF-β2 (4 ng/mL), and LIF (20 ng/mL).[19] Cultures were maintained for 1 week, and the medium was renewed after every 48 hours. The experiment was performed in triplicate reactions.

Immunocytochemistry

Differentiation of MSCs towards nephrogenic lineage was analyzed by the expression regulation of stem cells and renal markers. Briefly, for immunocytochemistry, cells grown in 24 well plates were fixed in 4% paraformaldehyde in Dulbecco phosphate-buffered saline (Gibco-BRL). Wnt4, Wt1, Pax2, Vim, Oct4, Sox2, E-cad, and ZO1 antibodies (Santa Cruz replacement antibodies, Aviva Systems Biology, USA) were used at 1:50 to 1:200 dilutions. Primary antibodies were detected by using labeled antimouse, antirabbit, and antigoat secondary antibodies (Jackson, USA). Slides were counterstained with 4,6-diamidino-2-phenylindole for nuclear detection. cells were mounted with Vecta-shield mounting medium and examined under

fluorescence microscope Olympus IX-51 equipped with Digital Camera DP-70 (Olympus, Japan). Six images were taken per field of triplicate wells.

Gene Expression Analysis

Total RNA was extracted from differentiated and control ADMSCs and BMDMSCs using TRIzol (Invitrogen Inc, USA). RNA was quantified by Spectrophotometer (NanoDrop, ND-1000). cDNA was synthesized from 1 µg of each RNA sample using M-MLV reverse transcriptase (Invitrogen, USA). For gene expression analysis, real-time polymerase chain reaction was used to analyze the mRNA expression levels of pronephrogenic (Wnt4, Wt1, and Pax2), epithelial (E-cad and ZO1), and mesenchymal (Vim) markers to track differentiation of both ADMSCs and MSCs towards nephrogenic lineage. Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to design gene specific primers. Sequences were taken from National Center for Biotechnology Information and University of California Santa Cruz Genome Browser. The oligonucleotide sequences specific for the selected genes corresponding to pronephrogenic, epithelial, and mesenchymal markers are presented in the Table.

Estimation of Cell Survival of Differentiated Stem Cells Against Ischemic Injury

In vitro ischemic injury. To produce an in vitro ischemic injury model, control and differentiated MSCs were cultured at 10^4 cells per well of a 6-well plate in triplicate. Cells were washed with phosphate-buffered saline and subjected to 100 µg of oxygen peroxide solution for 2 hours to induce ischemic injury.

Senescence assay. Cells exposed to ischemic injury with 100 µg of oxygen peroxide were further subjected to senescence assay to analyze the cell survival. Cells were washed with phosphate-buffered saline, fixed for 3 to 5 minutes in 4% formaldehyde at room temperature. After washing, cells were incubated at 37°C with fresh senescence-associated stain solution (Abcam). Staining was observed after 4 hours. Cells with blue-stained nuclei were considered senescent. Stained and unstained cells were counted and the data were expressed as a percentage of senescent cells.

Trypan blue assay. Cell apoptosis was determined with a trypan blue-exclusion assay and cell senescence assay after treating differentiated cells with oxygen peroxide in 6 well plates for 2 hours. The medium was removed from the culture plates, cells were washed with phosphate-buffered saline and 4% trypan blue solution (Sigma Aldrich, USA) was added. The plates were incubated at 37ºC for 10 minutes. Cells were again washed with phosphate-buffered saline and analyzed under phase contrast microscope. Six high-power fields of each well were selected. Total number of cells was counted along with cells stained positive for trypan blue. Stained cells were considered nonviable and the data were expressed as a percentage of nonviable cells.

Statistics Analysis

The 1-way analysis of variance and the paired *t* test were performed for comparisons. Values of continuous variables were expressed as mean ± standard deviation. A significance level of .05 was adopted.

