Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/7afq8s81

MESENCHYMAL STROMAL CELLS FROM WHARTON'S JELLY EXPLANT CULTURE NOT ONLY CHARACTERIZES EMBRYONIC STEM CELLS BUT ALSO HAS GERM LAYER MARKERS

T Paras Singh[1*], M L Sherpa[2], Anup Pradhan[3], T A Singh[4]

[1*]Associate Professor, Department of Biochemistry, Shija Academy of Health Sciences, Imphal West, Manipur, India
[2]Professor, Department of Biochemistry, Sikkim Manipal Institute of Medical Sciences, Sikkim Manipal University, Gangtok, Sikkim, India
[3]Professor, Department of Obstetrics and Gynecology, Sikkim Manipal Institute of Medical Sciences, Sikkim Manipal University, Gangtok, Sikkim, India
[4]Professor, Department of Biochemistry, Shija Academy of Health Sciences, Imphal West, Manipur, India

***Corresponding author-** Dr T Paras Singh
*Associate Professor, Department of Biochemistry, Shija Academy of Health Sciences, Imphal West, Manipur, India

Abstract:

Background and Objectives: Mesenchymal stromal cells (MSCs) have a unique property of immunomodulatory, homing and regenerative potential. It can also be isolated from several neonate tissues like placenta, umbilical cord and umbilical cord blood as they are considered primitive tissues. Human umbilical cord Wharton's Jelly (hUCWJ) is a rich and promising source of MSCs due to its close association with embryonic tissue. It is a non-controversial and easily available source of human tissue. The aim of the study was to examine the expression of embryonic stem cells and germ layers markers in the neonate derived MSCs from hUCWJ.

Methods: The ex-plant isolated MSCs from hUCWJ were sub-cultured and the study was conducted at 3rd passage. After defining the isolated cells as MSCs (by morphology analysis, immunophenotyping by flowcytometer and tri-lineage differentiation), a semi-quantitative two-step reverse transcription polymerase chain reaction (RT-PCR) was performed to synthesize complementary DNA (cDNA). Finally using specific primers for embryonic stem cells and germ layer markers, PCR (Polymerase Chain Reaction) was performed on the cDNA and the products analyzed.

Results: A distinct expression of germ layer markers (nestin, TGFβ (transforming growth factor beta), GATA4) and embryonic stem cells markers (OCT4 (Octamer-binding transcription), NANOG (Nanog homeobox gene) and SOX2 (SRY (sex determining region Y)-box 2)) were observed on hUCWJ-MSCs

Interpretation & conclusions*:* A strong expression of endodermal marker could be observed compared to ectodermal and mesodermal markers. It also expresses embryonic stem cells markers supporting the pluripotency and primitive nature of the explant derived hUCWJ MSCs.

Keywords: Explant, Germ layer markers, Mesenchymal stromal cells, Primitive nature, Wharton's jelly.

Introduction:

The Mesenchymal stromal cells (MSCs) are emerging to be more popular than the others stem cells. Isolations of adult MSCs from different sources have been reported by many studies during the last decade. The adult MSCs derived from different sites have different characteristics in terms of regeneration and differentiation. The MSCs first isolated by Friedenstein *et al* in bone marrow is still the most frequently investigated cell type which he differentiated in adipocytes, osteocytes and chondrocytes[(1)]. As the research advance, in vitro differentiation of mesenchymal lineages to osteoblasts, chondrocytes and adipocytes from bone marrow are not limited, but can also be isolated and differentiated from other sources like adipose tissue[(2)], menstrual blood[(3)], amniotic fluid[(4)], umbilical cord [(5)], cord blood[(6)], dental pulp[(7)]and placenta[(8)]. MSCs also have unique properties of immunomodulatory and homing. It has been reported that in tissue-specific homing of leukocytes, the chemokines receptor with their ligands and adhesion molecules play an important role by implicating of hematopoietic precursors into and through tissue[(9)]. It has been observed that MSCs do not express costimulatory molecules such as CD80, CD86 or CD40 and constitutively express a low level of major histocompatibility complex-I molecules, thus lacking immunogenicity. While comparing younger versus aged human-derived MSCs different studies have stated that there are decreased proliferation rates, low potential chondrogenic and osteogenic differentiations and increased senescent features [(10)]. Therefore, with higher proliferation and expansion potential, the neonates derived MSCs like Wharton jelly, placenta, umbilical cord blood, are considered primitive when compared with MSCs obtained from other adult tissues like adipose tissue and Bone Marrow[(11)].

