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Original Article

Acute toxicity study of Mesenchymal Stromal cells derived from Wharton's Jelly in mouse by intravenous and subcutaneous route

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ABSTRACT:

Aim: The present study was to evaluate the acute toxicity of WJ-MSCs in mouse by intravenous and subcutaneous route and to assess their potential for side effects, MLD, MTD and LD_{50}. **Objectives:** Wide ranges of clinical and preclinical trials have suggested exploitation of adult MSCs for the cell-based reparative therapeutic approach; considering pros and cons of embryonic stem cells. However, for the clinical use existing adult stem cells source such as bone marrow, adipose tissue may be detrimental due to invasiveness in the procedure, less number of initial isolation and unsuitability for allogenic transplants. Recently fetal tissues such as Placenta, WJ have attracted as a good stem cell source due to its easy accessibility, ethical safety, immunological tolerance and large number of initial isolation of homogenous population necessary for increasing current market demand. **Methods:** In present study, we tried to work on complete characterization and up-scaling profiling of cells isolated from WJ, along with assessment of possible toxic effects of these cells when administered *in-vivo* and optimizing the route of administration with other clinical evaluation been addressed. **Results:** We confirmed that cells isolated from WJ exhibit morphologically and phenotypically similar properties as MSCs. The animal study also reveled that no mortality, no abnormal clinical signs and no remarkable pathological changes. **Conclusion:** Our animal toxicity study along with attempted rapid expansion of these cells to meet large clinical demands would allow them to be a lucrative candidate for clinical therapy.

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INTRODUCTION

Since the past decade cell based alternative therapeutic approach has gained considerable interest as a potential and lucrative alternative for variety of deadly diseases worldwide. Accumulated evidences from various clinical trials are supporting the existence of fundamental cells named as "Stem Cells" harbored by some of the post-natal tissues and are capable of multi-lineage differentiation and providing new pathways of alternative treatment options beyond current practices. Previously embryonic stem cells obtained in the blastocyst stage were considered the sole reserve of these pluripotent cells. However, with the recent investigation adult stem cell's universality, ability to manufacture in a bulk and long term cryostorage efficiency present a unique opportunity to produce an "off-the-shelf" hassle free cellular therapeutic source ready for the treatment of diseases in acute settings [1].

Mesenchymal Stromal Cells (MSCs) are more potent due to some of the significant characteristics such as, ability for tissue reparation through direct or paracrine effects, immunomodulation properties and supportive cell engraftment [2]. Hassle free isolation, lack of any ethical issues, expansion, multilineage differentiation capacity are other functional advantages altogether making them more rewarding alternative for stem cells therapy and regenerative medicines. Some of the reviews have also documented effective homing ability of these MSCs to damaged tissues where they will respond to the microenvironment and perform regulatory immune and inflammatory responses at target sites thereby facilitating repair

of damaged tissue [1]. So far bone marrow is the most widely accepted, classic source of MSCs. However, for clinical use bone marrow may not be a good option due to its highly invasive procedure, reported decline in cell number and differentiation potential with the increasing age and limited potency [3].

Collective experimental data suggest that MSCs isolated from extra embryonic perinatal tissues represent an intermediate cell types that partially combine some pluripotent properties of embryonic stem cells and some immunoregulatory properties of adult stem cells. It is observed that because of its closed ontogenetic relationship with embryonic stem cells they exhibit immuno-privileged characteristics due to the presence of Human Leukocyte Antigen G (HLA-G) and lack of class II HLA, possess broader multipotent plasticity, greater expansion capacity and faster doubling time *in-vitro* may be due to their longer telomere than adult MSCs [4].They are also shown to express slightly different cytokine profile than adult MSCs. Moreover, because these tissues are discarded after birth they indulge into minimum ethical issues. In summary, MSCs isolated from fetal tissues is more attractive and alternative source to embryonic as well as post-natal adult tissue due to their unique physiological characteristics and expansion potential which may be associated with their relative naive status.

Functionally human placenta can be seen as the feto-maternal organ serving as an immunological barrier [5-7] whereas umbilical cord (UC) is an elastic cord connecting fetus and placenta during pregnancy protecting enclosed vessels from compression. Literature mentioning about Wharton's jelly (WJ) expressing mesenchymal stromal cell population were first obtained more than twenty years ago [8]. In one regard they meet the minimal criteria for defining adult MSCs like morphology, plastic adherent, self-renew and in vitro multi-lineage differentiation potential [9] and in other regard they possess typical properties of pluripotent embryonic stem cells such as presence of pluripotency markers like OCT-4, presence of HLA-G for immuno-tolerance, good telomere length; based on which these cells can be characterized as amenable, plentiful and inexpensive alternative for therapeutic application[10].

Currently many efforts are being performed for the development of different cell-based systems as test objects for determination of therapeutic effects in pharmacological screening using MSCs. However further study is necessary to determine clinical limitations and realistic clinical protocols. It is unknown till date whether WJ-MSCs engraft in the targeted area for a longer period and whether they display self-renewal as well as multi-lineage potency *in-vivo*. Since the amount of WJ-MSCs needed in the tissue regeneration is very high; scaling up is necessary for the successful clinical application. For this reason, establishing parameters for GMP related expansion and routine clinical application of expanded cells as well as minimum doses required to avoid thrombo-embolic complications and infections are critical aspects to be determined.

