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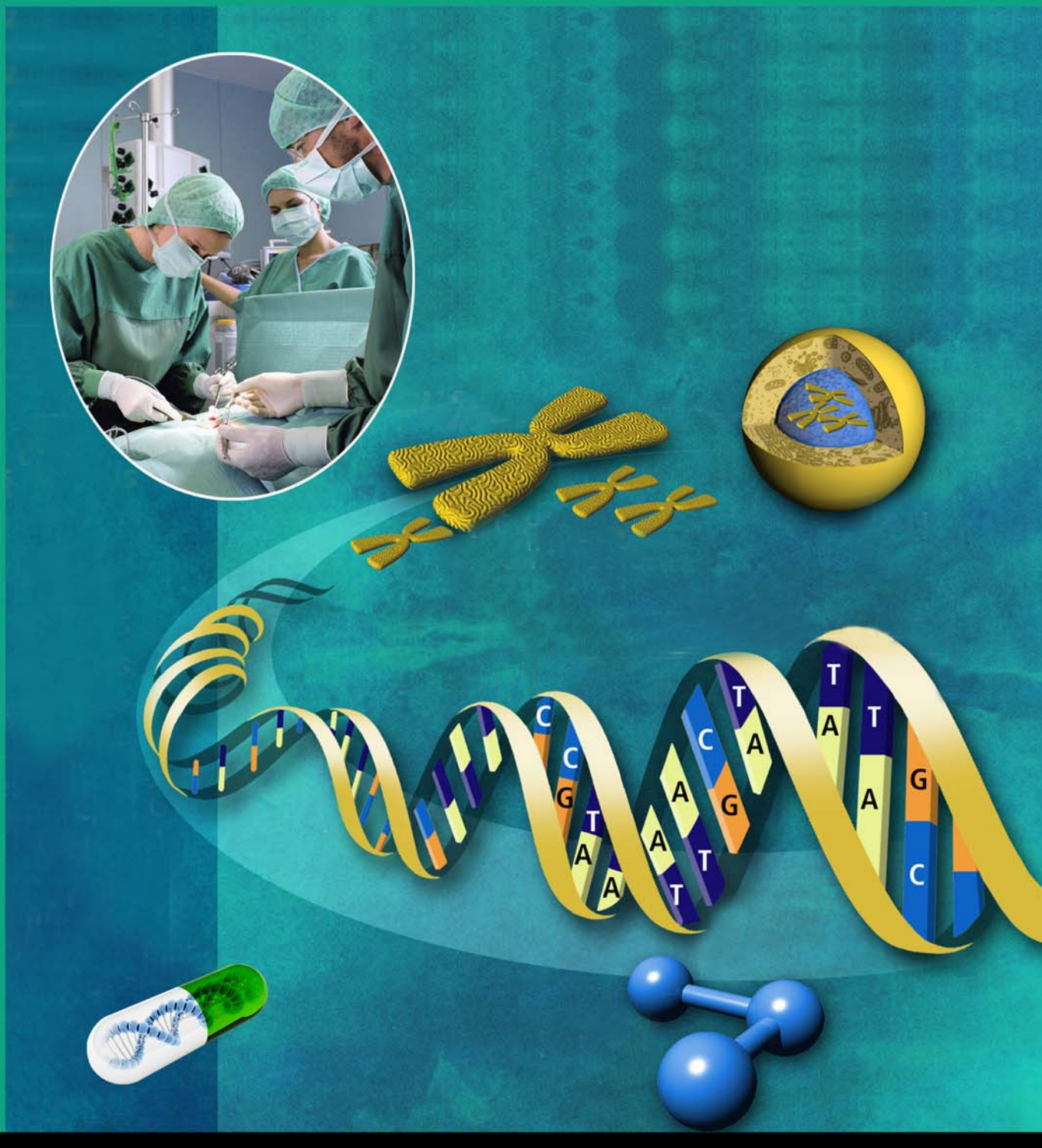

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Research Paper

CARDIOMYOGENIC DIFFERENTIATION OF FETAL DERIVED MESENCHYMAL STEM CELLS: A TRANSFORMATIVE APPROACH FOR CARDIAC TREATMENT

Shraddha Singh Gautam[1]* and Jaianand Kannaiyan[1]

*Corresponding Author: ***Shraddha Singh Gautam*** ✉ shraddhs80@gmail.com

There have been tremendous advances since past decade in the field of regeneration medicines with a hope of having potential to improve recovery from the most widespread diseases; cardiovascular disorder is one of them. Since then continuous preclinical and clinical trials have allowed to identify some important issues such as immune rejection, number of cells required, route of administration, engraftment, etc. These are to be addressed by appropriately focused bench work to find out a stem cell source having prerequisites as easily available, phenotypically well characterized, genotypically stable, consistent, scalable and sterile for an effective therapeutic application. In the current investigation we tested whether MSCs isolated from two fetal tissue sources such as Wharton's jelly and Umbilical cord blood can be coaxed *in vitro* into cardiomyogenic lineages with the exposure to 5'Azacytidine and whether regeneration of *in vivo* mouse myocardial infarcted heart is triggered by transplantation of these differentiated cells. Cardiac progenitor formation from guided *in vitro* MSC differentiation is the major determinant of *in vivo* cardiac regeneration in murine model. We could also observe that Wharton's Jelly is more potent source as compared to cord blood as far as extent of cardiac regeneration is considered.