To maintain the properties of stem cells which include self-renewal and pluripotency, expression of the three transcription factor Oct4, SOX2 and Nanog are essential[(12)]. The expression of Nanog is up-regulated by Oct4 together with SOX2[(13)].The transcription factor SOX2 and Nanog are the initial transcription control which offers an initial clarification of multipotent capacity of MSCs. Suppressor genes for differentiation like Dnmt3b, Foxd3, Id4, Jmjd2c and Suz12 are transcriptionally activated along with SOX2 and Nanog. The genes responsible for differentiation are activated when the transcription factors are suppressed[(14)]. Oct4 belongs to the family of Pou-domain transcriptional factors, and it is found in developing embryos, developing endoderm as well as developing neurectoderm[(15)]. SOX2 is a member of the SRY-related HMG-box (SOX) transcription factor family with a diverse role in stem cell potency and maintenance, embryonic development and cancer[(16)]. Transcription factor Nanog forms a part of a regulatory complex network and its function is based on regulating cell death, proliferation and determining cell fate[(17)]. Expression of the transcription factors SOX2, Nanog and Oct4 has been reported in adult tissue implicating the maintenance and differentiation of adult stem cells[(18)]. However, there is a controversy among different research groups regarding the expression of pluripotency regulators in adult tissues[(19)].

The question that needs to be answered with explant derived Wharton's jelly cells is if these cells express one kind of committed cells (multipotent) or are these cells pluripotent. Because if the latter is true then this would be a large source for obtaining pluripotent stem cells for research as well as for therapeutic. Novelty and impact: this work sets for the first time a framework to understand the pluripotency /multipotency of hUCWJ derived by explant and has on importance from point of view of the biology of cell differentiation. Also to know if Wharton's jelly can be a largely untapped source of stem cells from which tissue models for disease biology can be created. Besides this, it is the first time in the cell differentiation literature that characterization to search for pluripotency of Wharton's jelly has been undertaken. In addition, it presents a general model for cell differentiation that can be used to analyze similarities and differences between different cell types in the discarded tissue from obstetrics units. The issue of differential capability is difficult to interpret. If characterizations show good results then large scaling up of separation of potential cells followed by industrial level cell

cultures can be done from Wharton's Jelly cells. This characterization will help to optimize bench work, speeding up biologically relevant cells and reduce costs.
Therefore the aim of the study was to isolate the MSCs from hUCWJ by explant culture, then see if they can be defined as per criteria [20]. A semi-quantitative two-step RT-PCR was performed where RNA was extracted and the first strand cDNA was synthesized. Finally, confirm the expression embryonic stem cells and germ layer markers in the above cells by PCR.

Materials and Methods:
This experimental study was conducted for a period of 24 months (16.07.2015 to 15.07.2017) in the Departments of Biochemistry and Obstetrics and Gynaecology, Sikkim Manipal Institute of Medical Sciences & Central Referral Hospital, SMU, Gangtok, Sikkim after the due approval of the Institutional Review Board. A total number of 22 human umbilical cord tissue samples were collected from the placental ends after the delivery of the baby. Informed consents were obtained from all participating mothers.

***Collection of UCWJ*:**
Around 10 cm long hUCWJ was collected from the placental end in 50 ml tube containing 32 ml of Hank's Balanced Salt Solution (Gibco, USA) supplemented with 300 U/ml penicillin, 300 µg/ml streptomycin, 150 µg/ml gentamicin and 1µg/ml fungizone (Gibco, USA).

Defining MSCs as per criteria
Isolation of UCWJ MSCs: Under aseptic laminar flow hood cabinet (ESCO, Singapore), the umbilical cord was transferred to a tissue culture dish containing phosphate buffered saline (Sigma, USA) and washed 3-4 times to remove any blood and blood clots. The umbilical cord was dissected vertically (Fig 1A) and the vein and arteries were removed (Fig 1B). The remaining part of the cord was diced to make 2-3 mm^2/pieces (Fig 1C). Around 8 diced pieces were transferred to a 35mm culture plate (BD Falcon, USA) and allowed to stand for 10 min. Transfer 2 ml DMEM low glucose, Glutamax (Gibco, USA), containing 20% Fetal bovine serum (Gibco, USA) and antibiotics. The culture plate was incubated in a CO_2 incubator (New Brunswick, Eppendorf, USA) at 37°C and 5% CO_2 for 4 days and the medium was changed every 3 days with 3 ml DMEM supplemented 10 % FBS and antibiotics. The explants were allowed to migrate until it reached 50-60% confluence. Then the explant was removed and the cells attached to the culture plate was trypsinized by trypsin, EDTA (Gibco, USA) then split in 1:3 ratio.