We here in the current investigation, have tried to expand Wharton's jelly derived stromal cells at minimum passage level for successful therapeutic application.

We could isolate WJ-MSCs and under controlled culture condition could upscale large number which would be sufficient for clinical application at passage 2 (P2). We carried out different quality analysis to check the efficacy of isolated cells at various stages of expansion. We also carried out toxicity study in rats to determine a Minimum Lethal Dose (MLD) for human use, to get the preliminary identification of target organ toxicity and to check the feasibility of intended route for human administration.

We observed that WJ-MSCs isolated were expanded up to P2 to get as many as $1.3x10^9$ cells from a single cord with no significant change in viability, stemness, karyotyping and sterility. These cells when administered through intravenous and subcutaneous route showed no signs of mortality, toxicity and pathological changes in Swiss albino mice of both the sexes. Minimum Tolerated Dose (MTD) and Minimum lethal dose were estimated to be higher than $10x10^6$ MSCs/kg body weight, which is 10 times higher than the proposed human therapeutic dose. The present study thus will be useful in establishing clinically relevant criteria for isolation characterization, long term cultivation, maintenance of WJ-MSCs in regenerative medicine application.

MATERIALS AND METHODS

Isolation of Mesenchymal stromal cells:

Collection and Transport of Umbilical Cord: Human umbilical cords (n = 5) from both the sexes were collected from full-term births after either cesarean section or normal vaginal delivery with informed consent using guidelines approved by the Institutional Committee for Stem Cell Research (IC-SCR) and Institutional Ethical Committee (IEC) at CelluGen Biotech Pvt. Ltd, formerly Unistem Biosciences Pvt. Ltd, Gurugram, India. Mothers with abnormal medical history were excluded from the study. Maternal blood samples were screened for infectious diseases like HIV 1& 2, HCV antibodies, HBs-Ag, Anti-HBC, HTLV 1&2, CMV-IgM, Malaria and Syphilis as per existing regulatory guidelines.

Maternal blood reactive for any of the infectious diseases was not considered for the study. Approximately 25 to 38 cm of the clamped cord was cut and transferred into a labeled tube containing collection medium with antibiotics. Collection medium used was Dulbecco's modified Eagle medium -Nutrient Mixture Ham's F-12 (1:1) with L-Glutamine (1X); 2.438g/L Sodium Bicarbonate (DMEM/F12; Gibco, USA) and 0.125 mg/ml Cefoperazone sodium (MP Biomedicals, LLC). After collection sample was transferred to the processing facility within 36 hrs. The shipment temperature was maintained at 4–15°C.

Isolation and Primary expansion: Processing length and weight of cord were as per the inclusion criteria. The cord was initially washed with Dulbecco's Phosphate-Buffered saline (DPBS) (HiMedia, India), disinfected with 70% ethanol for 2 minutes and again washed twice with DPBS. The tissue was cut into small pieces, exposed transversely and blood vessels were removed. WJ was picked up and cut into small pieces, in a sterile tube. Approximately 35-40 jelly explants of about

0.5mm size each were plated in tissue-culture-grade T-75 flask (Nunc, Denmark) containing culture medium.

Culture medium used was 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 10% fetal bovine serum (FBS; Gibco, US) supplemented with 3ng/mL bFGF (Sigma, USA), 0.125 mg/ml Cefoperazone sodium (MP Biomedicals, LLC). All flasks were left undisturbed in 5% CO_2 incubator 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 a medium at an interval of 5 days. Cells were harvested and counted at 70–80% confluency using 0.25% trypsin (Gibco, USA)and were re-plated at a density of 4500cells/$cm^2$for Passage 1and 3000 cells/cm^2 were plated at passage 2 in T-175 tissue culture flasks (Nunc, Denmark). All harvested cells were suspended in a cryoprotectant solution composed of 90% complete media and 10% dimethyl sulfoxide (DMSO) (Origen Biomedical, USA) and stored in the vapor phase of liquid nitrogen tank; wherein cells were maintained for 1 year.

In-Process and Release Testing:

Immunophenotyping: Harvested cells from passage 2 were analyzed for immuno phenotypic characterization.1x10^5 Cells were incubated with specific mouse anti-human antibodies conjugated to flurochromes. Cells were stained with antibodies CD90-FITC (2µl), CD73-APC (5µl), CD105-PE (20µl), CD45-FITC (20µl), CD34-PE (20µl), CD79a-APC (20µl) and HLA-DR (5µl). All antibodies were procured from BD Pharmingen, NJ. Cells incubated with identical concentrations of FITC-(5µl), PE- (5µl), PercpCyc.5.5 (5µl), APC-(5µl) conjugated mouse IgG isotype antibodies (BD Pharmingen, NJ) served as isotype controls and cell viability was measured using 7AAD. After incubation for 10 mins at 37℃, cells were acquired by flow cytometer (FACSCalibur, BD Biosciences, USA). Approximately 10,000 events were detected and data analysis was done using the Cell Quest Prosoftware (BD Biosciences, USA).