Keywords: Mesenchymal Stem Cells, *In vitro* Cardiomyogenic Differentiation, Engraftment, Cardiac Regeneration

INTRODUCTION

Over the past decade, study of stem cells has gained tremendous interest in the field of regenerative medicines. They being the most naive cells in the body have known to possess ability to self-renew and differentiate into different lineages. This unique characteristic makes them the subject of intense experimental and clinical research for the treatment of many challenging diseases; Ischemia is one of them. Initially for the treatment of myocardial infarction which is a leading cause of cardiac injury, organ

[1] Stem Cell Division, Unistem Biosciences Pvt. Ltd., Gurgaon-122016, Haryana, India.

transplantation was the only available cure apart from prevention; but due to the scarcity of organs and other reported issues like immune rejections there have been advances in searching other options such as drug therapy, revascularization and resynchronization devices. However the proportion of cardiovascular deaths in the group of heart failure patients with depressed left ventricular function has not improved over the time (Menasche, 2009). This has motivated researchers to find out an alternative therapeutic major for cardiovascular disorders. The paradigm of heart being a post mitotic organ was challenged by the discovery of Cardiac Progenitor Cells (CPC's) clustered in niches throughout the myocardium in adult heart. These are present at distinct stages of commitment with the ability to produce cardiomyocytes responsible for normal wear and tear of the tissue (Dey *et al*., 2013; Beltrami *et al*., 2001). Myocardial infarction engenders an irreversible loss of these functional cardiomyocytes which do not replicate and cannot be replaced with consequent loss of myocardial cell mass. However direct transplantation of cardiac progenitor cells as precursors of cardiomyocytes is complicated due to the difficulty either in obtaining myocardial samples from patients or their expansion in quantities of therapeutic significance. Alternatively only cell-based therapies comprising of transplantation of *in vitro* differentiated cardiomyocytes which can contribute to homing at a targeted site and thereby increasing the number of viable and functional cardiomyocytes population holds considerable clinical significance (Leri *et al*., 2008; Paul *et al*., 2009).

Pluripotent stem cells can provide cellular precursors for cardiomyocytes *in vitro*. As a consequence; many stem cell types have been subjected to challenges to demonstrate a cardiomyocytes differentiation potential. Apart from therapeutic implications and obviating ethical concerns aroused by embryonic stem cell lines, Mesenchymal Stromal Cells (MSCs) have attracted significant attention for the repair of damaged heart tissue, this attention is partly due to their *ex vivo* expansion potential while maintaining their plasticity, and partly due to their immunosuppressive properties, trophic effect resulting from the paracrine secretion and capacity to promote neovascularization (Hoover-Plow *et al*., 2012; Razban *et al*., 2012). Furthermore it has also been evident that MSCs are present in virtually all post natal organs, afford easy handling, possess enormous expansion potential and comply with the allotransplantation. Hence, these MSCs being isolated in abundance, having differentiation potential and immunomodulatory properties even across xenogenic barriers; can be exploited for directed *in vitro* differentiation into cardiomyocytes and thus can be transplanted *in vivo* for effective cardiac regeneration.

Out of many known MSCs sources, fetal tissues have come out to be the highly concentrated source recently. With an annual global birth rate of over 100 million per year, these fetal tissues are large under utilized stem cells source with innate advantages. One paramount advantage is that they possess a primitive ontogeny and have not been exposed to immunologic challenges. Owing to this property of immune modulation, MSCs isolated from fetal tissues are considered as double edged sword which can not only bring about tissue repair but also attenuate adverse inflammatory reaction in an allogenic setting (Weiss *et al*., 2008; Wolbank *et al*., 2007; Van *et al*., 2007). So far there are

many studies demonstrating multi lineage differentiation potential of these fetal tissues derived MSCs (Ohnishi and Nagaya, 2007; Oswald *et al*., 2004). They have been reported to acquire cardiomyocytes phenotype *in vitro* after treatment with 5' Azacytidine which proved to significantly enhance regeneration in infarcted area in a mouse model (Xu *et al*., 2004; Qian *et al*. 2012; Moscoso *et al*., 2005).

The study will improve our understanding related to *in vitro* differentiation potential into cardiac lineages, route of administration for effective homing and therapeutic potential of these fetal derived MSCs. MSCs are found to be isolated from several compartments of Umbilical Cord (UC) but Wharton's Jelly (WJ) and Umbilical Cord Blood (UCB) are among the most common. Both of them have shown to be relatively short doubling time, require greater number of passages to senescence (Karahuseyinoglu *et al*., 2007) may be due to their naive status and relative youth. Both of these sources allow rapid initial isolation of large number of cells, avoiding the necessity of extensive multiplication and potential epigenetic damage. They even have advantages that cells isolated from fetal structures in the perinatal period and hence better tolerated following transplantation with less incidence of graft rejection.

In this regard, we in the current investigation have chosen above mentioned fetal tissues Wharton's Jelly and UCB as sources. MSCs isolated will be studied both for their guided *in vitro* cardiomyogenic differentiation potential and *in vivo* ability to reverse ventricular remodeling. This proposed differentiation will be carried out by exposing isolated MSCs to 5' Azacytidine and will be confirmed byrt-PCR for the presence of cardiac specific markers such as NkX 2.5, GATA-4 and β-actin(Matsuura *et al*., 2004). *In vivo* post infarct analysis of murine hearts will be done on the C57/BALB mice with respect to infarct size and improved cardiac regeneration (Gaebel *et al*. 2011).

