

Acute Toxicity Evaluation and Safety Study of Allogeneic Wharton's Jelly-Derived Mesenchymal Stromal Cells Therapy in the Animal Model

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Abstract: *In-vitro* and *in-vivo* investigations using ex vivo-expanded human Wharton's jelly-derived mesenchymal stromal cells (WJMSCs) isolated from single donors has shown therapeutic advantages. Clinical grade expansion, safety, and acute toxicity of Wharton's jelly-derived mesenchymal stromal cells isolated from five healthy donors were evaluated in Wistar Albino Rats via intravenous and subcutaneous routes in this study. The expression of several cell surface markers, differentiation capacity, and immunomodulatory activities of the isolated WJMSCs were all evaluated. In a series of preclinical investigations, the clinical grade expanded cells were given to rodents by intravenous and subcutaneous methods to examine their *in-vivo* safety, overall toxicity, and tumorigenic potential. We confirmed that WJ cells exhibit morphological and phenotypic features that are comparable to MSCs. In rats, the study found no mortality, aberrant clinical symptoms, or significant pathological alterations at varied dosages administered intravenously or subcutaneously. Furthermore, rats tolerated repeated treatment of WJMSCs well, with no evidence of prenatal harm in the same animal species. The WJMSCs were shown to be non-toxic, non-teratogenic, and non-tumorigenic in Wistar Albino Rats. More research is needed to see if it can be safely supplied to human patients.

Keywords: Wharton's Jelly, Umbilical Cord Mesenchymal Stromal Cells, Intravenous, Subcutaneous, Safety, Toxicity

1. Introduction

MSCs (mesenchymal stromal cells) have been touted as a potential regenerative therapy tool. Because of their ability to proliferate and migrate, as well as their multilineage differentiation into osteoblasts, chondroblasts, and adipocytes, MSCs have gotten a lot of attention. Recent research has found that MSCs play a critical role in controlling practically all stages of inflammation and tissue regeneration, as evidenced by *in vitro* experiments, animal model studies, and clinical trials [1]. Furthermore, WJ-MSCs are a reliable alternative to MSCs since they are a globally available tissue with no ethical concerns because they are normally thrown as human waste [2]. As a result, Wharton's jelly mesenchymal stem cells have emerged as a viable

alternative source of MSCs for tissue repair and cell treatment in the field of regenerative medicine.

The stability of MSCs in large-scale manufacturing to meet clinical demand is one of the safety difficulties [3, 4]. Pal et al. [5] investigated upscaling of MSC production in several culture media up to 25 passages in 2009 and discovered minute differences in characterization, population doubling time, and differentiation potential at every 5th passage. Variations in MSCs from different sources are another challenge of standardizing. When compared to BM-MSC, the WJ-MSC was discovered to exhibit a distinct predilection for age-independent proliferation [6, 7]. After revival, the release of growth factors, differentiation potential, and surface indicators was modest to non-existent after long-term preservation of these cells using biobanking

[8, 9]. Cooper and Viswanathan (2011) found that the recovered Wharton's Jelly UC-MSC remained alive after three years of cryopreservation [10]. From the beginning of the culture, all five cord MSC maintained cell viability over 95% and consistently expressed CD73 and CD105 markers at a rate of 98 percent. The UC-MSC injected in their clinical study was shown to be safe and effective in correcting perfusion abnormalities in patients following coronary bypass surgery [11].

Although years of research demonstrate MSC-based treatment's low immunogenicity and malignancy, incidences of adverse reactions have been described [4, 12-14]. MSCs lack the MHC-I surface protein [15], allowing them to move safely to the site of inflammation. The subsequent differentiation and production of MHC-I and MHC-II surface proteins, on the other hand, might contribute to the extinction of these cells [14]. In this study, we attempted to increase Wharton's jelly-derived stromal cells to the minimal passage level required for effective therapeutic use. We were able to isolate WJ-MSCs and upscale a considerable number of them under controlled culture conditions, which would be suitable for clinical use at passage 2. (P2). To assess the effectiveness of separated cells at various phases of growth, we performed numerous quality analyses. We also conducted rat toxicity research to calculate a Minimum Lethal Dose (MLD) for human usage, to acquire a preliminary identification of target organ toxicity, and to see if the chosen route for human administration was feasible.

We found that WJ-MSCs could be grown up to P2 to yield as many as 4.74×10^9 cells from a single cord without affecting viability, stemness, karyotyping, or sterility. When these cells were given to Swiss albino mice of both sexes via intravenous and subcutaneous routes, they exhibited no symptoms of mortality, toxicity, or pathological alterations. The Minimum Tolerated Dosage (MTD) and Minimum Lethal Dose (MLD) were calculated to be greater than 10×10^6 MSCs/kg body weight, which is ten times the suggested human therapeutic dose. As a result, the current work will be valuable in defining clinically relevant criteria for WJ-MSC separation, characterization, long-term culture, and maintenance in regenerative medicine.

2. Materials and Methods

2.1. Research and Animal Ethics

All chemicals were of analytical reagent grade and used without further purification. The Umbilical cord tissue (n = 5) irrespective of the sex of the baby was collected from full-term births after the cesarean section was obtained from the C-section delivery process with the donor consent form and institutional guidelines. The experimental study was approved by the Institutional Animal Ethics Committee of Nandha Pharmacy, Erode (NCP/IAEC/2020-21/39), and performed under the conditions recommended by the 'Committee for the Purpose of Control and Supervision of Experiments on Animals (688/PO/Rs/S/02/CPCSEA).