Tri-lineage Differentiation: Adipogenic differentiation was initiated in a culture medium (DMEM F12/ 10%FBS) supplemented with 200 M indomethacin, 0.5mM 3-isobutyl-1-methylxanthine, 10 g/ml insulin, and 1 M dexamethasone (all reagents from Sigma Aldrich, USA). After 18 days cells were fixed with 4% formaldehyde for 30 minutes. After fixation, cells were rinsed once with DPBS and stained with oil red O for 30 minutes. Hemotoxylin was used as a counterstain. Chondrogenic differentiation was induced in confluent monolayer cultures of WJ-MSCs using chondrogenesis differentiation kit (Millipore, US). After 14 days chondrogenic cultures were fixed with 4% formaldehyde for 30 mins and stained with 1% Alcian blue (Sigma, USA) in 0.1N HCL solution for 30 minutes. Osteogenic differentiation was induced in the DMEM F12/ 10%FBS medium supplemented with 2mM L-glutamine (Millipore, US), 0.1µM dexamethasone, 10mM β-glycerophosphate, 0.2mM ascorbic acid (all reagents from Sigma Aldrich, USA). Mineralized deposits were visualized by alizarin Red S staining after 21 days. Cells were fixed with 4% Formaldehyde (Sigma Aldrich, USA) for 30 min and exposed to 1% silver nitrate (Sigma Aldrich, USA) under bright light for 60 min. Images were captured using CKX41 Olympus inverted microscope (Olympus, Japan).

Endotoxin Assay: Endotoxin levels were determined by the gel clot Limulus Amebocyte Lysate test method (LAL), Endotoxin produces a gelation reaction with amebocytes or circulating blood of *Limulus polyphemus* (American Horseshoe crab). Limulus Amebocyte Lysate was reconstituted with reagent water and mixed in equal parts of the sample (Lonza, USA). After incubation in the presence of endotoxin, gelatinous occurs; but in the absence of endotoxin, gelatinous does not occur. The LAL test requires serial dilutions with neutral pH. LAL is limited to aqueous solutions or extracts of test specimen hence endotoxin testing was performed on the cell supernatant obtained at the time of P2.The lysate sensitivity used in the assay was 0.125EU/ml.

Cell Counts and Viability: Cell numbers were determined by using a hemocytometer and viability was assessed by 0.4% trypan blue dye (Gibco, USA) exclusion method.

Sterility and Mycoplasma Assay: Sterility (aerobic, anaerobic, and fungal) and mycoplasma assays were performed before cryo preservation and during release by random testing of culture supernatants to exclude microbiological contamination. The sample was injected into aerobic and anaerobic BacTAlert bottle (Biomerieux, INC, Derham) and incubated for 7 days. Mycoplasma detection assay was done by Lucetta Luminometer method; the commercially available kit, MycoAlert (Lonza, USA) was used for the detection of mycoplasma in the cell culture supernatant.

Animal study:

Experimental animals: The study design and allotment of animals was approved by Institute for Toxicological Studies (INTOX). In brief, **s**amples of five different batches of MSCs were administered by a single subcutaneous injection to groups of five or three Swiss albino mice per sex at the dose of 10x10^6MSCs/kg body weight which is 10 times the maximum human therapeutic dose. A concurrent vehicle control group of mice was similarly treated with 0.9% sodium chloride injection as diluent. Body weights of all animals were recorded prior to dosing (day 1) and weekly thereafter. They were observed for mortality and signs of toxicity for a period of 14 days post-dosing. All mice were sacrificed at the termination of the study and subjected to a complete necropsy. The study was also performed for intravenous injection in a similar method described.

Administration: The suspension was administered to each mouse by a slow intravenous injection, made in the lateral tail vein and subcutaneous injection made in the flank region as a single bolus. The injections were made using sterile hypodermic syringe and stainless-steel needle (26G). The dosage volume administered to each individual mouse was adjusted according to its body weight recorded on the day of dosing, and was 10ml/kg body weight.

Mortality: The observations were made on the mice from the control and treatment groups. All animals were observed

periodically for deaths during first 4 hours of injection (At an interval of 30 minutes, 1hr, 2hrs, and 4 hrs) and thereafter they were observed once a day for 14 days. Any instances of pre-terminal death were recorded.

Clinical signs: All animals were observed periodically for signs of toxicity during first 4 hours of injection (At an interval of 30 minutes, 1hr, 2hrs, and 4 hrs) and thereafter they were observed once a day for 14 days. The appearance, progress and disappearance of these signs if any were recorded.

Body Weights: Body weights of mice were individually recorded for grouping purpose prior to dosing (day 1) and at weekly intervals thereafter (days 7 and 15). Weight changes and group mean values were computed (over day 1 bodyweights).

Necropsy and Histopathology: At the end of a study (day 15) all surviving animals were weighed and humanely sacrificed by carbon dioxide asphyxiation. All animals in the study were subjected to complete necropsy and the gross pathological changes were recorded, preserved and subjected to microscopic examination.

Statistical analysis: The body weight data of different groups were compared by Bartlett's test for homogeneity; the data were transformed using appropriate transformation, wherever required. The data with homogeneous intra-group variances were subjected to one-way analysis of variance (ANOVA-Snedecor and Cochran, 1980). Dunnett's pair wise comparison (Scheffe, 1953) of means of treated groups with control mean was done individually. The variance was evaluated at 5% level of significance.

Estimation of MLD, MTD and LD50: Where ever possible LD_{50} value with fiducial at 95% confidence level was calculated following the method of Litchfield and Wilcoxon (1949). Minimum lethal dose (MLD) and maximum tolerance dose (MTD) was determined.

RESULTS

Expansion of MSC: Approximately 9.8-14.8 $x10^6$ cells were isolated primarily from the tissue (n=5, range 25-38cm) at the end of Passage 0 after 14-18 days. Cells were trypsinized and were seeded into 4500cells/cm^2 in T-175 flasks.