We reported that the selected sources of MSCs displayed significant phenotypic and genotypic alterations after *in vitro* exposure to 5'-Azacytidine. Apart from this we have also confirmed that these differentiated cells engrafted efficiently in a murine model leading to improved regeneration in the infarcted area, reduced Left Ventricular (LV) dysfunction. But the extent of regeneration is observed more with WJMSCs than in CBMSCs.

This again necessitates a need to do more source-oriented study to reach to final conclusion. Apart from this retention of sufficient number of transplanted cells at the site of injury is another major issue for which strategies to enhance effective homing of transplanted stem cells are of utmost important. In this regard present investigation will be a truly transformative approach to cardiac therapeutics.

MATERIALS AND METHODS

Collection and Transport of Umbilical Cord

Human umbilical cords (n = 2) and Cord blood (n = 2) were collected from full-term births after normal vaginal delivery with informed consent using guidelines approved by the Institutional Committee for Stem Cell Research and Therapy (ICSCRT) at Unistem Biosciences Pvt. Ltd., India. A mother's medical history was reviewed and maternal blood sample was screened for infectious disease markers like HIV 1 & 2, HCV

antibodies, HBsAg, CMV-IgM, Malaria and Syphilis as per existing regulatory guidelines. Blood sample reactive for any of the infectious diseases excluded from the study. The cord was clamped, cut about 25 to 38 cm in length and collected in a labeled tube containing F12 Dulbecco's modified Eagle's medium (DMEM/F12) with Cefoperazone sodium 0.125 mg/ml (Jaianand *et al.,* 2013) and transferred to the processing facility within 36 h. The shipment temperature maintained at 4-15°C.

MSC Isolation and Culture from Wharton's Jelly

In a biosafety cabinet tissue was washed once with PBS; decontaminated thoroughly with 70% alcohol and again washed twice with PBS to remove all traces of blood samples. Approximately 25 Wharton's jelly explants of about 1 mm size each were plated in tissue-culture-grade T-75 flask (BD) with culture medium. Culture medium consists of Dulbecco's modified Eagle medium -Nutrient mixture Ham's F-12 (1:1) with L- Glutamine (1X); 2.438 g/L Sodium Bicarbonate (DMEM/F12; Gibco, USA) and 10% fetal bovine serum (FBS; Gibco, US) supplemented with 2 ng/mL bFGF (Sigma, USA), 0.125 mg/ml Cefoperazone sodium (MP Biomedicals, LLC). The flasks were left undisturbed in a 5% CO_2 incubator maintained at 37°C for 4-5 days after which fresh culture medium was added to the flasks. Adherent cells were allowed to expand for 14-17 days by changing the media at an interval of 5 days. The cells were harvested at 70-80% confluency using 0.25% trypsin (Gibco, USA). Cell count was performed after harvesting and cells were replated at a density of 3000 cells/cm^2 for expansion in T-175 tissue culture flasks (Nunc, Denmark).

MSC Isolation and Culture from Cord Blood

For the processing of CBMSC's, mononuclear cells were obtained by negative immunodepletion of CD34, CD45 cells using a commercially available kit from cord blood samples (BD Pharmingen, USA), followed by Lymphoprep (Fresenius KabiNorge, AS) density gradient centrifugation (1.077 ± 0.001 g/ml) and plated on tissue culture flasks (Nunc, Denmark) with an expansion medium. Cells were allowed to adhere. Medium was changed after every 5 days. Expansion medium consists of Dulbecco's modified Eagle medium -Nutrient mixture Ham's F-12 (1:1) with Glutamax (1X); 2.438g/L Sodium Bicarbonate; Sodium Pyruvate (DMEM/F12+; Gibco, USA) and 15% fetal bovine serum (FBS; Gibco, US) supplemented with 4 ng/mL bFGF (Sigma, USA), 0.125 mg/ml Cefoperazone sodium (MP Biomedicals, LLC). Once adherent cells reached approximately 70% to 80% confluency, they were detached with 0.25% trypsin and subcultured under the same culture conditions.

Confirmation for Surface Markers by Flow Analysis

Passage 2 cells from both the sources were then subjected to flow analysis for MSC specific markers such as CD90, CD105 and Hematopoietic markers such as CD34, CD45. Approximately 2 x 10^4 cells were utilized per marker. Trypsinized cells were washed with PBS to remove traces of media and trypsin. Cells were collected in a centrifuge tubes and incubated with specific antibodies or CD markers (BD Biosciences, USA) tagged with fluorescent colors for 45 min at 4°C. After incubation; tagged cells were centrifuged and suspended in PBS for analysis.

In Vitro Myogenic Differentiation

To study in vitro cardiomyogenic differentiation potential; MSC's at P2 obtained from both the sources were exposed to 5' Azacytidine (Sigma, LLC) in 6-well plates (Corning, USA) at a density of 1 x 10^4 each with growth medium Dulbecco's Modified Eagle Medium Nutrient Mixture F12 (DMEM/F12) (Gibco, USA) +10% Fetal Bovine Serum (FBS) (Gibco, US). After 24 h medium was changed to induction medium containing DMEM/F12+10%FBS+10µM 5'Azacytidine. Incubation with induction medium was carried out for 24 h; after which the medium was replaced with maintenance medium as too much exposure to 5' Azacytidine could be harmful to cells. Maintenance medium used was DMEM/F12+2% FBS. Medium was changed every 3 days for week. Cells were observed under microscope daily to visualize morphological changes associated with the extent of differentiation. MSC's without addition of an induction medium were used as controls.