Immunophenotyping by Flow Cytometry:
International society for cellular therapy[20] has described a panel of markers which need to be expressed by the adherent cell population to label them as Mesenchymal stromal cells. We used BD Stemflow Human MSC Analysis Kit (Becton, Dickinson, USA) where the following antibodies were provided CD73-APC, CD105-PerCP-Cy5.5, CD90-FITC and CD44-PE as positive markers and the PE channel open to use in combination with the supplied negative MSC cocktail (PE CD45, PE CD34, PE CD11b, PE CD19 and PE HLA-DR). It also included antibody conjugate for immunophenotyping the MSCs.

In vitro differentiation:
In-vitro differentiation was observed when the MSCs expressed differentiation to Mesenchymal lineages like Osteoblast, Chondrocytes, and Adipocytes under proper induction conditions used to functionally ascertain "mesenchymalness" of adult MSCs according to International Society for Cellular Therapy (ISCT) norms [20].

Adipogenic differentiation:
The cell containing medium was aspirated from 50-60% confluence cells at the 3rd passage and rinsed with 2 ml PBS. A 2 ml of adipogenic differentiation medium, (Gibco, USA) was added to the culture plate then incubated at 37°C and 5% CO_2 for 4 days. The medium was replaced with pre-warm adipogenic differentiation medium every 3-4 days and incubated. MSCs continue to undergo limited expansion as they differentiated under adipogenic condition. After 14 days of cultivation, the adipogenic culture was stained with Oil Red O staining, (Sigma, USA) to confirm that the MSCs had differentiated to adipocytes.

Chondrogenic differentiation:
The medium was aspirated from 70-80% confluence cells from 3rd passage and rinsed twice with 2 ml PBS and 0.5ml of trypsin was added to the culture plate followed incubation for 7 min at 37°C. The cell suspension was transferred to a 15 ml centrifuge tube and pelleted at 1500 rpm for 10 min. The pellet was re-suspended in 200 µl of complete medium and micromass culture was generated by seeding 3 to 4 5µl droplets on the culture plate. After cultivating micromass cultures for 2 hrs, 2 ml of pre-warmed chondrogenic differentiation medium, (Gibco, USA) was added to the culture plate and incubated at 37°C and 5% CO_2. The culture was re-fed with chondrogenic medium every 2-3 days. After 14 days of cultivation, chondrogenic pellets were stained with Alcian Blue Stain, (Sigma, USA) to confirm MSCs had differentiated to chondrocytes.

Osteogenic differentiation:
The cell containing medium was aspirated from 50-60% confluence cells from the 3rd passage and rinsed with 2 ml PBS. Then, 2ml of osteogenic differentiation medium, (Gibco, USA) was added on to the culture plate and incubated at 37°C and 5% CO_2 for 4 days. The culture was re-fed every 3-4 days with pre-warmed osteogenic differentiation medium and incubated. The MSCs continue to undergo limited expansion as they differentiated under osteogenic condition. After 21 days of cultivation, the osteogenic culture was stained with Alizarin Red S, (Sigma, USA) to confirm that the MSCs had differentiated to osteocytes.

***RNA extraction and Quantitative Reverse Transcription PCR*:**
Using TRIzol reagent, (Sigma, USA) the RNA was extracted from the MSCs in the culture plate as per the manufacturer's instructions. RNA quality was assessed by the ratio of absorbance at 260 and 280 nm using Biospectophotometer, (Eppendorf, USA). A total of 3 µg of total RNA was reverse transcribed to cDNA using Superscript III First-Strand Synthesis System, (Invitrogen, Lithuania) for RT-PCR according to the manufacturer's instructions. The polymerase chain reaction, (PCR) (Genei, India) was performed by using the PCR master mix, (Puregene, USA) with the sets of primers (Table-I). PCR conditions included an initial denaturation of 5 min at 94°C, followed by 35 cycles of 30 sec denaturation at 94°C, 45 sec at annealing temperature and 30 sec extension at 72°C, with a final elongation step at 72°C for 7 min. Amplified products were separated by electrophoresis, (Genei, India) on a 2% agarose gel, (Promega, USA) and observed by ethidium bromide, (Promega, USA) staining under Gel Doc – it 310 imaging system, (UVP, USA)