Once confluency achieved 96.37-133.2$x10^6$ adherent cells were harvested. Adherent cells with fibroblastic morphology could be observed as early as 24h after seeding (Fig. 1). After successive passages with a 1:3 split ratio, 7.4 $x10^8$- 13.4x 10^8 P2 MSCs were harvested in 4-5 days and cryopreserved at the end of first expansion period. Viability of the freshly harvested cells was greater than 95% in all cases. The harvested cells from one UC using current method yields 2.61-3.95 x 10^4cells/cm^2 at P0, 5.53-6.74 x 10^4cells/cm^2 at P1 and 5.24-6.89 x 10^4cells/cm^2 at P2 accordingly.

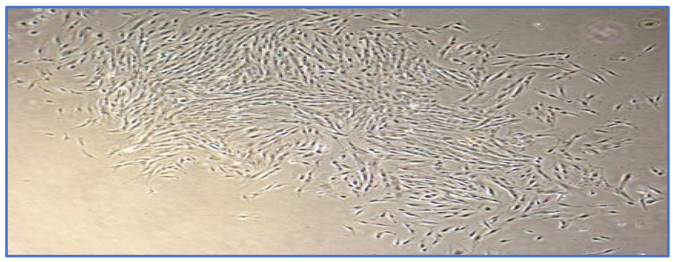
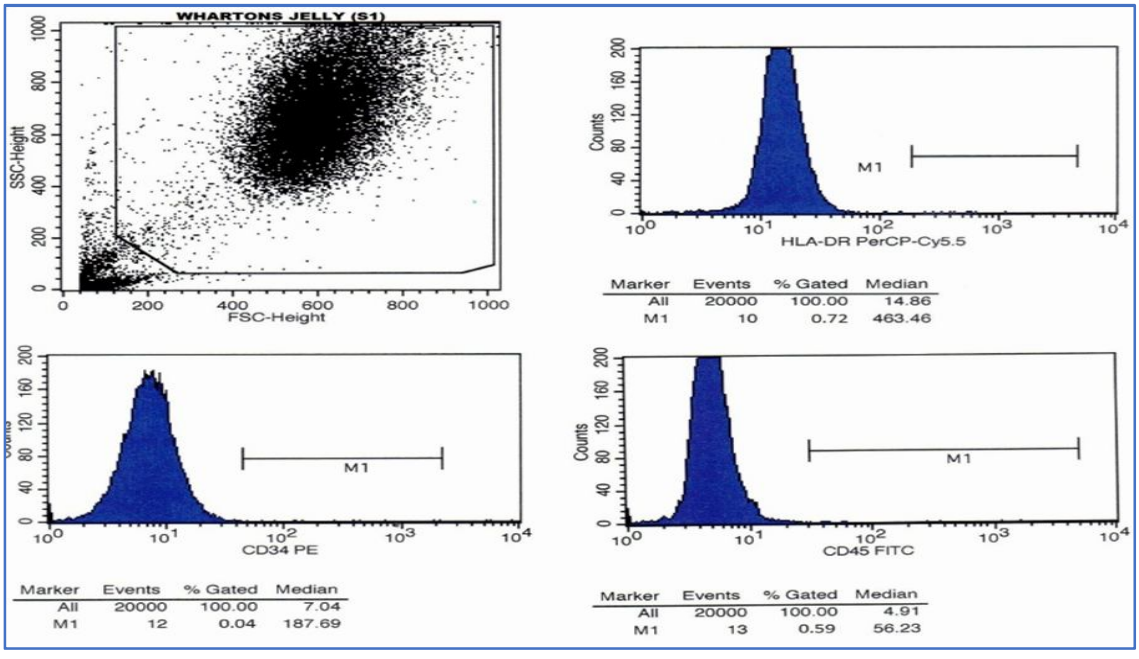


Fig. 1:Morphology of Umbilical cord – Wharton's jelly derived Mesenchymal stem cells from phase-contrast microscope form a monolayer of adherent fibroblat-like cells by day 20. Scale bar = 50µm.

Phenotype and Purity: Flow cytometry studies showed that more than 90% of the WJMSCs displayed uniform mesenchymal stem cell markers such as CD90, CD105 and CD73. WJMSCs were negative for markers such as CD45, CD34 and CD79a (Fig. 2). Expression of HLA-DR antigen was not expressed although cells expressed HLA-ABC antigen. Viability of the cells was determined to be more than 90% by 7-AAD dye exclusion method using flow cytometry.