2.2. In-vitro Studies and Characterization

2.2.1. Stem Cells Culture

The human umbilical cord was retrieved after cesarean section with informed consent. Explant culture was used to extract WJ-MSCs from the umbilical cord, as stated in the previous article. Briefly, 35-40 jelly explants with a diameter of 0.5 mm were transferred into a tissue-culture-grade T-75 flask containing a culture medium (Nunc, Denmark). Non-adherent cells were removed and a new medium was added after incubation at 37°C for 3-5 days. MSCs were cultured and expanded in Dulbecco's modified Eagle medium-Nutrient mixture Ham's F-12 (1:1) with Glutamax (1X); 2.438 g/L Sodium Bicarbonate; Sodium Pyruvate (DMEM/F12+; Gibco, USA) with 10% PLTMax Human Platelet Lysate (SCM141, Merck) supplemented with 2 ng/mL basic fibroblast growth factor in this study (bFGF; Sigma-Aldrich, UK). The cells were kept at 37°C in a 5% CO₂ incubator (Thermo Scientific, USA), with the media replaced every five days. Cells were dissociated with TrypLE Express (Gibco, USA) after achieving 70-80% confluency and passaged 1:3 onto new T175/ Hyper culture flasks.

2.2.2. Characterization of Stem Cells

WJ-MSCs from all passages were used in all of the investigations. Cells were flow cytometrically analyzed for surface antigen expression (FACSCalibur, BD Biosciences, USA) and data were analyzed using the CellQuest Pro software (BD Biosciences, USA) [15]. CD90-FITC, CD73-APC, and CD105-PE (BD Pharmingen, USA) were used to determine cells that were positive for cell surface markers and were negative for CD45-FITC, CD34-PE, CD79a-APC, and HLA-DR (BD Pharmingen, USA). As per our earlier research [15, 16], mesodermal differentiation was investigated at the capacity of osteogenic, chondrogenic, and adipogenic lineages to differentiate utilizing distinct culture conditions. The tests are carried out according to the International Society for Cellular Therapy (ISCT) standards for defining human MSCs [17].

2.2.3. Cell Proliferation Assay

Based on the total CPDL, the WJMSCs' estimated growth efficiency and proliferation potential were calculated using the formula: Where Ni is the initial number of cells sown, Nf is the final number of harvested cells, and ln is the natural log, $CPDL = \ln(Nf/Ni) \ln 2$. Cells were plated in culture flasks at a density of 3000 cells per cm² and subcultured for 4-5 days. The cells were then counted and replated at a density of 3000 cells/cm². To compute the CPDL, the population doubling levels for each passage were calculated and then added to the preceding passages' population doubling levels.

2.2.4. Colony-Forming Unit (CFU) Assay

About 100 MSCs were seeded in duplicate on a 100 mm² cell culture dish in DMEM/F12+ supplemented with 10% FBS and cultured for 14 days. A fresh complete medium was used to replace the cultures every three days. For ten minutes,

the plates were tinted with crystal violet. The cells were washed twice with distilled water, and all visible colonies were enumerated before photos were taken using an Olympus CKX41 Microscope (Japan).

2.2.5. Sterility and Mycoplasma Assay

Before cryopreservation, sterility (aerobic, anaerobic, and fungal) and mycoplasma tests were done, as well as random testing of culture supernatants the following release to rule out microbiological contamination. The sample was injected into a BacTAlert bottle (Biomérieux, INC, Derham) and incubated for 7 days. The Lucetta Luminometer technique was utilized to detect mycoplasma in the cell culture supernatant, and the commercially available kit MycoAlert (Lonza, USA) was employed to accomplish so. The cell supernatant collected at the time of P2 was tested for endotoxins using an endotoxin assay. The sensitivity of the lysate utilized in the experiment was 0.125EU/ml.

2.3. In-vivo Studies

2.3.1. Experimental Animals

Institutional Animal Ethics Committee (IAEC), institute for toxicological investigations, endorsed the study design and animal allocation (Nandha Pharmacy). In a nutshell, samples of five distinct batches of MSCs were given to groups of five or three Wistar albino rats each sex through a single subcutaneous injection at a dosage of 10×10^6 MSCs/kg body weight, which is 10 times the maximal human therapeutic dose. A 0.9% NaCl injection was used as a diluent in a contemporaneous vehicle control group of rats. All animals' body weights were measured before treatment (day 1) and regularly afterward. For 14 days after the dose, they were monitored for death and indications of toxicity. At the completion of the study, all rats were euthanized and exposed to a thorough necropsy. A similar approach was used to conduct the trial for intravenous injection. All of the tests were carried out in accordance with CPCSEA criteria.

2.3.2. Administration

The dispersion was injected into each rat as a single bolus by slow intravenous injection in the lateral tail vein and subcutaneous injection in the flank area. A sterile hypodermic syringe and a stainless steel needle were used to administer the injections (26G). The dose amount provided to each individual rat was 10ml/kg body weight and was modified according to its body weight measured on the day of treatment.

2.3.3. Mortality

The experiments were conducted on rats from both the cases and controls. During the first four hours following injection, all animals were monitored for mortality (at intervals of 30 minutes, one hour, two hours, and four hours), and then once a day for the next 21 days. Any cases of death that occurred before the end of existence were documented.

2.3.4. Clinical Signs

During the first four hours following injection, all animals

were monitored for indications of toxicity (at intervals of 30 minutes, one hour, two hours, and four hours), and then once a day for the next 21 days. These symptoms' presence, progression, and disappearance, if any, were documented.

2.3.5. Body Weights

Before treatment (day 1), and at weekly intervals afterward, rat body weights were separately recorded for grouping purposes (days 7, 14 and 21). Changes in weight and group mean values were calculated (over day 1 bodyweights).