Primer Sequences

Primers	Sequences	Product Size	Annealing Tm
Pax2 (F)	TGGGCAGGTACTACGAGACTG		
Pax2 (R)	ACGCTGGGAACTGTATCATTG	190	60.0
Wnt4 (F)	CCTTTGCAGTGACAAGAGCAT		
Wnt4 (R)	TGCTTCTCTCTCGGACGTCTA	275	60.0
Wt1 (F)	GATACAGCACGGTCACTTTCG		
Wt1 (R)	CAACTGTCGGTAGGGGTGTG	184	61.0
ECad (F)	AATCAGATCCGATGTCTCTGC		
ECad (R)	CGGGGATTAGGCTCAGATAAG	176	60.0
ZO1 (F)	GGAATTGCAATATCTGGTGGA		
ZO1 (R)	TAGCTGCTGAACAGCAAAAGC	183	61.0
GAPDH (F)	CCAAGGTCATCCATGACAACT		
GAPDH (R)	GGATACATTGGGGGTAGGAAC	242	60.0

RESULTS

Morphological Alteration of Mesenchymal Stem Cells After Treatment With Growth Factors

Mesenchymal stem cells isolated from both bone marrow and adipose tissues were preconditioned with cocktail of growth factors. Phase contrast microscopy showed a morphological alteration of both types of MSCs. Before treatment, cells were more like fibroblast with extensions adherent to plastic surface. After 1 week of treatment, extensions started to disappear and cells became epithelial like with appearance of a few rounded colonies (Figure 1).

Differentiation of Mesenchymal Stem Cells Towards Renal Lineage

The cocktail of growth factors has been reported to induce nephrogenesis. Treated ADMSCs and BMDMSCs were analyzed for expression of pronephrogenic, epithelial, and stem cell markers. Immunofluorescence staining showed an enhanced expression of pronephrogenic markers Wnt4, Wt1, and Pax2 (Figure 2) in both ADMSCs and BMDMSCs after 1 week of differentiation. Similarly, expression of renal epithelial markers, Ecad and ZO1, was also increased in both types of cells showing their successful differentiation towards nephrogenic lineage (Figure 3).

Both types of MSCs were further analyzed to observe whether expression of mesenchymal stem cell markers was altered or not before and after differentiation. Immunofluorescence staining illustrated that in contrast to nephrogenic markers, expression of mesenchymal stem cell markers Oct4 and Vim were reduced in both types of MSCs after treatment with cocktail compared to control cells