Total RNA Extraction and rt-PCR

To confirm successful cardiomyogenic differentiation, cells were analyzed for genetic markers specific for cardiac origin such as Nkx-2.5, GATA4 which are well known cardiac transcription factors and β-actin: a cardiac structural protein. RNA was extracted with Trizol reagent (Invitrogen, US) from differentiated cells. 1 x 10^4 cells were utilized for extraction. cDNA was synthesized by rt-PCR kit (Quiagen). The reverse- transcribed cDNAs were amplified by PCR using oligonucleotide primers as per Table 1. Thermal profile used for PCR was 94°C for 2 min followed by 35 cycles of 30 s at 94°C, with 30 s annealing followed by 1 min extension at 72°C. Additional 10 min incubation at 72°C was performed after a last cycle. PCR products were size fractionated by electrophoresis using 1% agarose gel for chosen primers which were compared with molecular weight markers.

Tube Formation

For angiogenesis or tube formation assay we used BD Matrigel Angiogenesis kit. For the test we utilized 1 x 10^4 differentiated cardiac progenitors from both the sources for each well coated with matrigel. The 4 welled matrigel plates were activated by overnight refrigeration at 4°C as per the manufacturer's instruction. Growth medium used for the assay was Endothelial Growth Medium (EGM) (Lonza, Singapore) +20% FBS and maintenance medium was EGM +2% FBS. Induction medium used was EGM+20%FBS+5 ng/ml Vasular Endothelial Growth Factor (VEGF) (Sigma, LLC). For first 30 min cells were incubated with matrigel matrix using Growth Medium. After 30 min growth medium was replaced with Induction medium for 24 h followed by which cells were replaced by

Table 1: Primers for Cardiac Markers

Marker	Forward Primer	Reverse Primer	Product Size (bp)	Annealing Temperature (°C)
Nkx-2.5	5'AGTGGAGCTGGACAAAGCC3'	5'TAGCGACGGTTCTGGAATTT3'	216	55
GATA-4	52 CTGTCATCTCACTATGGGCA32	52 CCAAGTCCGAGCAGGAATTT32	275	60
β-Actin	5'GGACCTGGCTGGCCGGGACC3'	5'GCGGTGCACGATGGAGGGGC3'	583	60

maintenance medium for 48 h. Cells were observed under microscope hourly to visualize morphological changes associated with the extent of angiogenesis.

Development of Experimental Animal Model

All animal studies were performed as per the protocol finalized by the institutional committee for the use and care for animal. C57/BALB mice weighing minimum of 20 g at an age of 8-12 weeks were used for the study. The room temperature was controlled at 25°C. All mice were anesthetized with isobutane. All animals were placed in a supine position by tying their legs and the upper jaw. The heart was exposed by minimum left sided thoracotomy; the left anterior descending coronary artery (LAD) was permanently ligated. Immediately after ligation animals were grouped to receive intramyocardial infusion of 20 µl basal DMEM/F12 with 2 x 10^5 MSC's per sources such as WJ and CB as test (n=5) and plain 20 µl DMEM/F12 as a control (n=5). Along the border of the blanched myocardium 4 x 5 µl injections were given. Heart function was determined by histological analysis, six weeks post treatment. Results were compared with the normal mice.

Histological Studies

Six weeks after surgery mice were euthanized. Each heart was removed and embedded in paraffin. For analysis such as measurement of infarction size, cardiac remodeling; infarcted area of the tissue was cut transversely at 6 µm thickness.

Infarction Size: Heart sections with major infarct level were selected and were analyzed statistically using computerized planimetry (Axis Vision Zeiss, Germany). Data was expressed as Mean+/- SEM.

Fibrosis: To evaluate fibrosis, the Sirius red positive regions of collagen deposition were examined using digital imaging.

RESULTS

Morphology Analysis: An outgrowth of small spindle shaped cells was observed in less than 7 days in the culture. With more days passing in the culture; cells were doubled forming small to large colonies at $P_{(0)}$ (Figure 1). Thespindle morphology was changed to elongated fibroblast like morphology at $P_{(1)}$ and was maintained throughout subsequent passages. Once 70-80% confluency achieved; cells were then subjected to trypsinisation. As evident from (Figure 2) passage 2 (P_2) cells when employed for flow analysis showed negative for CD45 and CD34 specific hematopoietic markers. More than 98% WJMSCs were observed to be positive for CD90 and CD105; mesenchymal specific markers. 90% CBMSCs population was observed to be positive for CD90 and CD105.