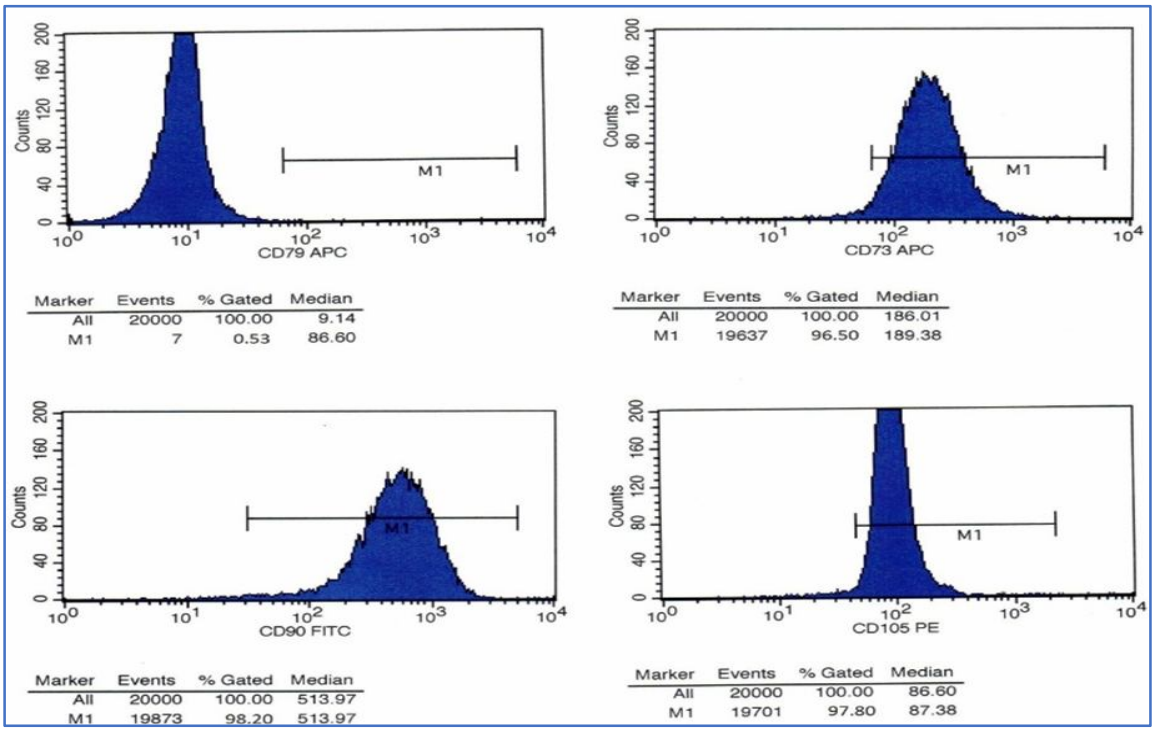


Fig. 2: Immunophenotype of Wharton's jelly derived MSCs. Representative flow cytometry analysis of WJMSCs after expansion when labeled with antibodies against human antigens CD34(PE), HLA-DR(PerCP-Cy5.5), CD45(FITC), CD79(APC) as negative markers and CD105(PE), CD73(APC), CD90(FITC) as mesenchymal specific markers; Color shaded histogram represents positive reactivity with the indicated antibody

Differentiation: The expanded MSCs were subjected to Adipogenic, Osteogenic and Chondrogenic differentiation. Induction of osteogenesis in the expanded MSCs resulted in extracellular matrix mineralization which was confirmed by Alizarin Red staining. Adipogenic differentiation was confirmed when accumulated lipid vacuoles stained positive with oil red O staining. Positive staining of collagen fibers with alcian blue indicated that MSCs were successfully differentiated to chondrocytes (Fig. 3).No changes were observed in undifferentiated or control cells.

Microbiological tests: Cultures were observed to be sterile and free of any aerobic, anaerobic and fungal contamination when checked periodically with the supernatants. Endotoxin levels of final products were lower than 0.2EU/ml.

Cell count in the dosing formulations:MSCs were processed to prepare dosing suspensions for each batch of the test article with final target viable cell concentrations of $1x10^6$ MSCs/ml, with an allowable variation of ±20% (I.e. between 0.8-$1.2x10^6$viable MSCs/ml). These dosing suspensions, when subjected to verification of their viable cell concentrations indicated that viable counts of MSCs for each batch of the test article was found to be within the desired range and the acceptable criteria.

Mortality and Clinical signs: Frequent clinical examinations at an interval of 30mins, 1hr, 2hrsand 4hrs after intravenous/ subcutaneous administration on the day of dosing and daily thereafter till 14th day not revealed any incidence of abnormal clinical signs or preclinical deaths among treated mice as per Table 1 dosing.