Table 1 Shows Primer sequence for PCR reactions

Name	Sequence	Product size	Genebank reference no.
nestin	F – GCCCTGACCACTCCAGTTTA R – GGAGTCCTGGATTTCCTTCC	220 bp	NM_006617.1
GATA4	F – TCATCTCACTACGGGCACAG R – GGGAAGAGGGAAGATTACGC	233 bp	NM_002052.2
TGF-β1	F – GCGTGCTAATGGTGGAAAC R – CGGTGACATCAAAAGATAACCAC	275 bp	NM_000660.3

SOX2	F – GTATCAGGAGTTGTCAAGGCAGAG R – TCCTAGTCTTAAAGAGGCAGCAAAC	77 bp	NM_003106.2
NANOG	F – CCTGTGATTTGTGGGCCTG R – GACAGTCTCCGTGTGAGGCAT	77 bp	NM_024865.2
OCT4	F – GTGGAGGAAGCTGACAACAA R – ATTCTCCAGGTTGCCTCTCA	119 bp	NM_002701.3

Results:

Human neonates derived Mesenchymal stromal cells were isolated from UCWJ by explant method and the cultures were studied in early passages. Wharton's Jelly MSCs isolated by enzymatic digestion and explant possess same characteristics. However, explant method has several advantages over enzymatic isolation as there is no proteolytic stress on cells. It also increases the chance of high yield and successful isolation as the piece of tissue origin in the primary culture comes in an association. Furthermore, it is cost effective and reduces the risk of biological contaminations.

***Explant isolation of MSCs and morphology analysis*:**

It has been observed that in an average of 5-7 days, the MSCs start migrating from UCWJ explant (Fig 1D). In optimal culture condition, it is adherent to plastic as monolayer cells and the migrated cells have a heterogeneous cells population (Fig 2A) with prominent nuclei (Fig 2B). By 3rd passage, it becomes more uniformly spindle shape (Fig 2C) with a homogeneous population of MSCs which were confirmed by the ISCT criteria.

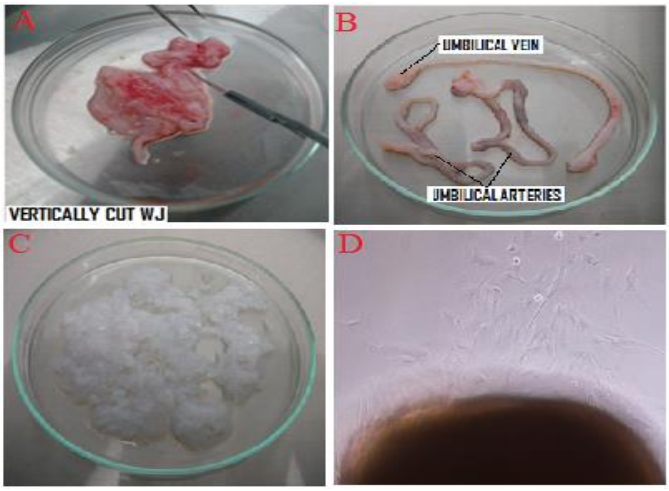

Fig: 1 shows isolation of hUCWJ by ex-plant method

(A) Vertically cut umbilical cord Wharton jelly.
(B) Removed vein and arteries for umbilical cord
(C) Chop umbilical cord.
(D) Cell migration from the ex- plant