2.3.6. Necropsy and Histopathology

At the end of the 22nd day, 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.

2.3.7. Safety Study

In the safety investigation, both genders of Wistar albino rats (180-200 gm) were examined. The vehicle (1ml saline/kg) was delivered intravenously to Group 1 as a control. MSCs were given alone from Group 2 to Group 5. To Group 2 and Group 3, the complete test substance was also injected intravenously once (1×10^7 cells/head/body kg weight). The rats were monitored for 13 weeks after injection, daily for clinical symptoms, and twice weekly for tumors. If a tumor founds or, grows to a pre-determined size or by the end of the limited period, the rat is euthanized and the xenograft removed for further examination. Animals were starved overnight 13 weeks following inoculation, and blood samples were obtained through sinus puncture for hematological examination under Ketamine (50mg/kg, i.p.) anesthesia. The suspension was given to each rat in Groups 4 and 5 through a slow subcutaneous injection in the flank area as a single bolus. A sterilized hypodermic syringe and a stainless-steel needle were used to administer the injections (26G). The dose amount supplied to each individual rat was 10ml/kg body weight and was modified according to its body weight reported on the day of treatment.

2.3.8. Statistical Analysis

The homogeneity of the bodyweight data of different groups was tested using Bartlett's test, and the data were converted using suitable transformations where necessary. One-way analysis of variance was used on the data with homogenous intra-group variances (ANOVA-Snedecor and Cochran, 1980). Dunnett's pairwise comparison of treatment group means with control group means (Scheffe, 1953) was done individually. At a 5% threshold of significance, the variance was assessed.

2.3.9. Estimation of MLD, MTD, and LD50

Wherever possible, the Litchfield and Wilcoxon technique was used to determine the LD₅₀ value with fiducial at a 95% confidence level (1949). It was determined what the minimum lethal dosage (MLD) and maximum tolerated dose (MTD) were.

3. Results

3.1. Expansion of MSCs

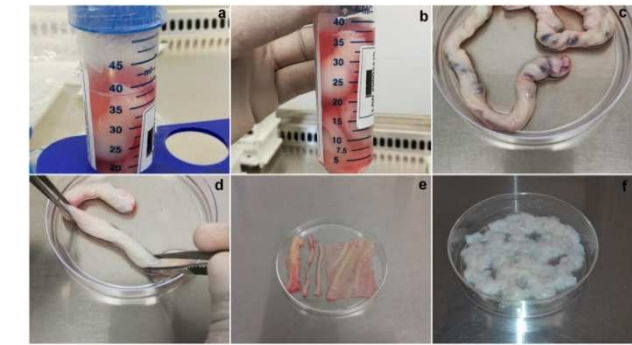


Figure 1. Harvesting of Wharton’s jelly: (a). Collection of Umbilical cord and processed within 24 hours, (b). An umbilical cord was transferred under Biosafety cabinet for processing by blunt dissection, (c). Analysis of the primary test parameters of umbilical cord tissue for further process (Before Washing), (d). Processed cord tissue and separated blood clots, epithelial layers (e). The blood vessels are removed from a cord and (f). Wharton’s jelly was picked up and cut into small pieces.

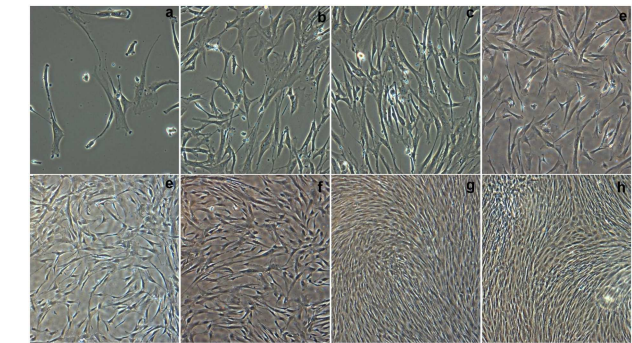


Figure 2. Morphology of Wharton’s jelly MSCs from Phase-Contrast Microscope: Morphology of WJ-MSCs of one representative donor at passage 2 cultured at different days of interval: WJ-MSCs form a monolayer of adherent fibroblast-like cells by (a to h): 18 hours, 24 hours, 36 hours, 48 hours, 54 hours, 60 hours, 72 hours, and after 78 hours at Scale bar of 10x respectively.

The growth of the cells was observed for the first 24 hours, at which time the cells were mostly round and oval in shape. After 24 hours of incubation, most of the cells were adherent to the flask and became spindle in shape. Approximately 10.9 to 14.7 x10⁶ cells were isolated primarily from the Wharton’s jelly (n=5) at the end of Passage 0 after 15 to 17 days. Adherent cells with fibroblastic morphology could be observed as early as 24h after seeding. Cells were trypsinized and were seeded into 3000cells/cm² in T-175 flasks. Once confluency was achieved 1.4x10⁸-2.6 x10⁸ adherent cells were harvested in 5 to 6 days. After successive passages with a 1:3 split ratio, 1.95 x10⁹- 4.74 x 10⁹ P(2) MSCs were harvested in 7 to 9 days and cryopreserved at the end of the first expansion period. The viability of the freshly harvested cells was greater than 90% in all cases. The harvested cells from isolated Wharton’s jelly from one cord using current method yields 1.82-2.45 x 10⁴cells/cm² at P(0), 3.77-5.43 x 10⁴ cells/cm² at P1, 4.19-5.47 x 10⁴cells/cm² at P(2) and 4.06-5.71 x 10⁴cells/cm² at P(10) accordingly. Figure 1

shows the harvesting and clinical scale expansion of Wharton’s Jelly derived mesenchymal stem cells. The growth of the cells (Figure 2) after different hours incubation, at which time the cells were mostly fibroblast-like cells in shape; (WJ1-WJ5) of the Wharton’s jelly samples respectively.