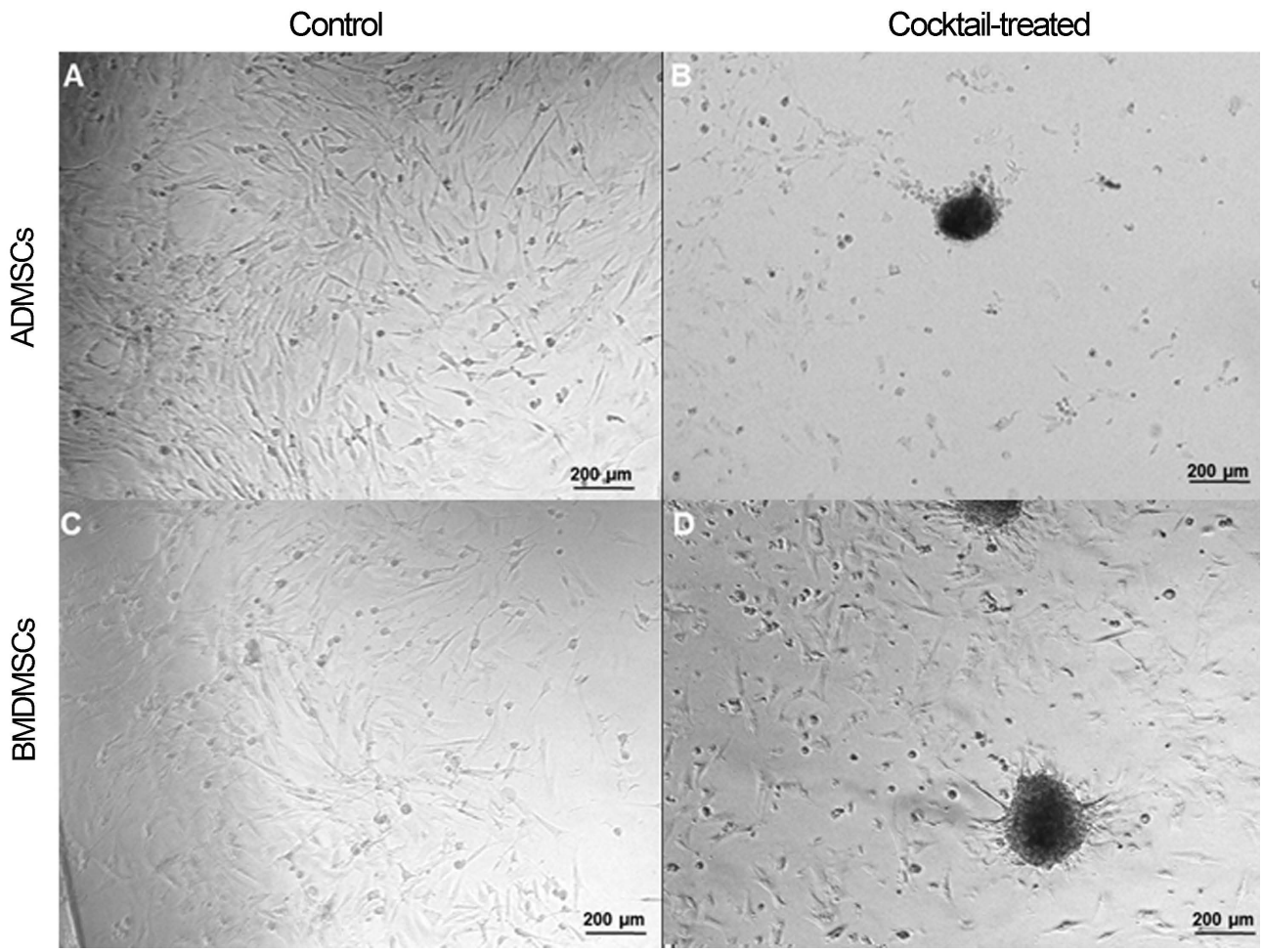

Figure 1. Morphological alterations of mesenchymal stem cells after treatment with cocktail of growth factors. A, Phase contrast microscopy of control adipose-derived mesenchymal stem cells (ADMSCs). B, ADMSCs after 1-week treatment with cocktail. C, Control bone marrow-derived mesenchymal stem cells (BMDMSCs). D, BMDMSCs after 1-week treatment with cocktail. Both types of mesenchymal stem cells show significant and comparable morphological alteration after differentiation treatment with cocktail.

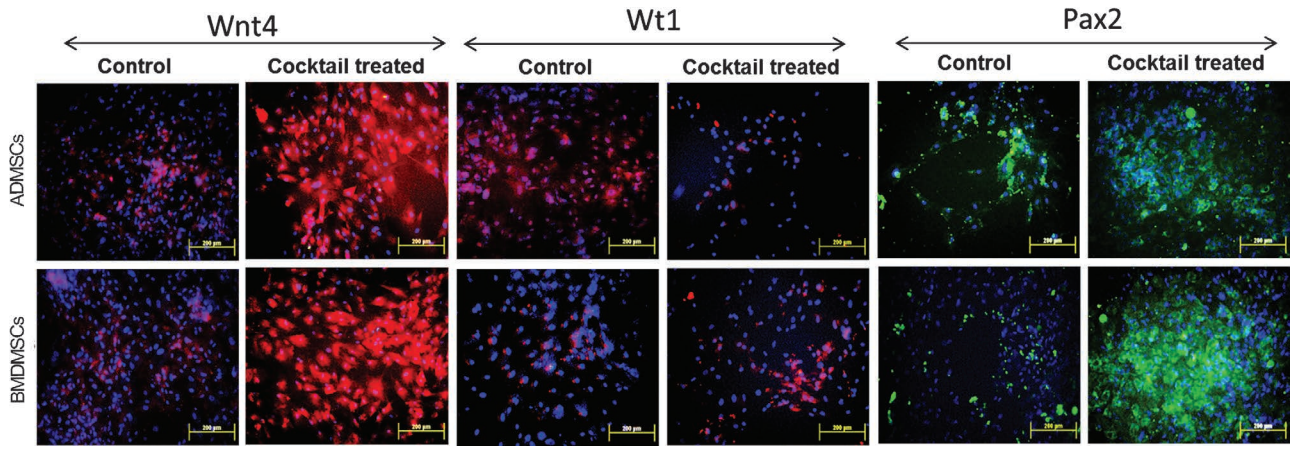

Figure 2. Induction of pronephrogenic markers Wnt4, Wt1, and Pax2 in mesenchymal stem cells treated with growth factors. Immunofluorescence staining shows significant enhanced expression of Wnt4, Wt1, and Pax2 in cocktail-treated adipose-derived mesenchymal stem cells (ADMSCs) compared to control cells (upper panel) and cocktail-treated bone marrow-derived mesenchymal stem cells (BMDMSCs) compared to control cells (lower panel).