Figure 1: Representative Phase-contrast Images of MSCs Showing Morphology After Expansion (A) Morphology of Wharton's Jelly Derived MSCs (*n*=2). (B) Morphology of Cord Blood Derived MSCs (*n*=2)

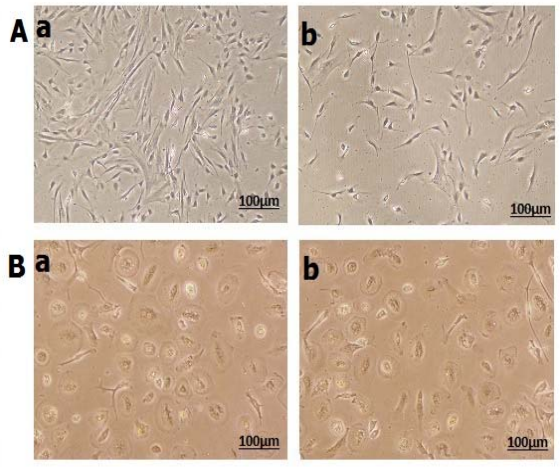
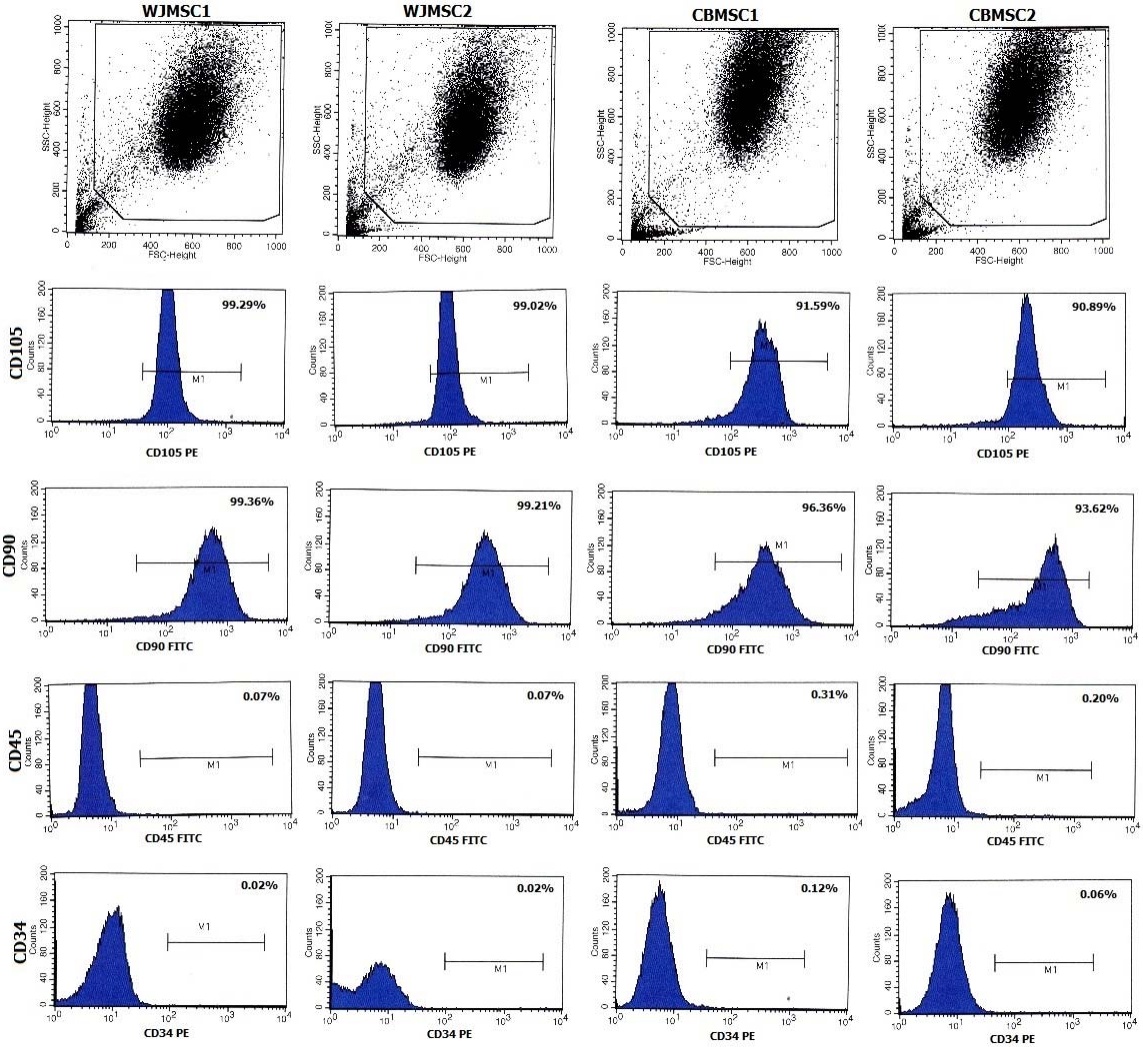