3.2. Phenotype and Purity

Flow cytometry analysis revealed that more than 90% of WJMSCs had consistent mesenchymal stem cell markers such as CD90, CD105, and CD73. CD45, CD34, and CD79a were all shown to be absent in WJMSCs were illustrated in Figure 3. Although cells expressed HLA-ABC antigen, there was no expression of HLA-DR antigen. The cells' viability was verified to be greater than 90% utilizing the 7-AAD dye exclusion technique and flow cytometry.

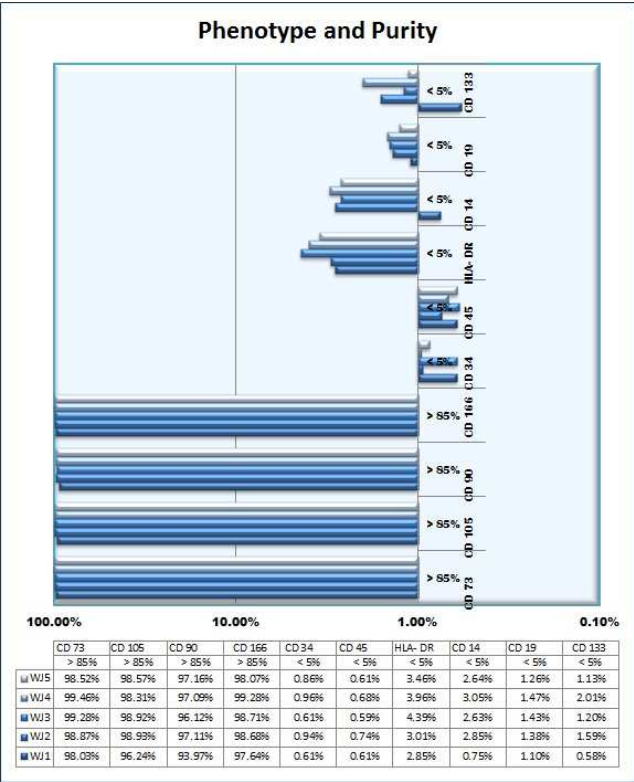


Figure 3. Immunophenotype of Wharton’s jelly derived MSCs: Representative flow cytometry analysis of WJMSCs after expansion when labeled with antibodies against human antigens CD34 (PE), HLA-DR (PerCP-Cy5.5), CD45 (FITC), CD79 (APC) as negative markers and CD105 (PE), CD73 (APC), CD90 (FITC) as mesenchymal specific markers; Color shaded histogram represents positive reactivity with the indicated antibody.

3.3. Differentiation

Adipogenic, Osteogenic, and Chondrogenic differentiation were performed on the expanded MSCs. Extracellular matrix mineralization was observed in the expanded MSCs after osteogenesis was induced, as validated by Alizarin Red staining. When accumulating lipid vacuoles were stained with oil red O, adipogenic differentiation was verified. Alcian blue staining of collagen fibers revealed that MSCs

had effectively differentiated into chondrocytes (Figure 4). Undifferentiated and control cells showed no alterations.

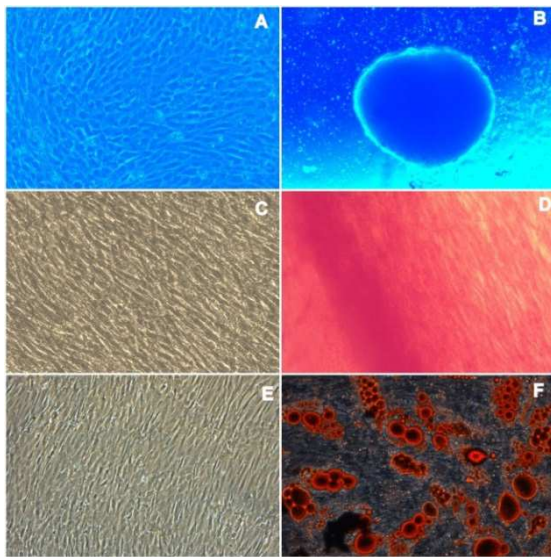


Figure 4. Tri-lineage differentiation of WJ-MSCs: WJ-MSCs derived after expansion were induced to differentiate along (B) Chondrogenic proteoglycans formation (alcian blue), (D) osteogenic (alizarin red S) lineages, (F) adipogenic (oil red O staining). (A, C, E) corresponding uninduced control cultures were stained for comparison. Scale bar = 50µm.

3.4. Cumulative Population Doubling Level (CPDL) Analysis

The CPDL was evaluated to determine the isolated WJ-MSCs' proliferation potential. WJ-MSCs were sown in a culture plate at a density of 3000 cells/cm² and subcultured after 4-5 days. The method was continued until passage 10 was reached, at which point the CPDL was measured. Against various passage levels, a consistent growing graph of cell development was found (Figure 5).