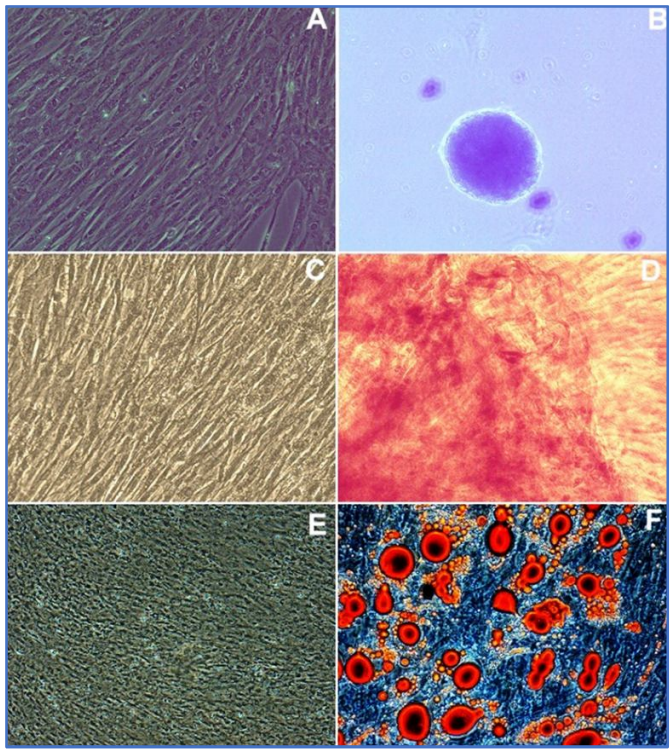


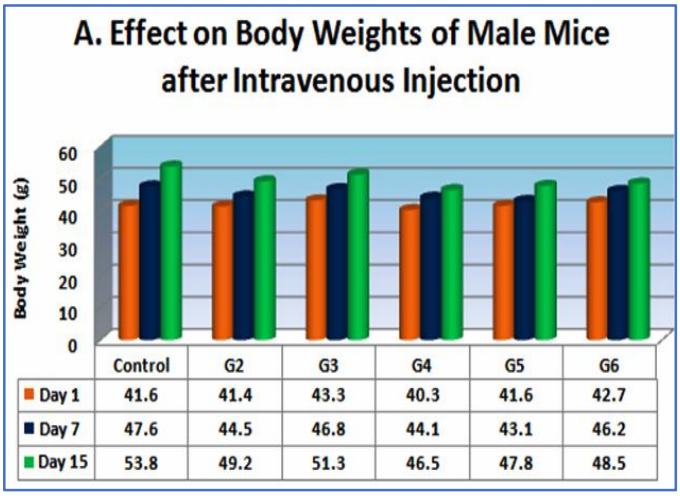
Fig. 3: Tri-lineage differentiation of WJ-MSCs. WJ-MSCs derived after expansion were induced to differentiate along (B) Chondrogenic proteoglucans formation(alcian blue), (D) osteogenic (alizarin red S) lineages, (F) adipogenic (oil red O staining). (A,C,E) corresponding uninduced control cultures were stained for comparison. Scale bar = 50µm

Table 1: Summary of Mortality

Group	Dose (10x10□ MSCs/kg body weight)	Incidence of Mortality					
		Male		Female		Male & Female (Pooled)	
		Absolute	%	Absolute	%	Absolute	%
G1(Vehicle control)	10 ml/kg Sodium Chloride Inj.	0/5	0	0/5	0	0/10	0
G2	10	0/5	0	0/5	0	0/10	0
G3	10	0/3	0	0/6	0	0/6	0
G4	10	0/3	0	0/3	0	0/6	0
G5	10	0/3	0	0/3	0	0/6	0
G6	10	0/3	0	0/3	0	0/6	0

Absolute mortality is presented as number of animals died/numbers treated.

Body Weight: No adverse effects of treatments were observed on body weights of the treated mice during 14 days of observation period of the study. As evident from a graph; body weights recorded prior to dosing (day 1) and at weekly intervals thereafter (days 7 and 15) of all male and female mice treated with MSCs at 10x10□MSCs/kg body weight, continued to gain weight till they were sacrificed on day 15 (Fig. 4 and Fig. 5).



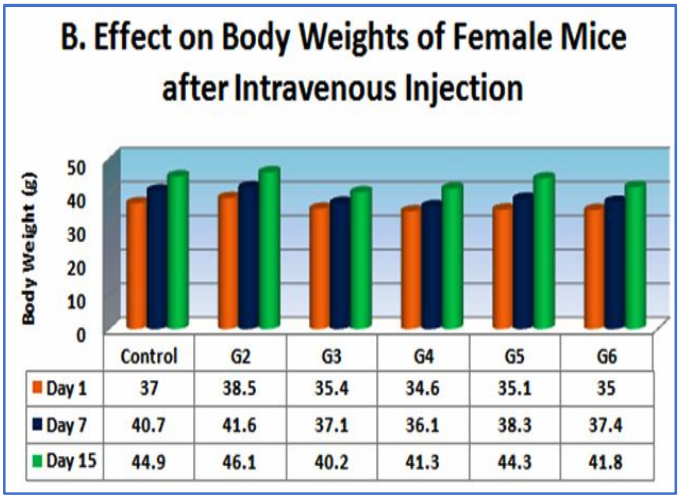
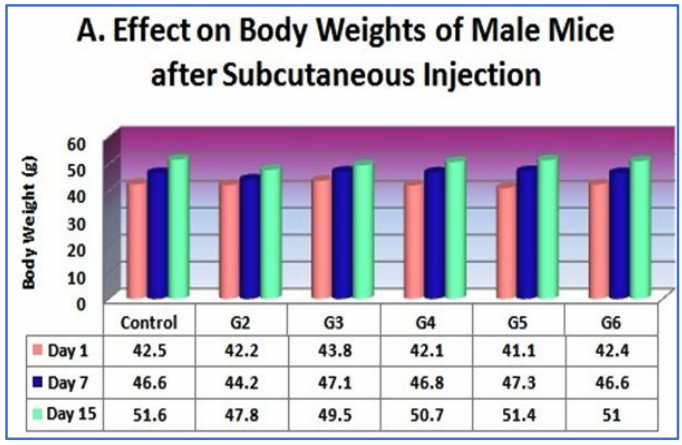


Fig.4: Graphical representation of average body weights of (A) male mice and (B) female mice after intravenous MSCs injection showing continuous weight gain from day 1 to day 15