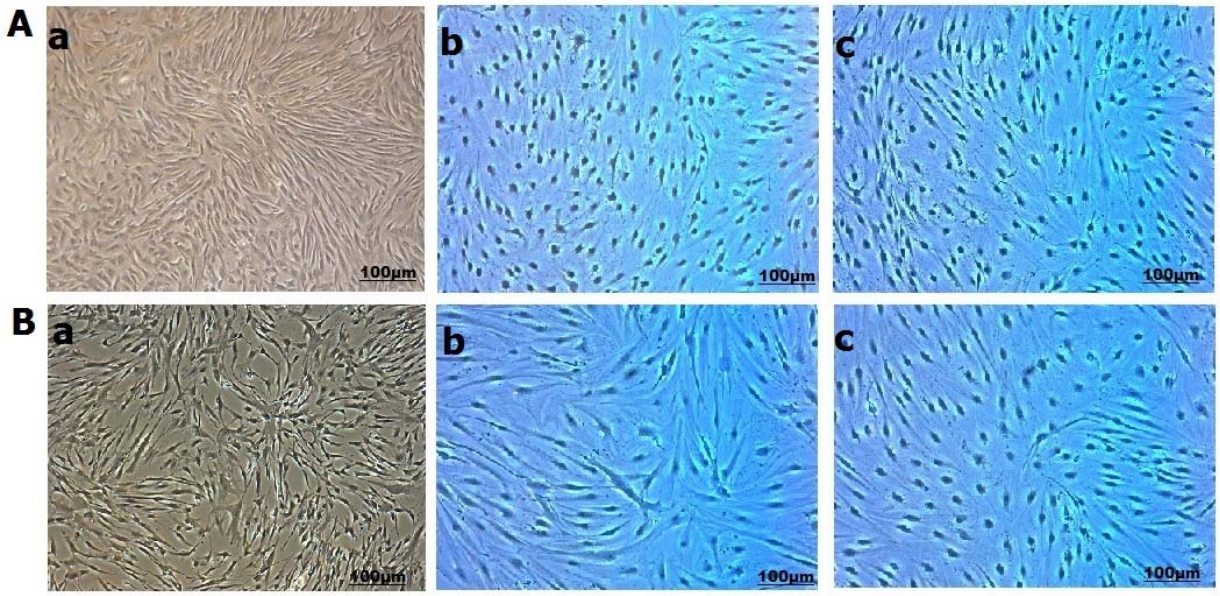
Figure 2: Immunophenotype of Wharton's Jelly Derived MSCs (WJMSC) (*n*=2) and Cord Blood Derived MSCs (CBMSC) (*n*=2). Representative Flow Cytometry Analysis of MSCs After Expansion When Labeled with Antibodies Against Human Antigens CD 34(PE), CD 45(FITC) as Negative Markers and CD 90(FITC), CD 105(PE) as Mesenchymal Specific Markers; Color Shaded Histogram Represents Positive Reactivity with the Indicated Antibody



Differentiation: As hypothesized in the beginning of the study MSCs obtained from both the sources were subjected to differentiation to cardiomyocytes after exposure to 5' Azacytidine. Small percentage of the cells was started floating on the surface of the media, 24 h post exposures. Adhered cells began to proliferate with alteration in the morphology after approximately 6-7 days in the culture media. Cells were observed to undergo characteristic cardiac progenitor morphology from long fibroblast to round ball like cells (Figure 3). The differentiation was confirmed by rt-PCR for cardiac markers.

PCR Analysis: Total RNA was isolated from induced as well as non-induced culture from both the sources. It has been observed that 5'

Figure 3: In vitro myogenic differentiation of umbilical cord stem cells one week after treatment with 5′ Azacytidine, some cells gradually increased in size and formed a ball-like round in appearance. (A) Morphology analysis of WJMSCs (a) Control for WJMSCs without addition of induction medium, (b,c: WJMSCs are differentiated to cardiac progenitor cells after treated with 5′ Azacytidine (*n*=2). (B)Morphology analysis of CBMSCs (a) CBMSCs control without addition of induction medium, (b,c: Differentiated CBMSCs to cardiac progenitor cells after treated with 5′ Azacytidine (*n*=2)



Azacytidine treatment strongly induced expression of NKX2.5, GATA4 and β-actin; which are cardiac specific markers (Figure 4). The expression was compared with base pair of the molecular weight marker using 1% agarose gel electrophoresis. Non induced or controlled cells were observed to be negative for these markers. Results confirmed successful differentiation of MSCs to cardiomyocytes when exposed to 5'Azacytidine.

Tube Formation: Capillary network formation a distinct mesh like structure is observed when differentiated WJMSCs were cultured and seeded on matrigel and exposed to VEGF. After around 1-2 h cells started migrating and began to align themselves to form tube like structures. About 11-14 h later closed polygonic structure began to appear which turned to complex mesh like structure within next 24 h (Figure 5). However in case of CBMSCs no network formation is observed when cultured on matrigel; which has to be further studied.

Infarct size: Six weeks post treatment, analysis of the ventricular cross sections of non-transplanted animals showed damaged area of LV, which is evident from the formation of the scar tissue, thinning of left ventricular valve and extensive collagen deposition (n=5) (Figure 6A). Sections treated with WJMSCS showed significantly reduced LV wall thinning and evident reduction in the myocardial damage. Improvement with CBMSCs was observed not to be up to the mark as compared to WJMSCS. Statistical analysis showed (Figure 6B and Table

Figure 4:5'Azacytidine induced cardiac specific genes β-Actin, GATA4 and Nkx2.5. Expression of β-Actin (216bp), GATA4 (275bp) and Nkx2.5 (583bp) genes were analyzed by rt-PCR; Lane 1: Molecular Marker; Lane 2,3: expression of cardiac specific genes was detected with WJMSCs (*n*=2). Lane 4,5: expression of cardiac specific genes was detected with CBMSCs (*n*=2)