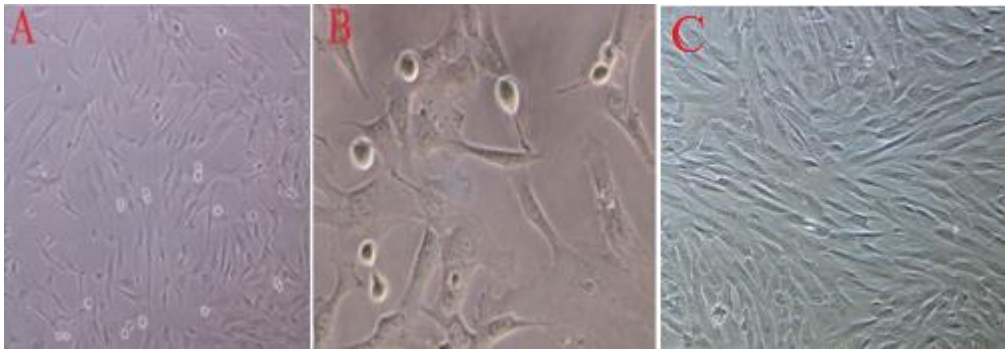

Fig 2 shows

(A) Heterogeneous cells population of hUCWJ explant migrated cells at P0 (10X).
(B) Large round nucleus at P0 of MSCs (40 X).
(C) Spindle/fibroblastoid structures of MSCs at P3 (10X)

***Characterization of MSCs by Immunophenotypic*:**

Explant derived MSCs were analyzed for flowcytometry, at 3rd Passage and it expressed positive markers CD73, CD105, CD90 and CD44 but lacked expression of MSC cocktail (PE CD45, PE CD34, PE CD11b, PE CD19 and PE HLA-DR) for all the cells population (Fig 3).

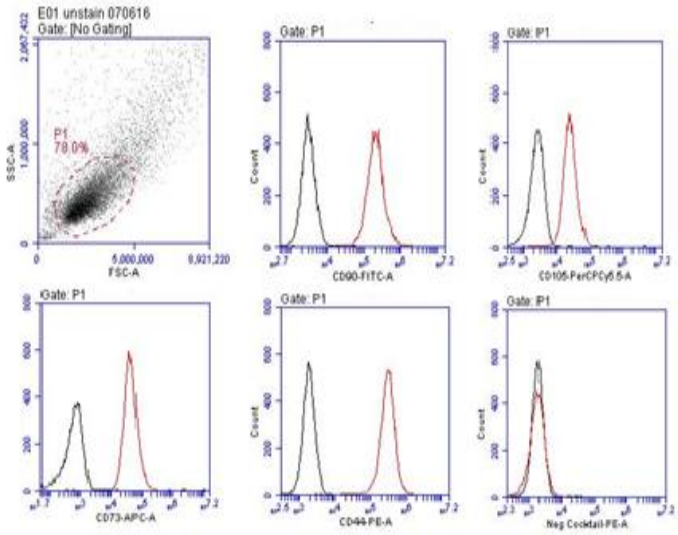

Fig: 3. Expression of the cell surface markers (CD90, CD105, CD73, CD44) and lack expression of negative cocktails (PE CD45, PE CD34, PE CD11b, PE CD19 and PE HLA-DR) (red lines) when compare with the positive and negative iso-type control cocktails (black lines). The plots were derived from gated events based on light scattering characteristics of the MSCs at passage 3. Cells were analyzed using BD Accuri C6 flow cytometer.

***Tri-lineage differentiation of MSCs*:**

Adipogenic differentiation was seen as early as 7 days in adipogenic differentiation medium with the cells showing the presence of lipid vacuoles under the microscope. After 14 days the cells were fixed with 4% formaldehyde and adipogenesis was confirmed by Oil Red O stain (Fig 4A and 4B).

Under chondrogenic differentiating medium, the cells start aggregating within 2-3 days. After 14 days of culture, a spheroid cell mass was formed which was fixed with 4% formaldehyde solution and stain with Alcian Blue. The blue staining indicated the synthesis of proteoglycans by chondrocytes (Fig 4C and 4D).

Osteogenic differentiation was seen from day 10 in osteogenic differentiation medium and the MSCs started showing mineralization under the microscope. The MSCs were further cultured for 21 days and fixed with 4% formaldehyde. Calcium depositions were checked using Alizarin Red S (Fig 4E and 4F) and osteogenic differentiation was confirmed.