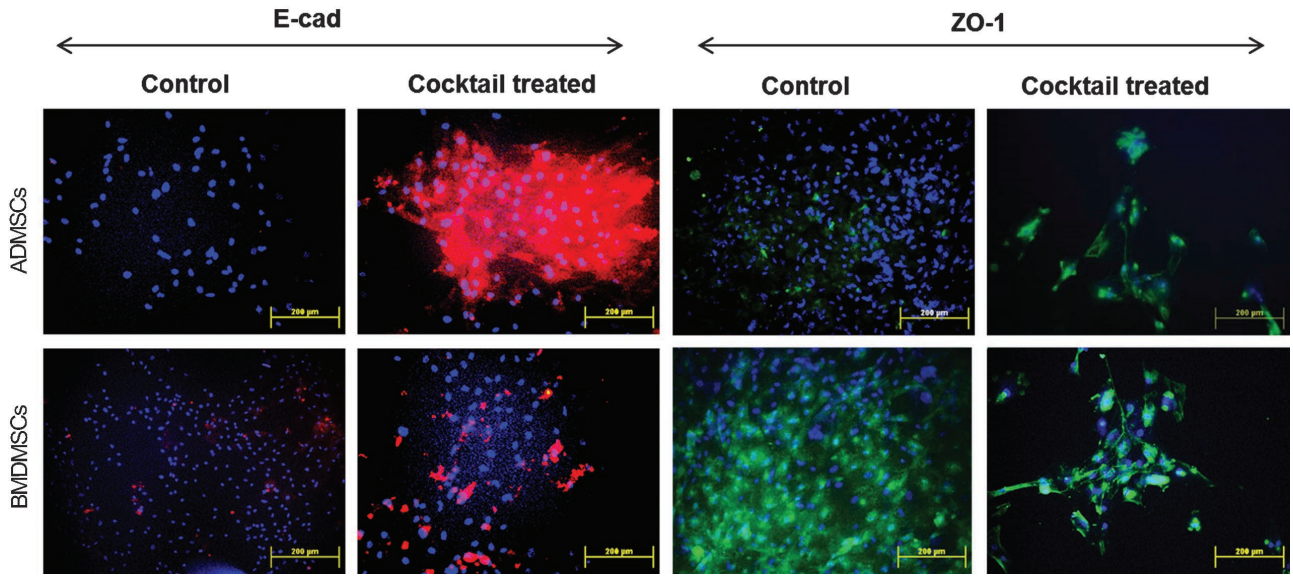

Figure 3. Induction of renal epithelial markers Ecad and ZO1 in mesenchymal stem cells treated with growth factors. Immunofluorescence staining shows significant expression of Ecad and ZO1 in cocktail-treated adipose-derived mesenchymal stem cells (ADMSCs) compared to control cells (upper panel) and cocktail-treated bone marrow-derived mesenchymal stem cells (BMDMSCs) compared to control cells (lower panel).

(Figure 4).

In order to further confirm the altered expression of several relevant genes, semiquantitative real-time polymerase chain reaction was performed with RNA isolated from both differentiated and control cells. The genes, including Wnt4, Wt1, Pax2, and Ecad, were significantly upregulated in differentiated ADMSCs and BMDMSCs cells compared to controls. Whereas, MSC markers Oct4 and Vim were downregulated. Expression of β-actin, an internal control, remained unaltered (Figure 5).