Cell Growth Curve of Wharton's jelly Mesenchymal Stromal Cells

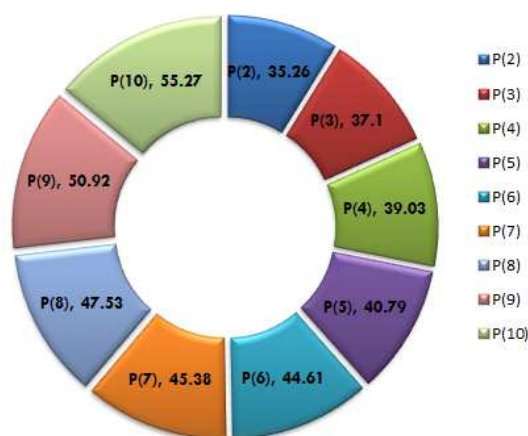


Figure 5. Cell Growth Curve of Wharton's Jelly Mesenchymal Stromal Cells: CPDL values 35.26, 37.1, 39.03, 40.79, 44.61, 45.38, 47.53, 50.92, 55.27 was measured respectively for Wharton's jelly from passage 2 to passage 10, and evaluated that cells grew consistently until passage 10.

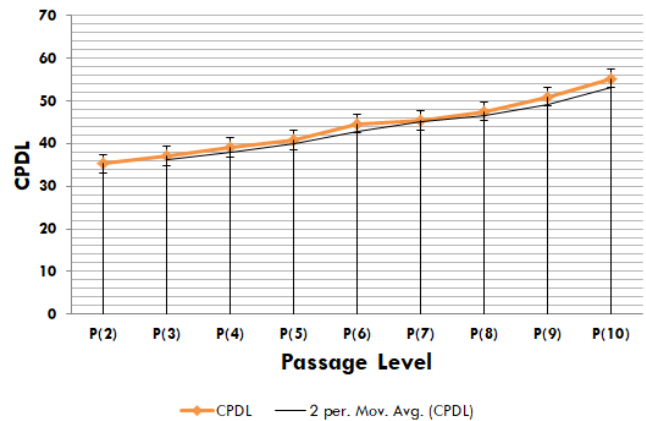


Figure 6. Cell Growth Curve of Wharton's Jelly Mesenchymal stem cells.

Seeding density seems to have an effect on the growth of WJ-MSCs in the medium composition. The nutrient mixture Ham's F-12 (1:1) with Glutamax (1X); 2.438g/L Sodium Bicarbonate; Sodium Pyruvate; and 10% fetal bovine serum supplemented with 3ng/mL bFGF were evaluated in Dulbecco's modified Eagle medium. WJ-MSC were grown at 3000 cells/cm² in T75, T175, and Hyper flasks starting at passage-1 and continuing until passage-10, as shown in Figures 5 and 6. The seeding density of 3000 cells/cm² resulted in greater cell proliferation and total yield ($P < 0.05$).

3.5. Colony-Forming Unit (CFU)

Umbilical cord tissue contained Wharton's jelly-derived MSCs grown in DMEM/F12+ supplemented with 10% FBS, according to data from five distinct samples. On day 14, the cultures were stopped and stained with 5% crystal violet for CFU-F determination. Under optimum settings, the CFU potential of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) reveals the efficiency of fetal sources, which were examined with each sample of WJ-MSCs demonstrated with distinct colony-forming unit ability, is high. Figure 7 shows that the CFU formation was investigated using DMEMF-12 and FBS as culture plate additives, which were stained with crystal violet.

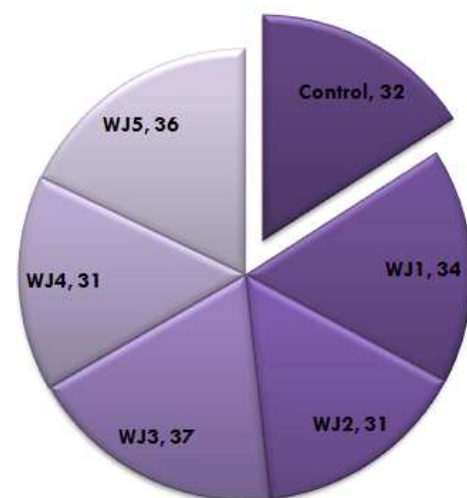


Figure 7. Colony Forming Unit Analysis of Wharton's Jelly MSCs.

Cells were cultivated until the tenth passage level, which shows that greater colony-forming units in Wharton's jelly plates and each cord plate (n=5) WJ-MSCs demonstrate a different colony-forming unit ability, as shown in Figure 7. The colony-forming unit study revealed that Wharton's Jelly Mesenchymal Stem Cells of five donors (WJ1 to WJ5) had a clonogenic potential of CFU 34, CFU 31, CFU 37, CFU 31, and CFU 36, respectively. Figure 7 illustrates the test values compared to the colony-forming units of the treated control, which are closely matched with the positive control value CFU 32 at the tenth passage level.

3.6. Microbiological Tests

When the supernatants were tested frequently, the cultures were found to be completely sterile and devoid of any aerobic, anaerobic, or fungal contamination. Final product endotoxin levels were less than 0.2EU/ml.

3.7. Mortality and Clinical Signs

On the day of dosing and daily thereafter until the 21st day, frequent clinical examinations at intervals of 30 minutes, 1 hour, 2 hours, and 4 hours after intravenous/subcutaneous administration did not reveal any abnormal clinical signs or preclinical deaths among treated rats as per Table 1 and Table 2 dosing.

3.8. Body Weight

During the 3-week observation period of the investigation, no deleterious effects of treatments on the body weights of the treated rats were noticed. Body weights of all male and female rats treated with MSCs at 10×10^6 MSCs/kg body weight were recorded before dosing (day 1) and at weekly intervals afterward (days 7, 14, and 21) and continued to gain weight until they were euthanized on day 22.

Table 1. Summary of Mortality: Absolute mortality is presented as the number of animals that died/numbers treated.