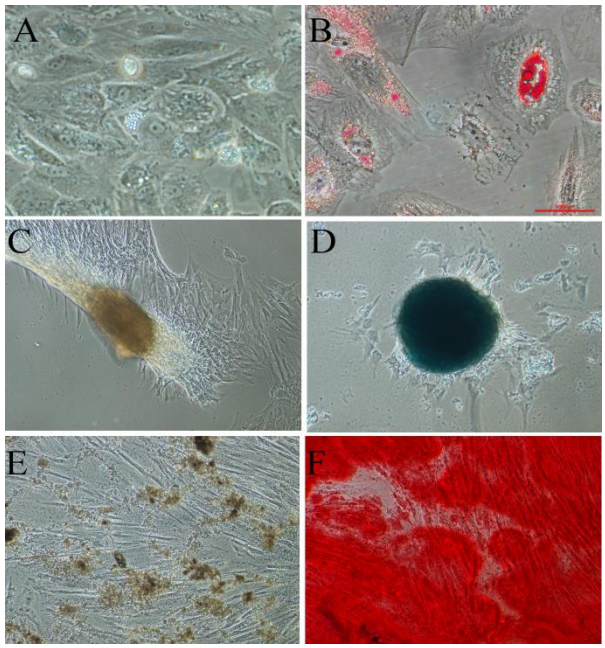

Fig 4: Trilineage differentiation of hUCWJ:

Adipogenic differentiation (4A) unstained lipid droplets (4B) lipids droplet stain confirmed with Oil Red O for adipogenic differentiation. Chondrogenic differentiation. (4C) unstained chondrocytes. (4D) Glycosaminoglycans in cartilages for chondrogenic differentiation confirmed with Alcian Blue staining. Osteogenic differentiation. (4E) unstained osteogenic differentiation (4F) Calcium salts binding in osteogenic differentiation confirmed with Alizarin Red S.

***Reverse transcriptase PCR*:**

Mesenchymal stromal cells isolated from hUCWJ were used to perform RT-PCR for embryonic stem cell and germ layer markers using specific primers (Table 1). The hUCWJ-MSCs expressed the transcriptional factors nestin, TGFβ, GATA4, Oct4, Nanog and SOX2 (Figure 5), thereby confirming the pluripotency. This is shown in Figure 5.

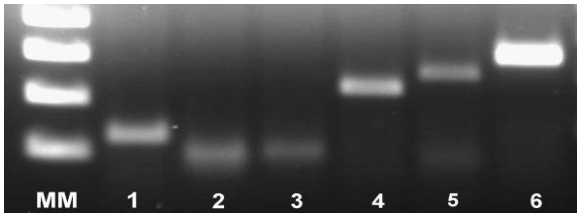

Fig 5: Reverse transcriptase PCR

Analysis of embryonic stem cells markers lane 1-OCT4, 2- NANOG, 3- SOX2 and germ layer markers lane 4- nestin, 5-TGFβ1 and 6-GATA4 in human umbilical cord Wharton's jelly by reverse transcriptase polymerase chain reaction (RT-PCR)

Discussion:

Mesenchymal stromal cells or stem cells are interchangeably used in the literature. Arnold Caplan mentions that even labeling it as Mesenchymal Stromal cell is incorrect but suggested it be named as Medicinal Signaling Cells (MSCs) because of these cells home in on sites of the injury or disease and secrete bioactive factors that are immunomodulatory and trophic[21]. MSCs can be isolated from different adult tissue like bone marrow, adipose, heart, dental pulp etc. and neonatal tissue like the umbilical cord, placenta, amniotic fluid which were the later considered primitive as they are isolated from extra embryonic tissue. Remarkable differences between neonatal and adult MSCs have been

observed by many researchers. The hUCWJ cells are present in relatively high number, with an average of 400,000 cells isolated per umbilical cord[22] which is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow, emphasizing a primary advantage of stem cells harvested from extra embryonic sources[23]. MSCs isolation by explant has more advantages compared to proteolytic digestion because proteolytic stress on cells is excluded and a piece of the tissue origin come in companion which raises the chance of successful and high yield isolation. Migrated cell population at P0 will have heterogeneous cells population but this is limited to primary culture phase only. The unattached cells are constantly removed after subsequent media change and during the first subculture, non-stem adherent cells will also be eliminated due to their confined ability to proliferate and divide[24]. In this study, we tried to observe the expression of embryonic stem cells markers i.e. Oct4, Nanog and SOX2 and expression of germ layer markers i.e. nestin, TGFβ and GATA4 in neonatal derived hUCWJ by explant method. All the cultured cells from the hUCWJ samples show typical fibroblastoid morphology and differentiated into adipogenic, chondrogenic and osteogenic lineages as per criteria[20]. The immunophenotyping of explant derived Wharton's Jelly MSCs expressed more than 99% of CD90, CD73, and CD44 and the expression of CD105 were above 92% of the cell population that was run in the flowcytometer. The cocktail of negative marker CD45, CD34, CD11b, CD19 and HLA-DR were expressed by less the 2% of the total cell population.