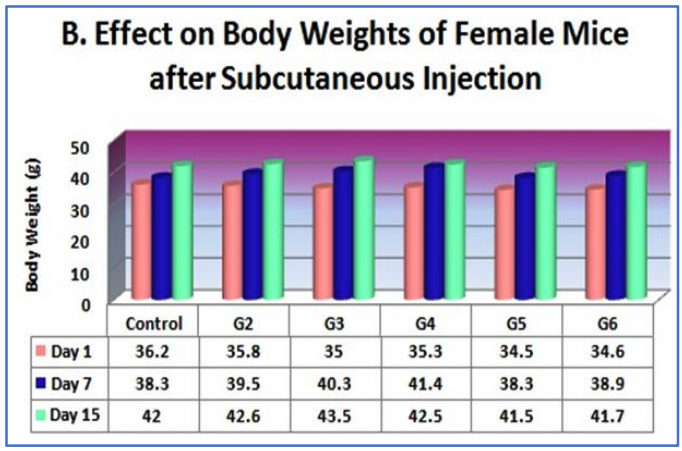


Fig. 5: Graphical representation of average body weights of (A) male mice and (B) female mice after subcutaneous MSCs injection showing continuous weight gain from day 1 to day 15.

***Statistical Analysis*:** After intravenous injection; values of body weights and body weight changes of the treatment group mice did not differ significantly from those of the control group (P>0.05), except for a few values (Table 2) which were found to be slightly lowered (but statistically significant) than in control group. However, this apparent lowering was of small magnitude and of little biological significance since it was comparable to the normal pattern of the body weight gain by mice. The statistical finding was more likely to be incidental considering the small sample size (three) per sex.

Table 2: Summary of male and female mice body weights after intravenous injection

Intravenous mode	Body Weights (g)		Male mice					Female mice				
	Groups	Observation	Day 1	Day 7	Change (1-7)	Day 15	Change (1-15)	Day 1	Day 7	Change (1-7)	Day 15	Change (1-15)
Control (10 ml saline/kg)	G1	Mean	41.6	47.6	6	53.8	12.2	37	40.7	3.7	44.9	7.9
		± S. D.	2.1	1.9	3.1	3.4	5.1	1.4	2.8	2.5	3	2.7
		n	5	5	5	5	5	5	5	5	5	5
MSCs Treated mice (Test group) 10 x 10□ MSCs/kg	G2	Mean	41.4	44.5	3	49.2	7.8	38.5	41.6	3.1	46.1	7.6
		± S. D.	2.3	2.9	1.1	3.9	2	1.3	1	1.9	2.6	3.7
		n	5	5	5	5	5	5	5	5	5	5
	G3	Mean	43.3	46.8	3.4	51.3	8	35.4	37.1^{S-}	1.7	40.2	4.8
		± S. D.	1.2	2.2	1.2	3.4	2.2	0.6	0.7	0.6	1.6	1
		n	3	3	3	3	3	3	3	3	3	3
	G4	Mean	40.3	44.1	3.8	46.5^{S-}	6.3	34.6	36.1	1.5	41.3	6.7
		± S. D.	0.2	1	0.8	0.4	0.5	2	2.4	1.5	2.7	1.3
		n	3	3	3	3	3	3	3	3	3	3
	G5	Mean	41.6	43.1^{S-}	1.5	47.8	6.1	35.1	38.3	3.2	44.3	9.2
		± S. D.	1.2	0.3	1.1	2.2	1.1	1.7	2.9	1.7	3.2	2.5
		n	3	3	3	3	3	3	3	3	3	3
	G6	Mean	42.7	46.2	3.5	48.5	5.8^{S-}	35	37.4	2.4	41.8	6.8
		± S. D.	1.1	0.6	0.7	1.7	1.7	2.2	3.2	1	2.2	0.1
		n	3	3	3	3	3	3	3	3	3	3

S-: Values significantly lower than those of control group, with P < 0.05

After subcutaneous injection; values of the body weights and body weight changes of the treatment group mice did not differ significantly from those of the control group (P>0.05), (Table 3)except in groups G3, G4 and G6 of female mice where the body weight gain in first week was found to be higher than in control group. However, this observation could be a result of difference in stocking density (five in a cage in G1 v/s three in a cage for these treatment groups) and has no toxicological significance.

Table 3: Summary of male and female mice body weights after subcutaneous injection

Subcutaneous mode	Body Weights (g)		Male mice					Female mice				
	Groups	Observation	Day 1	Day 7	Change (1-7)	Day 15	Change (1-15)	Day 1	Day 7	Change (1-7)	Day 15	Change (1-15)
Control (10 ml saline/kg)	G1	Mean	42.5	46.6	4.1	51.6	9.1	36.2	38.3	2.1	42	5.8
		± S. D.	1.4	1	1	1.1	1.5	1.1	1.2	0.4	1.6	0.7
		n	5	5	5	5	5	5	5	5	5	5
MSCs Treated mice (Test group) 10 x 10□ MSCs/kg	G2	Mean	42.2	44.2	2	47.8	5.6	35.8	39.5	3.8	42.6	6.8
		± S. D.	1.6	2.5	1.8	3.3	3.3	0.6	1.2	1	2.5	2.3
		n	5	5	5	5	5	5	5	5	5	5
	G3	Mean	43.8	47.1	3.4	49.5	5.7	35	40.3	5.3 $^{S+}$	43.5	8.5
		± S. D.	2.2	0.6	1.7	0.6	2.7	1.5	1.9	0.5	3.2	1.7
		n	3	3	3	3	3	3	3	3	3	3
	G4	Mean	42.1	46.8	4.7	50.7	8.6	35.3	41.4	6.1 $^{S+}$	42.5	7.2
		± S. D.	2.1	2.4	1	3.3	1.8	0.8	2.7	2	3.7	2.9
		n	3	3	3	3	3	3	3	3	3	3
	G5	Mean	41.1	47.3	6.2	51.4	10.3	34.5	38.3	3.8	41.5	7
		± S. D.	0.8	0.8	0.3	1.4	0.6	1.7	2.1	0.5	2.9	1.3
		n	3	3	3	3	3	3	3	3	3	3
	G6	Mean	42.4	46.6	4.1	51	8.6	34.6	38.9	4.3 $^{S+}$	41.7	7.1
		± S. D.	2.5	2.6	0.6	2	0.6	0.3	0.1	0.3	0.4	0.5
		n	3	3	3	3	3	3	3	3	3	3