Enhanced Resistance of Differentiated Cells Against Ischemic Injury

Trypan blue and senescence assays were performed to estimate the cellular resistance of both differentiated and undifferentiated cells against ischemic injury. Results revealed a significant and comparable decrease of cell death and cellular senescence under ischemic conditions in both types of differentiated MSCs compared to control undifferentiated cells (Figure 6). This indicated that differentiated cells were more stable and suitable to be transplanted in enhanced resistance

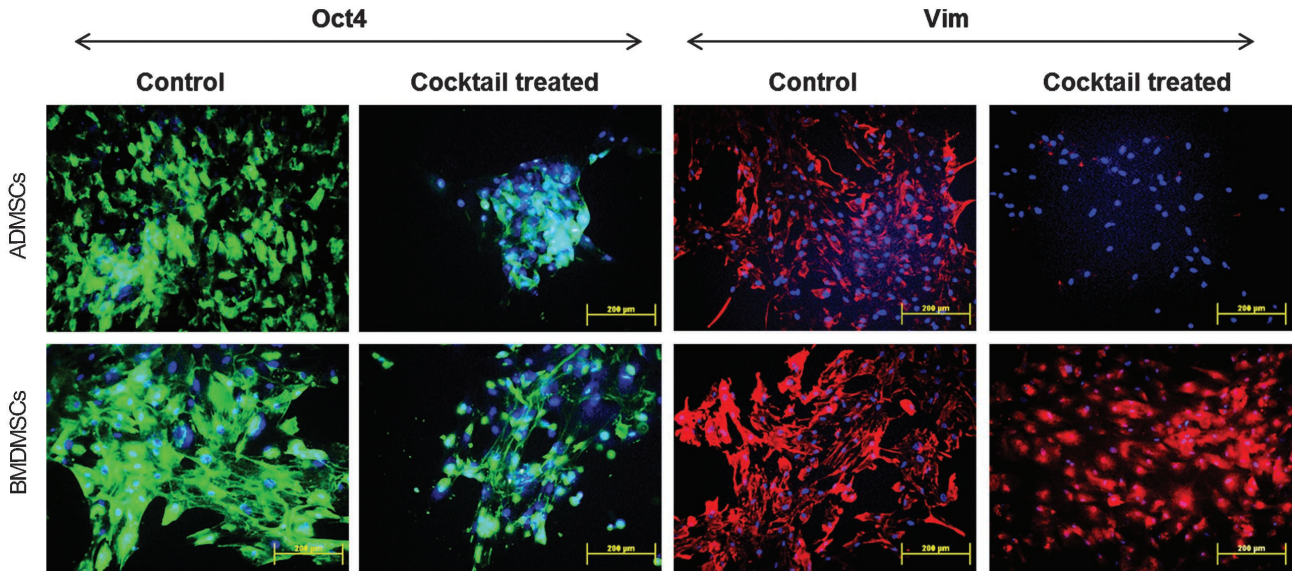

Figure 4. Induction of stem cell marker Oct4 and mesenchymal marker Vim in mesenchymal stem cells treated with growth factors. Immunofluorescence staining shows significant expression of Oct4 and Vim in cocktail-treated adipose-derived mesenchymal stem cells (ADMSCs) compared to control cells (upper panel) and cocktail-treated bone marrow-derived mesenchymal stem cells (BMDMSCs) compared to control cells (lower panel).

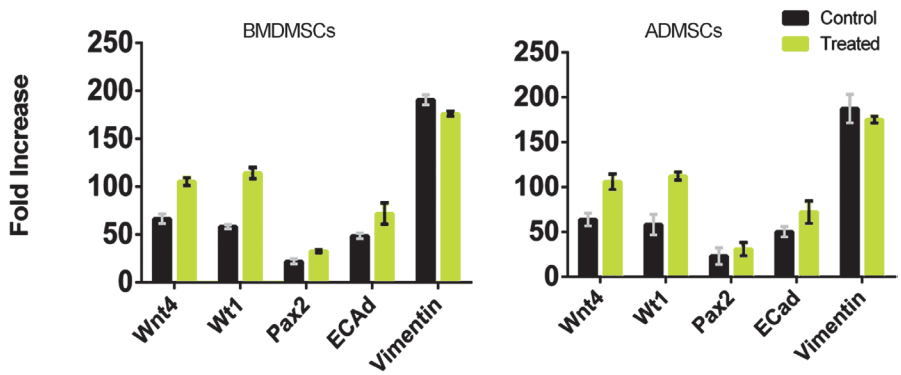

Figure 5. Semi-quantitative real-time polymerase chain reaction analysis for expression regulation of genes after treatment of mesenchymal stem cells with growth factor cocktail. The differential expression regulation of pronephrogenic, renal epithelial, and stem cell and mesenchymal markers were further confirmed with polymerase chain reaction. ADMSCs indicates adipose-derived mesenchymal stem cells and BMDMSCs, bone marrow-derived mesenchymal stem cells.

to ischemic injury.