Group	Drug Treatment	Intravenous administration Incidence of Mortality					
		Male Rat		Female Rat		Male & Female Rat	
		Absolute	% Mortality	Absolute	% Mortality	Absolute	% Mortality
G1	Normal Saline (Vehicle Control)	0/5	0	0/5	0	0/10	0
G2	1 million cells	0/5	0	0/5	0	0/10	0
G3	2 million cells	0/5	0	0/5	0	0/10	0
G4	4 million cells	0/5	0	0/5	0	0/10	0
G5	6 million cells	0/5	0	0/5	0	0/10	0
G6	8 million cells	0/5	0	0/5	0	0/10	0
G7	10 million cells	0/5	0	0/5	0	0/10	0

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

Table 2. Effect of MSC on Mortality rate after subcutaneous administration in Wistar albino rats.

Group	Drug Treatment	Subcutaneous administration Incidence of Mortality					
		Male Rat		Female Rat		Male & Female Rat	
		Absolute	% Mortality	Absolute	% Mortality	Absolute	% Mortality
G1	Normal Saline (Vehicle Control)	0/5	0	0/5	0	0/10	0
G2	1 million cells	0/5	0	0/5	0	0/10	0
G3	2 million cells	0/5	0	0/5	0	0/10	0
G4	4 million cells	0/5	0	0/5	0	0/10	0
G5	6 million cells	0/5	0	0/5	0	0/10	0
G6	8 million cells	0/5	0	0/5	0	0/10	0
G7	10 million cells	0/5	0	0/5	0	0/10	0

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

Table 3. Summary of male and female rats body weights after intravenous injection.

Groups	Drug Treatment	Body Weight (gms)			
		Male Rat			
		1 st day	7 th day	14 th day	21 st day
Test Group Intravenous injection	G1 Normal Saline	183.25±2.22	191.20±4.5	198.22±3.8	205.64±3.57
	G2 1 million cells	181.10±1.58	189.22±3.3	197.62±2.9	204.23±3.75
	G3 2 million cells	179.50±1.68	186.23±4.55	193.20±3.05	203.20±2
	G4 4 million cells	182.22±2.1	190.64±3.3	196.40±3.3	204.37±3.5
	G5 6 million cells	179.21±1.85	186.75±4.7	194.55±4.08	202.75±3.34
	G6 8 million cells	181.36±2.27	188.25±3.71	195.66±3.35	204.75±2.75
	G7 10 million cells	180.65±1.85	187.50±3.3	196.50±3.22	204.66±3.25

Table 3. Continued.

Groups		Drug Treatment	Body Weight (gms)			
			Female Rat			
			1 st day	7 th day	14 th day	21 st day
Test Group Intravenous injection	G1	Normal Saline	185.75±3.04	192.35±2.5	197.60±2.75	203.45±3.3
	G2	1 million cells	179.42±2.8	184.62±2.27	191.45±3.05	199.22±2.47
	G3	2 million cells	178.61±2.44	183.75±3.5	190.70±2.65	198.65±2.47
	G4	4 million cells	180.50±3.2	187.30±2.95	194.54±3	202.34±2.22
	G5	6 million cells	180.55±2.6	185.56±3.22	193.75±2.27	200.45±3
	G6	8 million cells	180.54±3.71	186.54±2.3	194.42±3.15	200.90±2.24
	G7	10 million cells	181.30±2.25	186.54±2.5	194.70±3.61	201.75±3.54

*Values are in Mean±SEM (n=10).

Table 4. Summary of male and female rats body weights after subcutaneous injection.

Groups		Drug Treatment	Body Weight (gms)			
			Male Rat			
			1 st day	7 th day	14 th day	21 st day
Test Group Subcutaneous injection	G1	Normal Saline	184.33±2.58	192.36±7.58	201.21±5.45	220.35±6.32
	G8	1 million cells	176.55±1.45	184.75±5.22	192.75±4.35	215.57±5.75
	G9	2 million cells	182.32±2.69	190.55±3.74	200.75±5.9	218.75±4.85
	G10	4 million cells	175.54±3.85	186.66±5.27	198.36±5.98	216.35±5.55
	G11	6 million cells	181.20±3.3	191.66±5.5	199.36±4.65	219.32±4.75
	G12	8 million cells	180.56±2.15	189.25±4.56	202.45±6.35	215.45±6.5
	G13	10 million cells	182.33±2.27	191.32±5.22	202.35±5.54	218.35±6.75

Table 4. Continued.

Groups		Drug Treatment	Body Weight (gms)			
			Female Rat			
			1 st day	7 th day	14 th day	21 st day
Test Group Subcutaneous injection	G1	Normal Saline	176.87±2.45	186.22±2.08	195.36±5.44	205.47±3.67
	G8	1 million cells	181.33±2.66	192.20±3.35	200.45±5.75	209.45±4.45
	G9	2 million cells	182.24±3.95	194.65±4.2	202.58±4.25	210.35±5.44
	G10	4 million cells	180.66±3.37	190.47±4.5	198.54±4.25	207.65±3.36
	G11	6 million cells	182.22±2.2	191.27±3.55	198.32±3.65	210.17±4.47
	G12	8 million cells	179.95±4.86	190.44±4.25	197.56±2.22	204.78±3.6
	G13	10 million cells	181.22±2.78	192.47±4.45	200.31±4.55	211.77±5.37

*Values are in Mean±SEM (n=10).

3.9. Statistical Analysis

Except for a few values (Table 3) that were somewhat lower (but statistically significant) than those in the control group after intravenous injection, the treatment group rats' body weights and body weight changes did not vary substantially from those in the control group ($P>0.05$). However, because it was equivalent to the regular pattern of body weight growth in rats, this apparent reduction was of a tiny scale and had little biological relevance. Given the limited sample size (three) per gender, the statistical finding was more likely to be coincidental. Consequently, after subcutaneous injection, the treatment group rats' body weights and body weight changes did not differ substantially from the control group ($P>0.05$) (Table 4).