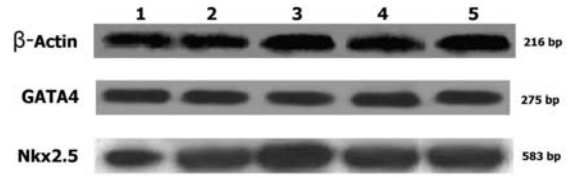


Figure 5: *In Vitro* Tube Formation of WJMSCs on Matrigel. (a) Alignment of Cells on Matrigelone Hour Post Incubation. (b-d) Extensive Network Formation Observed 6/ 12/24 Hours Post Incubation Respectively

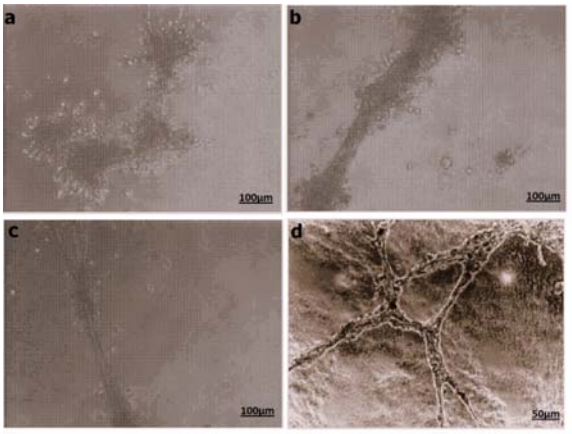


Figure 6A: Murine heart sections after cardiac damage. (a) Massive cardiac damage six weeks post infarction. Arrow is indicating significant thinning of LV and deposition of collagen. (b,c) Indicate reversal of cardiac remodeling associated with reduced wall thinning and reduced collagen deposition indicated by arrows. Evidently extent of regeneration is seen more in WJMSCs treated section (b) than CBMSCs treated section (c)

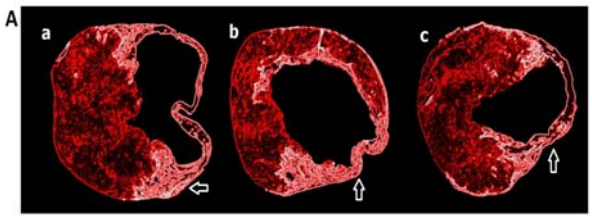


Figure 6(B): Statistical Analysis showing % Infarction in non-transplanted sections (40.82±5.05) which is reduced to great extent in WJMSCs treated sections (17.79±1.95) and to some extent in CBMSCs treated sections (32.1±2.73)

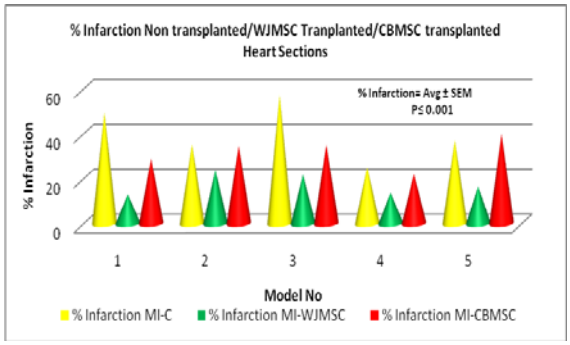


Table 2: Percentage of Infarction Calculated as Infarcted Area/Total LV Area x 100

Model No	% Infarction MI-Non Transplanted (MI-C)	% Infarction MI-WJMSC	% Infarction MI-CBMSC
1	49.4	13.01	28.89
2	35.34	23.92	34.59
3	57.24	21.8	34.94
4	25.08	13.72	22.14
5	37.05	16.5	39.92
Mean ± SEM	40.82 ± 5.05	17.79±1.95	32.1±2.73

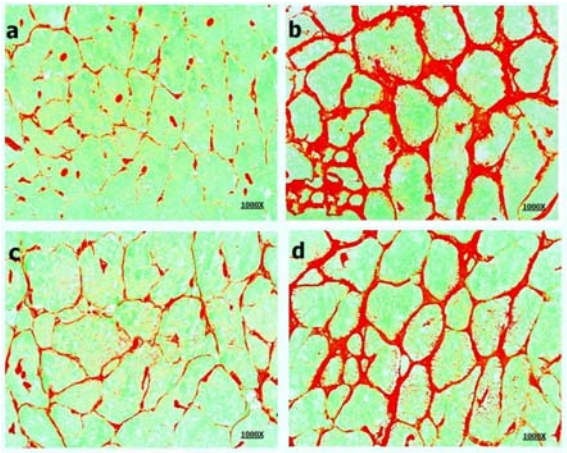
Table 3: Infarction Ratio Calculated as Infarcted Area/LV Area

Model No	Infarction Ratio MI-Non Transplanted (MI-C)	Infarction Ratio MI-WJMSC	Infarction Ratio MI-CBMSC
1	0.49	0.13	0.29
2	0.35	0.24	0.35
3	0.57	0.22	0.35
4	0.25	0.14	0.22
5	0.37	0.17	0.4
Mean ± SEM	0.41±0.18	0.18±0.08	0.32±0.14

2) visible decrease in the percent infarct as well as decrease in the ratio of infarction size to entire LV in transplanted hearts. The ratio was decreased from 0.41±0.12 to 0.18±0.08 after WJMSCs treatment and from 0.41±0.12 to 0.32±0.14 after CBMSCs (Table 3).