DISCUSSION

For the past several years, many studies have showed that MSCs proved their ability to protect different kidney injuries.[22,23] The primary therapeutic mechanism of MSCs is through release of paracrine factors and very less is known about the homing or their differentiation towards renal structures like tubule formation.[24] Studies further revealed that renal progenitor cells are comparatively more competent not only in immediate healing but also in their capacity to form new renal structures. One of the limiting factors in their use is low number and difficulty in isolation and purification.[25] However, the present study was planned to direct MSCs derived from bone marrow and adipose tissues towards nephrogenic lineage in vitro, and thereby establishing a system not only for studying renal cells differentiation but also for their potential use in kidney injury.

In the course of recent years, ADMSCs have gained extensive interest due to their comparable differentiation potential to that of BMDMSCs,

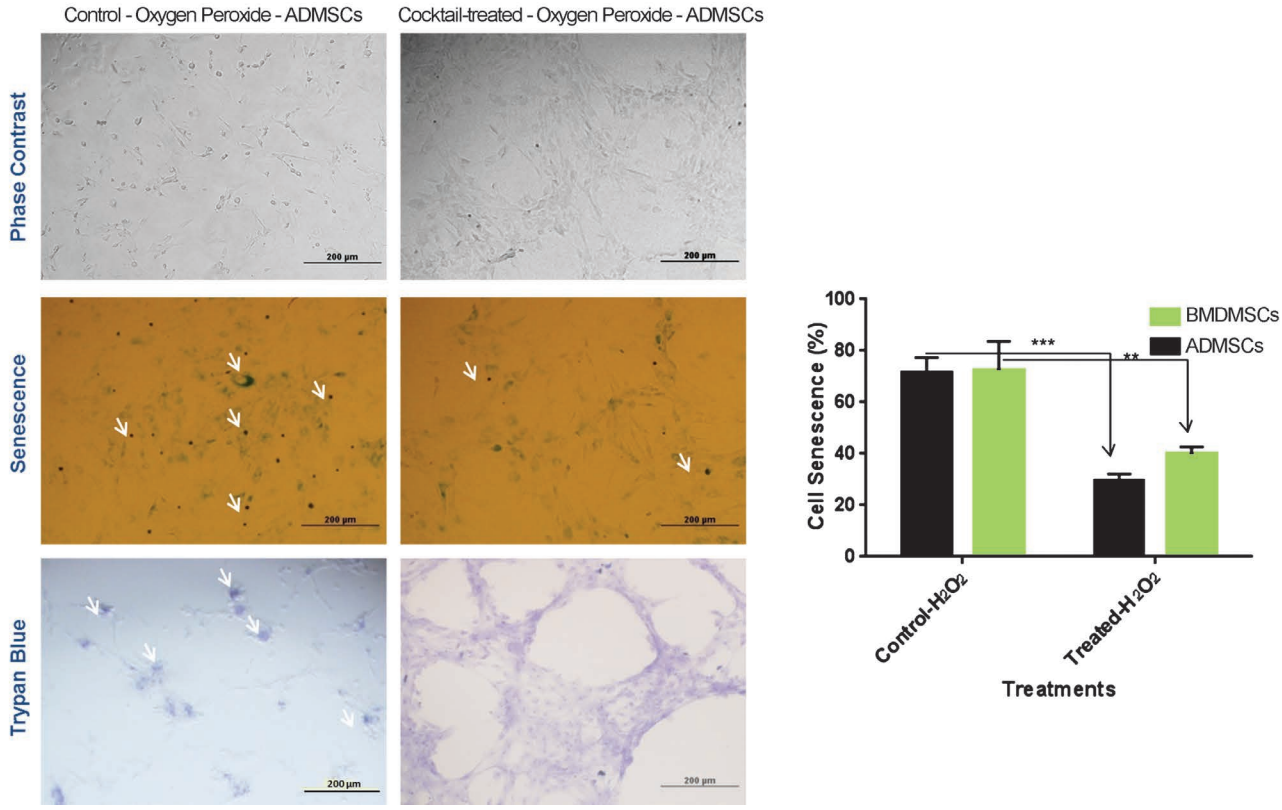

Figure 6. Trypan blue and senescence assay for the estimation of cell survival and viability.
Trypan blue and senescence assays were performed for cocktail-treated and control cells subjected to oxygen peroxide-mediated oxidative stress. Percentage of viable cells and senescent cells compared to total number of cells were shown under microscope and also measured. Significant results compared to the control are indicated with asterisks ($P < .05$).

less painful and easy availability.[26,27] Previously, various independent studies have been carried out comparing ADMSCs and BMDMSCs revealing that ADMSCs can be a suitable alternative source for BMDMSCs in therapeutic applications.[28,29] Moreover, studies further show that ADMSCs and BMDMSCs are comparable in their differentiation potential of towards hepatocytes, chondrogenesis, mineralisation and dentinogenesis, and hematpoesis.[30-35] The current study further confirms that ADMSCs are also comparable with BMDMSCs in their differentiation potential towards nephrogenic lineage and resistance to ischemic injury in vitro.

The addition of nephrogenic cocktail (FGF2, 50 ng/mL; TGF-β2, 4 ng/mL; and LIF, 20 ng/mL) to MSCs cultures was capable of inducing nephrogenesis as defined by the altered morphology and increased expression of pronephrogenic and nephrogenic epithelial markers. Previously, in a work done to understand the development of metanephric kidney, it has been reported that a combination of cytokines FGF2, LIF, and TGF-β2 cooperate to regulate nephrogenesis and induction of renal tubule formation.