3.10. Necropsy and Histopathology

At the 21-day observation period of the investigation, the

complete blood cell count and differential count of male and female rats revealed no aberrant effects of therapy on blood samples from intravenous injection and subcutaneous injection-treated rats.

Instead, Human umbilical cord tissue Wharton's Jelly Mesenchymal Stem Cells (MSCs) did not cause any obvious pathological alterations in the tissues or organs of the treated rats. When the rats were slaughtered and submitted to necropsy investigation at the end of the research on day 22, there were no aberrant results. MSCs from Wharton's jelly did not cause any noticeable gross pathological alterations in the treated animals' tissue/organs.

Three male rats from group G2 that had slight/mild pallor were slaughtered and submitted to necropsy analysis at the end of the research for intravenous injection on day 21. These livers showed a minor cytoplasmic rarefaction when examined under a microscope. Because there was no other observation in this study that could be linked to this discovery, and

because this finding could not be compared to historical control data, these observations were deemed to be coincidental and unrelated to MSC therapy.

The livers of one female rat from group G4 and one female rat from group G5 showed slight/mild pallor after subcutaneous injection. These livers showed a minor cytoplasmic rarefaction when examined under a microscope. Because there was no other observation in this study that could be linked to this discovery, and because this finding could not be compared to historical control data, these observations were deemed to be coincidental and unrelated to MSC therapy. There were no additional aberrant observations in male and female mice treated with Wharton's jelly-produced MSCs in this investigation. When the rats were slaughtered and submitted to necropsy investigation after the research on day 22, there were no aberrant results. MSCs did not cause any gross pathological alterations in the tissue/organs of the treated animals when injected intravenously or subcutaneously.

4. Discussion

The use of stem or progenitor cells in a cell-based therapy strategy has shown great promise in the treatment of a wide range of degenerative and age-related disorders. Despite its efficacy, the success of this pharmacological method is being hampered by a slew of challenges that must be overcome before they can be used in clinical settings. As an experimental process, the present market need is for a unique, easily available, and morally non-controversial source of stem cells that can be altered to meet all requirements for cell-based transplants. Because UC is thrown as medical waste after birth, it can be a technically and morally excellent source of Mesenchymal stromal cells [18-20].

Even though the efficacy of up-scaled clinical-grade mesenchymal stromal cells has been well understood based on accumulated clinical and previous published data [15, 16], it is still necessary to determine whether these cells are functionally important in homing capacities and to optimize the best route of administration based on the patient's pathophysiology. An animal study was conducted to determine the clinical toxicity of the therapeutical product as well as the optimal mode of administration to address the difficulties. A single dose of the test item was administered to five groups of rats of both sexes. MSCs were given to rats by subcutaneous and intravenous injections, as both procedures ensure systemic diffusion and might be a therapeutic option. For 21 days, the animals were monitored for symptoms of toxicity and death. In the absence of any hazardous reaction, the term was not prolonged. The trial was carried out at a limit dosage of 10×10^6 MSCs/kg body weights because this dose was thought to provide an appropriate safety margin given the proposed therapeutic use. MSCs are expected to have a therapeutic dosage of 1×10^6 MSCs/kg body weight [21-27].

The test dose was ten times more than what would be given to humans as a therapeutic dose. Because the limit of

10×10^6 MSCs/kg weight did not create any health issues in the treated rats, further dose levels were not explored. The lowest lethal dose, maximum acceptable dose, and median lethal dose of MSCs from UC tissue following acute intravenous and subcutaneous therapy in rats could not be determined because there were no adverse effects or death among the treated rats in this study. The MLD, MTD, and LD50 of MSCs from human umbilical cord tissue acute intravenous and subcutaneous toxicity in rats, on the other hand, were calculated to be more than 10×10^6 MSCs/kg body weight.

5. Conclusion

Cells isolated from WJ resemble fibroblast-like cells, have mesenchymal features including plastic adherence and tri-lineage differentiation, express phenotypically comparable markers, and can be scaled up for therapeutic use, according to the study. When MSCs are injected intravenously and subcutaneously into Wistar albino rats at a dose of 10×10^6 MSCs/kg body weight, the recorded observation shows that injected MSCs did not cause any mortality, abnormal clinical signs, adverse effect on body weights, or gross pathological changes in tissue/ organs by the treated rats during the preclinical study's observation period. However, human clinical trials to intend the therapeutic usages on this in the same direction is needed to be extended.

Disclosure of Potential Conflict of Interest

The authors declare that they have no competing interests.

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References

- [1] Singer NG, Caplan AI. "Mesenchymal stem cells: mechanisms of inflammation," *Annu Rev Pathol.* 2011; 6: 457-78.
- [2] Ding DC, Shyu WC, Lin SZ. "Mesenchymal stem cells. *Cell Transplant*," 2011; 20 (1): 5-14.
- [3] Hassan MNFB, Yazid MD, Yunus MHM, Chowdhury SR, Lokanathan Y, Idrus RBH, Ng AMH, Law JX. "Large-Scale Expansion of Human Mesenchymal Stem Cells," *Stem Cells Int.* 2020; 15; 2020: 9529465.