Fibrosis: In fibrosis analysis post infarct, extensive collagen deposition (Sirius Red) and increased distance between myocytes was observed in non-transplanted animals after 6 weeks infarction. Figure 7 indicating non-transplanted staining sections showing higher portion of collagen deposition. These sections when visually compared with transplanted sections showing reduced deposition which is similar to normal heart (Sham); Analysis confirms good improvement in myocardial damage occurring post infarction post WJMSC transplant as compared to CBMSCs

Figure 7: Fibrosis in murine heart sections. (a) Normal (Sham) section of the heart showing reduced deposition of collagen. (b) Infarcted sections showing significant collagen deposition. (c) WJMSCs transplanted sections showing again reduced deposition comparable with (a). (d) Minimized deposition of collagen observed in CBMSCs transplanted sections



DISCUSSION

Complicated severity of ischemic heart diseases has resulted in major worldwide death toll. The limited regenerative capacity of injured myocardium, negative remodeling and myocardial scar leads to gradual functional failure of the heart. This has prompted studies of cardiomyocytes. A foundation of such effort is to know what cells can be coaxed into cardiac fate and how this differentiation as well as homing can be governed. Recent reviews of completed clinical trials for stem cell therapy; report improvement of 10% or more in about half of the studies (George, 2010; Mozid *et al*., 2011; Sanz-Ruiz *et al*., 2010). Intensive studies are underway

to identify newer sources of stem cells in addition to established sources such as bone marrow, embryonic stem cells; but utilization of these cells pose lot of practical and ethical problems. However fetal tissues can be the best alternative stem cell source as in one regard they exhibit properties of embryonic stem cells due to their similar ontogenic relationship and in other regard they can be very easily obtained with minimum ethical issues. Current effort is thus to focus on establishing conditions for directed differentiation of cells isolated from fetal tissues by exposing them *in vitro* to altered chemical composition in the culture medium.

The two sources selected UCB and WJ proved to be a good choice of source due to their unlimited expansion potential and higher *in vitro* growth kinetics. These cells attained spindle morphology after about 4-5 days in culture. Isolated cells at passage 2 (P2) from both the sources when subjected to flow analysis observed to be positive for MSC markers such as CD90, CD105 confirming their mesenchymalorigin. Apart from this present study also addresses *in vitro* differentiation capacity of these cells after exposure to 5'-Azacytidine. 5'Azacytidine is a DNA demethylating compound which induces uncontrolled myogenic specification in mesenchymal stem cells by random demethylation (Antonitsis *et al*., 2007; Tomita *et al*., 1999; Tomita *et al*., 2002). The induced cardiomyogenic differentiation was observed to be successful with similar morphology such as rounded cell morphology, string bead like nuclei. In addition to this differentiated cells have also shown to express some cardiac specific markers such as GATA4, β-actin and NKx2.5; which were confirmed with reverse transcriptase PCR analysis. These markers were observed to be positive matching with the base pair of the positive control and thus supporting to our hypothesis of full differentiation into cardiac progenitors after 5'-Azacytidine treatment. The network forming potential of these differentiated cells was also confirmed using BD matrigel assay wherein angiogenesis was observed in WJMSCs only but not in CBMSCs. This needs to be investigated further to reach to the conclusion.

In vivo manipulation of the murine hearts was carried out to investigate chronic biological and physiological processes involved and thereby influencing the outcome. So far it has not been determined whether there has been a therapeutic difference in cells obtained from different sources. Hence combined Umbilical Cord Blood and Wharton's jelly stem cells have been utilized as treatment post MI to observe structural and functional changes. Analysis was done on the basis of comparison with non-transplanted as well as controlled (normal) mice. Post MI cardiac remodeling was observed in ventricular cross sections of all non-transplanted mice to be accompanied by structural changes in the LV such as wall thinning and fibrosis (collagen deposition) 6 weeks post MI. All sections treated with Wharton's jelly MSC showed decrease in the ratio of infarction size to LV confirming significant reduction in the damage post infarct on the contrary extent of myocardial regeneration in animals treated with CBMSCs was not as good as with the WJMSCs. Results were demonstrated different regenerative effects of the cells obtained from both the sources and also confirmed the need of detailed evaluation of different sources of MSCs prior to their clinical application.

Post Infarct cardiac remodeling is associated with the fibrosis (Wang *et al*., 2002; Hao *et al*., 1999) as a compensatory mechanism resulting in the extensive collagen deposition. This was confirmed by using Sirius Red 6 weeks after infarction. Analysis confirmed high portion of collagen deposition in the non-transplanted sections whereas in transplanted sections much reduced collagen deposition was observed. However animals transplanted with WJMSCs showed much reduced collagen deposition than with CBMSCs which was comparable with controlled sections.

CONCLUSION

Though the current investigation showed promising benefits of stem cell therapy to improve cardiac function post MI but no animal can fully duplicate the complex situation of patients. Hence, it is necessary to continue undertaking adequately designed and powered clinical trials provided that there experimental grounds are robust and consistent. In addition to this cell source also should be selected considering clinical indication. Further work is thus needed to confirm the proof of principle, to evaluate potential middle level and long term safety issues.

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Hyderabad, INDIA. Ph: +91-09441351700, 09059645577
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