Explant derived MSCs were also examined for the expression of germ layer markers where nestin, TGFβ and GATA4 show the expression pattern of ectodermal, mesodermal and endodermal markers respectively. Strong expression of endodermal markers GATA4 was observed in the Wharton jelly explant. However, in adult, MSCs Una Reikstina *et al* was unable to detect the expression of GATA4 in bone marrow, adipose tissue and dermal MSCs but they were able to detect GATA4 in heart MSCs [18]. Limana F *et al* suggest the expression of GATA4 transcription factor regulates the expression of genes involved in myocardial differentiation and function[25].

Many researchers showed inconsistent results in the expression of stem cell markers in adult stem cells. Deepa Bhartiya proposed that MSCs arise from very small embryonic-like stem cells (VSELs) and are multipotent which can give rise to various mesodermal cell types. She questioned the pluripotent and transdifferentiation properties of MSCs. She further added that the minimal transdifferentiation reported may actually be due to the existing subpopulation of VSELs[26]. But in our study, we cultured the MSCs, not the VSELs. We allowed the cells to reach till 3rd passage and thereafter defined them as MSCs as per criteria. We further confirmed the expression of embryonic and germ layer markers. The expression and role of transcriptional factors Oct4, SOX2 and Nanog had been confirmed in adult stem cells leading to many controversial results [18,27] because they are expressed in tumor cells[28]and it has also been proposed that it might be responsible for cancer stem cells resistant to chemotherapy[29]. Furthermore, Pierantozzi *et al*. found Nanog, but not Oct4 and SOX2 is expressed in cultured human adult MSCs[30].

Our study also showed the expression of transcription factors in the explant derived hUCWJ, MSCs by semiquantitative RT-PCR. Una Riekstina *et al* reported the expression of Oct4, Nanog and SOX2 in adipose tissue, dermal and heart MSCs. Detection of Oct4 and Nanog expression in human bone marrow and heart cells is consistent but they were not able to detect SOX2 expression in bone marrow MSCs[18]. Our study also supports the primitive nature of the neonates derives MSCs which are closely related to pluripotency by the expression of embryonic stem cells markers SOX2, Nanog and Oct4 from explant derived hUCWJ MSCs.

MSCs isolated from adult tissue have a significant clinical application but due to the age-related decrease in the growth and differentiation capacity and limited cell numbers lead to the search for alternatives. Explant derived hUCWJ MSCs culture has an advantage of non-invasive collection procedure with limited ethical concern and due to the expression of embryonic stem cells (Oct4, SOX2 and Nanog) and germ layers markers (nestin, TGFβ, GATA4) similar to that of embryonic stem cell make the right candidate for tissue regeneration and therapeutic potentials. The study is cross-sectional in design, therefore genomic and chromosomal test was not in the scope of the study to

determine the health of the neonates as a donor in advance. Another hindering issue is that each harvest gives non-uniformity in numbers and functions of progenitor cells. Further, expression of these transcription factors from other neonatal derived MSCs and its functional role as pluripotent stem cells markers are limited which need to be explored and the therapeutic potential of these cells need to be evaluated.

Compliance with Ethical Standard:

Funding:
We would like to sincerely thank Indian Council of Medical Research (ICMR) for funding our research project (ICMR No: 5/7/1268/2015-RCH).
And we would also like to thank IL Biotech Hub Project NER-BPMC (BT/04/NE/2009), Department of Biotechnology (DBT) Government of India, for its support.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethical approval: Studies involving human participants were accordance with the standard of the Institutional ethical committee (SMIMS/IEC/C/2016-069).

Inform consent: Informed consent was obtained from all participating mothers in the study.

References:

1. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. ExpHematol. 1974; 2: 83–92.
2. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. Methods. 2008; 45: 115–120.
3. Ulrich D, Muralitharan R, Gargett CE. Toward the use of endometrial and menstrual blood mesenchymal stem cells for cell-based therapies. Expert OpinBiolTher. 2013; 13:1387–1400.
4. Roubelakis MG, Pappa KI, Bitsika V, Zagoura D, Vlahou A, Papadaki HA et al. Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. Stem Cells Dev. 2007; 16: 931–952.
5. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. 2004; 22:1330–1337.
6. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood. 2004; 103:1669–1675.
7. Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM et al. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods. 2008; 14:149–56.
8. In 't Anker P, Scherjon S, Kleijburg-van der Keur C, de Groot-Swings G, Claas F, Fibbe W et al. Isolation of Mesenchymal Stem Cells of Fetal or Maternal Origin from Human Placenta. Stem Cells. 2004;22(7):1338-45.
9. Chamberlain G, Fox J, Ashton B, Middleton J. Concise Review: Mesenchymal Stem Cells: Their Phenotype, Differentiation Capacity, Immunological Features, and Potential for Homing. Stem Cells. 2007;25:2739–49
10. Marędziak M, Marycz K, Tomaszewski K, Kornicka K, Henry B. The Influence of Aging on the Regenerative Potential of Human Adipose Derived Mesenchymal Stem Cells. Stem Cells Internat. 2016;2016:1-15.
11. Troyer DL, Weiss ML.Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells. 2008;26:591-9
12. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, ZuckerJPetal.Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122:947-56.

13. Kim J, Jee M, Lee S, Han T, Kim B, Kang K et al. Regulation of Adipose Tissue Stromal Cells Behaviors by Endogenic Oct4 Expression Control. PLoS ONE. 2009;4(9):e7166.
14. Sharov A, Masui S, Sharova L, Piao Y, Aiba K, Matoba R et al. Identification of Pou5f1, Sox2, and Nanog downstream target genes with statistical confidence by applying a novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. BMC Genomics. 2008;9(1):269.
15. Reim G, Brand M. Spiel-ohne-grenzen/pou2 mediates regional competence to respond to Fgf8 during zebrafish early neural development. Development. 2002;129:917–33.
16. Fang X, Yoon J, Li L, Yu W, Shao J, Hua D et al. The SOX2 response program in glioblastoma multiforme: an integrated ChIP-seq, expression microarray, and microRNA analysis. BMC Genom. 2011;12(1).
17. Sun A, Liu CJ, Sun ZQ, Wei Z. NANOG: A promising target for digestive malignant tumors. World J Gastroenterol. 2014;20(36):13071.
18. Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I et al. Embryonic Stem Cell Marker Expression Pattern in Human Mesenchymal Stem Cells Derived from Bone Marrow, Adipose Tissue, Heart and Dermis. Stem Cell Rev. 2009;5(4):378-386.
19. Lengner C, Welstead G, Jaenisch R. The pluripotency regulator Oct4: A role in somatic stem cells?.Cell Cycle. 2008;7(6):725-8.
20. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
21. Caplan AI. Mesenchymal Stem Cells: Time to Change the Name. Stem Cells Transl Med. 2017;6(6):1445-51.
22. Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay G, Demiralp D et al. Biology of Stem Cells in Human Umbilical Cord Stroma: In Situ and In Vitro Surveys. Stem Cells. 2007;25(2):319-31.
23. Marcus A, Woodbury D. Fetal stem cells from extra-embryonic tissues: do not discard. J Cell Mol Med. 2008;12(3):730-42.
24. Hendijani F**.** Explant culture: An advantageous method for isolation of mesenchymal stem cells from human tissues. Cell Prolif. 2017; 50(2): e12334.
25. Limana F, Zacheo A, Mocini D, Mangoni A, Borsellino G, Diamantini A et al. Identification of Myocardial and Vascular Precursor Cells in Human and Mouse Epicardium. Circ Res. 2007;101(12):1255-65.
26. Bhartiya D. Are Mesenchymal cells indeed pluripotent stem cells or just stromal cells? OCT-4 and VSELs biology has led to better understanding. Stem Cells Int. 2013; 2013:547501.
27. Arnold K, Sarkar A, Yram M, Polo J, Bronson R, Sengupta S et al. Sox2+ Adult Stem and Progenitor Cells Are Important for Tissue Regeneration and Survival of Mice. Cell Stem Cell. 2011;9(4):317-29.
28. Cao D. The origin of cancer stem cells. Frontiers in Bioscience. 2012;S4(3):819-30.
29. Malik B. Cancer stems cells and resistance to chemo and radio therapy. Front in Bioscien. 2012;E4(1):2142.
30. Pierantozzi E, Gava B, Manini I, Roviello F, Marotta G, Chiavarelli M et al. Pluripotency Regulators in Human Mesenchymal Stem Cells: Expression of NANOG But Not of OCT-4 and SOX-2. Stem Cells Dev. 2011;20(5):915-23.