Values of treatment group mice do not differ significantly from those of the control group at 5% level significance.S+: values significantly higher than those of control group, with P < 0.05

Necropsy and Histopathology: MSCs from human umbilical cord tissue was not induced any remarkable gross pathological changes in tissue/ organs of the treated mice. When sacrificed and subjected to necropsy examination at termination of the study for intravenous injection on day 15, liver of three male mice from group G2 and one male mouse from G3 revealed slight/mild pallor. When subjected to microscopic examination, these livers revealed a mild cytoplasmic rarefaction. In absence of any other observation in this study which could be correlated to this finding, and comparability of this finding to the historical control data these observations were considered to be incidental and not related to treatment with the MSCs. There was no incidence of any other abnormal findings in this study in male and female mice treated with the MSCs. Also in the case of subcutaneous injection MSCs was not induced any gross pathological changes in tissue/ organs of the treated mice. There was no incidence of any abnormal findings in the study when the mice were sacrificed and subjected to necropsy examination at termination of the study on day 15.

DISCUSSION

Cell based therapeutic approach with the help of stem or progenitor cells has so far hold a huge potential for the treatment of vast array of degenerative and age-related diseases. Despite of its efficiency; the success of this medicinal approach is being challenged by many obstacles that must be addressed for exploitation of their clinical use. Being an experimental procedure current market demand is to obtain a source of stem cells which is unique, easily accessible, and ethically non-controversial can be manipulated to fulfill all criteria of cell based transplants. In this regard since UC is discarded after birth as a medical waste it can be technically as well as ethically good source of Mesenchymal stromal cells [11-13].

Current study has proved the contention that cells isolated from umbilical cord matrix are primitive stromal cells which are plastic adherent, can be differentiated to chondrocytes, adipocytes and osteocytes thus confirming their mesenchymal origin, showing similar phenotype as MSCs when analyzed by flowcytometry; and immunologically accepted when studied for acute toxicity in mouse model. In the study, we could successfully isolate mesenchymal stromal cells with fibroblast morphology. With an initial seeding density of 4500 cells/cm^2 we could expand these cells to a clinical quantity of $13.4x10^8$ cells within 30 days. Apart from these we also observed that this source allowed rapid initial isolation of large number of cells avoiding the necessity of extensive multiplication and potential epigenetic changes [14-16].

Though efficiency of up-scaled clinical grade mesenchymal stromal cells is well understood from accumulated clinical and previous published data [14-16], it is still very crucial to understand whether these cells are functionally important in homing capacities and to optimize the best route of administration depending upon patient's pathophysiology. To address the complications, an animal study was designed to determine clinical toxicity associated with the therapeutical product and suitable route of administration. Five group of mice of each sex were administrated the test article as a single dose.

The route of administration of MSCs to mice was subcutaneous and intravenous injection, since these routes ensures systemic dissemination and could be one of the therapeutic routes. Animals were observed for the incidence of mortality and signs of toxicity for 14 days. The period was not extended in absence of any observed toxic reaction. The study was conducted at the limit dose of $10x10^6$ MSCs/kg body weights, since this dose was considered to provide an adequate safety margin in the light of the intended therapeutic usage. The anticipated therapeutic dose of MSCs is $1x10^6$ MSCs/kg body weight [17-23].

The test dose was ten times the therapeutic dose anticipated for use with human patients. Since this limit of $10x10^6$ MSCs/kg weight did not induce any health issues among the treated mice, higher dose levels were not tested. The minimum lethal dose, the maximum tolerated dose and the median lethal dose of MSCs from UC tissue after acute intravenous and subcutaneous administration in mice could not be determined in absence of any adverse effects or mortality observed among the treated mice in this study. However, these numerical measures, viz. the MLD, the MTD and the LD_{50} of acute intravenous and subcutaneous toxicity of MSCs from human umbilical cord tissue in mice, were estimated to be more than 10x10 MSCs/kg body weight.

CONCLUSION

In the study, cells isolated from WJ resemble fibroblast like cells, exhibit mesenchymal properties such as plastic adherence, tri-lineage differentiation, expressing phenotypically similar markers and can be up scaled for clinical exploitation. For the acute toxicity assessment when MSCs are injected to Swiss albino mice at the dose of $10x10^6$ MSCs/kg body weight intravenously and subcutaneously, the recorded observation shows that injected MSCs not induced any mortality, abnormal clinical signs, adverse effect on the body weights, gross pathological changes in tissue/ organs by the treated mice during the observation period of the study. However, to determine long term engraftment and homing capability inside the host more work is needed in the similar direction.

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