- [4] Lukomska B, Stanaszek L, Zuba-Surma E, Legosz P, Sarzynska S, Drela K. "Challenges and Controversies in Human Mesenchymal Stem Cell Therapy. Stem Cells," *Int. 2019*; 9; 2019: 9628536.
- [5] Pal R, Hanwate M, Jan M, Totev S. "Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells," *J Tissue Eng Regen Med. 2009*; 3 (3): 163-74.
- [6] Peng L, Jia Z, Yin X, Zhang X, Liu Y, Chen P, Ma K, Zhou C. "Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue," *Stem Cells Dev. 2008*; 17 (4): 761-73.
- [7] Hu Y, Liang J, Cui H, Wang X, Rong H, Shao B, Cui H. "Wharton's jelly mesenchymal stem cells differentiate into retinal progenitor cells," *Neural Regen Res. 2013*; 5; 8 (19): 1783-92.
- [8] Wang Y, Han ZB, Song YP, Han ZC. "Safety of mesenchymal stem cells for clinical application," *Stem Cells Int. 2012*; 2012: 652034.
- [9] Thirumala S, Goebel WS, Woods EJ. "Clinical grade adult stem cell banking. Organogenesis," 2009; 5 (3): 143-54.
- [10] Cooper K, Viswanathan C. "Establishment of a mesenchymal stem cell bank," *Stem Cells Int. 2011*; DOI: 10.4061/2011/905621s.
- [11] Viswanathan C, Davidson Y, Cooper K, Tipnis S, Pujari G, Kurian VM. "Transplantation of autologous bone marrow derived mesenchymal stem cells trans-epicardially in patients undergoing coronary bypass surgery," *Indian Heart J. 2010*; 62 (1): 43-8.
- [12] Amer MH, Rose FRAJ, Shakesheff KM, Mado M, White LJ. "Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges," *NPJ Regen Med. 2017*; 10; 2: 23.
- [13] Huang XP, Sun Z, Miyagi Y, McDonald Kinkaid H, Zhang L, Weisel RD, Li RK. "Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair," *Circulation. 2010*; 7; 122 (23): 2419-29.
- [14] Musiał-Wysocka A, Kot M, Majka M. "The Pros and Cons of Mesenchymal Stem Cell-Based Therapies. Cell Transplant," 2019; 28 (7): 801-812.
- [15] Jaianand K, Balaji P. "Clinical Prospects of Scale-Up foetal Wharton's Jelly-Derived Multipotent Stromal Cells to fulfil the Therapeutic Demands," *Int J Pharm. Bio. Sci. 2015*; 6 (4): B882-B894.
- [16] Jaianand K, Balaji P. "Isolation, Characterization and Scale-Up of Foetal Amniotic Membrane-Derived Multipotent Stromal Cells for Therapeutic Applications," *Int J Pharm. Bio. Sci. 2015*; 6 (2): 376-385.
- [17] Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy. 2006*; 8 (4): 315-317.
- [18] Williams AR and Hare JM. "Mesenchymal stem cells: Biology, pathophysiology, translational findings and therapeutic implications for cardiac diseases," *Circulation Research, 2011*; 108 (8): 923-940.
- [19] Devang MP, Jainy S and Anand S. "Therapeutic potential of Mesenchymal Stem Cells in Regenerative Medicines," *Stem cell international. 2013*; 2013: 496218: 15.
- [20] Nekanti U, Mohanty L, Venugopal P, Balasubramanian S, Totev S and Ta. M. "Optimization and scale up of Wharton's jelly derived mesenchymal stem cells for clinical applications," *Stem cell research. 2010*; 5 (3): 244-254.
- [21] Young Bang O, Jin SL, Hyu Lee and Gwang Lee. "Autologous mesenchymal stem cell transplantation in stroke patients," *Annals of Neurology. 2005*; 57: 874-882.
- [22] Pal R, Venkataramana NK., Bansal A, Balaraju S, Jan M, Chandra R, Dixit A, Rauthan A, Murgod U and Totev S. "Ex vivo-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study," *Cytotherapy. 2009*; 11 (7): 897-911.
- [23] Martino G, Franklin RJ, VanEvercooren AB and Kerr DA. "Stem cells in multiple sclerosis (STEMS) consensus group. Stem cell transplantation in multiple sclerosis: current status and future prospects," *Nature Reviews Neurology. 2010*; 6 (5): 247-255.
- [24] Freedman MS, Bar-Or A, Atkins HL, Karussis D, Frassonni F, Lazarus H, Scolding N, Slavin S, Le Blanc K, Uccelli A; MSCT Study Group. "The therapeutic potential of mesenchymal stem cell transplantation as a treatment for multiple sclerosis: consensus report of the International MSCT Study Group," *Multiple Sclerosis. 2010*; 16 (4): 503-510.
- [25] Wang D, Akiyama K, Zhang H., Yamaza T, Li X, Feng X, Wang H, Hua B, Liu B, Xu H, Chen W, Shi S and Sun L. "Double allogenic mesenchymal stem cells transplantations could not enhance therapeutic effect compared with single transplantation in systemic lupus erythematosus," *Clinical and Development Immunology. 2012*; 2012: 273291.
- [26] Reinders ME, de Fijter JW, Roelofs H, Bajema IM, de Vries DK, Schaapherder AF, Claas FH, van Miert PP, Roelen DL, van Kooten C, Fibbe WE and Rabelink TJ. "Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study," *Stem Cells Translational Medicine. 2013*; 2 (2), 107-11.
- [27] Kim SJ, Moon GJ, Chang WH., Kim YH and O. Y. Bang. "Intravenous transplantation of mesenchymal stem cells preconditioned with early phase stroke serum: current evidence and study protocol for a randomized trial," *Stem cell Application Researches and Trials In Neurology. 2013*; 14 (1): 317.