[18] However, the combination of only LIF and FGF2 cooperates to induce nephrons within 7 days in culture. On the other hand, addition of TGF-β2 speeds up nephron induction to 2 to 3 days, comparable to their in vivo speed.[28,29] Furthermore, cocktail of the above 3 cytokines was being successfully used to induce renal cells in vitro.[19]

Differentiation of both ADMSCs and BMDMSCs has exhibited the successful induction of pronephrogenic markers expression such as Wnt4, Wt1, and Pax2. In the hierarchy of molecular events mediating nephrogenesis, Wnt4 is identified as a key autoregulator of the mesenchymal-to-epithelial transformation that reinforces tubulogenesis.[30-32] Wnt4 is further known to mediate tubulogenesis in the kidney though noncanonical calcium-Wnt pathway.[33] Since 1993, Wt1 is also known as an important marker of early nephrogenesis and its expression in our differentiated cells further confirms

the initiation of nephrogenesis in MSCs treated with cocktail of growth factors.[34,35] Pax2 is defined as a central regulator of kidney development and an important early marker of nephron progenitors. It is also recognized among the earliest markers for the renal epithelial cell lineage.[36,37]

Moreover, the differentiated cells have further revealed the expression of renal epithelial markers such as ZO1 and Ecad. On the other hand, stem cell marker, Oct4 and mesenchymal cells marker, Vim, expressions were decreased in differentiated cells. It has been already reported that MSCs can be differentiated towards renal epithelial cells when treated with conditioned medium from renal tubular epithelial cells. However, they have shown in vitro induced expression of ZO1 but not of Ecad.[38]

CONCLUSIONS

In summary, our findings indicate that MSCs derived from both bone marrow and adipose tissues can differentiate into functional renal-like cells in vitro exclusively with use of a simple protocol. Mesenchymal stem cells differentiated towards renal lineage are more resistant to ischemic injury showing an increased therapeutic potential. Moreover, ADMSCs are analogous with BMDMSCs in differentiation and resistance. It is concluded that differentiated MSCs could be a better choice to be used in different kinds of kidney injuries. However, further in vivo studies need to be performed before their application in human cellular therapies.

CONFLICT OF INTEREST

None declared.

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