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Therapeutic Consequence of Allogeneic Mesenchymal Stem Cell for Duchenne Muscular Dystrophy- Case Study

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ABSTRACT:*Duchenne muscular dystrophy (DMD) is an inherited disorder caused by mutation in gene that prevents the translation of dystrophin protein, which leads to massive skeletal muscle wasting. Any form of muscular dystrophy is considered as an incurable disease yet there are occupational treatments assisting patients with locomotion strengthening and to further extend the lifespan. Thus, emerging treatment strategy is warranted to improve patients' conditions from DMD. In the present study, we have evaluated the therapeutic outcome of human umbilical cord derived mesenchymal stem cell (hUC-MSC) in patients with DMD. In light of that, we have produced therapeutically safe (chromosomal stability, stable Immunopheotyping and mesodermal lineage differentiation ability) hUC-MSCs in our GMP compliance laboratory. Patients (24 and 27 years old) were administrated (intravenous [50X10^6] + intramuscular [50X10^6]) one single dose of hUC-MSC and followed up for 12 months. Therapeutic efficacy was measured as based on the level of creatinine phosphokinase (CK) in plasma. Both patients were highly responsive to hUC-MSC and the CK level was significantly dropped from 3rdmonth of post transplantaton. After 12 months of post transplantation the CK level was slightly increased in both patients as compared to 6th months. However, the level of CK level was still significantly lower than basal level. In conclusion, one single dose of hUC-MSCs treatment may not be sufficient to give better clinical outcome and also these results suggested that multi-dose of hUC-MSCs may give long term beneficial effects in patient with DMD.*
KEY WORDS: *human umbilical cord mesenchymal stem cell; Stem cell; Duchenne muscular dystrophy; Stem cell therapy.*

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I. INTRODUCTION

Duchenne muscular dystrophy (DMD) is a genetically determined X-linked musculo-degenerative disease characterized by lack of dystrophin production or synthesis of functionally impotent dystrophin [1]. This protein is essential to maintaining the integrity of the exoskeleton stability and function of the muscle cells [2].The nonattendance of dystrophin increases the phagocytosis and breakdown of muscle fibers, which eventually leads to the replacement of muscle tissue by adipose and connective tissues.[3].The lack of dystrophin, myofibers are unstable and fragile, which causes progressive skeletal muscle degeneration. The skeletal muscle consists of muscle stem cells named as satellite cells, which helps for muscle regeneration in-term of proliferation, differentiation and self renewal [4]. However, the proliferation, self renewal and regeneration capacity is reduced in DMD [5,6]

At present, there is no cure for DMD, nevertheless various strategies such as conservative management, medical management and replacement of the deficient protein have been devised to minimize inflammation muscle damage minimize fibrosis [7,8]. Recently, research has been probing for alternative therapeutic approaches for DMD. The new advancement in cell-based therapy alters the disease process and slows its progression [9].

More than a decade, mesenchymal stem cells (hUC-MSCs) have been explored as a potential source for stem cell[10]. Assorted approach has been explored to isolate stem cell from different sources. However, cells isolated form umbilical cord tissue has shown constant doubling time, stable Immunophenotyping and chromosomal structure [11]. Studies also have shown that multipotent stem cells derived from umbilical cord have more potential for tissue regeneration in various conditions and capable of differentiating into multiple cells types, such as adipogenic osteogenic, chondrogenic germ like cell, neuron like cell and myogenic cells [12-14]. In addition, hUC-MSCs abundant and easy to harvest, expansion and lesser ethical issues [15]. With these unique fundamental properties, in the present study we have evaluated the therapeutic effect of hUC-MSCs in patients with DMD.

II. METHODOLOTY

2.1 Case details

All procedures were followed in accordance with the ethical standards described by the Helsinki Declaration. The written informed consent was obtained from the study patients prior to treatment. This is a case report study and patients were recruited as per the inclusion and exclusion criteria set by treating physician. Two patients were enrolled in this study and there age range from 24 to 27 year old. DMD is confirmed by the physician and their plasma CK level was 1241 IU/L (24 year old) and 517 IU/L (27 year old) as the normal range is 38 to 174 IU/L.

1.2 Mesenchymal stem cell isolation and expansion

Umbilical cord (UC) was obtained from local maternity hospitals after normal deliveries with donor consent and complete medical history. The sample was collected in phosphate-buffered saline (PBS) supplemented with antibiotics, 100 U/ml penicillin and 100 μg/ml streptomycin and transported to the laboratory. The sample was washed with PBS several

time under a sterile contention and cut into 1cm in size. The segment was then cut longitudinally, and removed the blood vessels. Then the tissues was chopped into 2 mm in size and transferred into 75 cm2 culture flasks with MSC complete media and place it in 5% CO2 incubator. After 7 days, the UC tissues were removed and the adherent cells were allowed to expand until they reach about 70-80% confluence. Upon reaching 70-80% confluence, adherent MSCs were harvested (passage-0) by trypsinization (0.25% trypsin-EDTA, Invitrogen, BRL, Canada) and subculture MSCs until passage 3 for further use.

2.3 Immunophenotyping of hUC-MSCs

The phenotype of expanded hUC-MSC (at passage 3) were assessed with the following MSCs specific antibodies as described earlier (Subramani et al. 2015) CD73-PE (Mouse Anti-human), CD90-FITC (Mouse Anti-human), CD44-PE (Mouse Anti-human), CD105-PerCP-Cy(Mouse Anti-human, monoclonal, BD Biosciences). Staining was performed as per manufacture's instruction. Stained cells were re-suspended in PBS and analyzed by using flow cytometry (FC- 500 Beckman Coulter, USA). The acquired data were analyzed using CXP software provided by the manufacturer.

2.4 Growth kinetics of hUC-MSCs

The hUC-MSCs (~3 × 10 l/well) were plated in 96-well plates and incubated at 37 °C in a 5 % CO2 humidified incubator for 5 days lium was changed once in 2 days and cells were harvested and counted at every 24 h interval for 5 days. The growth curve of hUC-MSC was determined manually by performing live cell counting using trypan blue exclusion dye method. The growth curve was plotted as based on initial seeding, cell yields and days.

2.5 Doubling time of hUC-MSCs

The hUC-MSCs (3 × 10 l/well) were seeded in 96-well plates every day for 5 days and incubated at 37 °C in a 5 % CO2 humidified incubator. M m was changed once in 2 days and triplicates of hUC-MSC were maintained. On day 5, MTT (3-(4, 5-dimethylthiazol- 2, 5–diphenyl tetrazolium bromide) (Sigma Chemical Co., St. Louis, MO, USA) proliferation assay was carried out to assess the proliferation rate of hUC-MSC. MTT was dissolved in PBS at 5 mg/ml; added to the culture medium at a dilution 1:10 prior to be incubated at 37 °C for 4 h. The medium was aspirated; 100 µl of DMSO was added/well and the 96-well plate was read using a microplate reader (DYNEX Technologies, Chantilly, VA, USA) at the wavelength of 570 nm. The doubling time was determined using the Patterson Formula [Td = Tlg2/lg (Nt/N0)], Td is the doubling time (h), T is the time taken for cells to proliferate from N0 to Nt (hour), and N is the cell count.

2.6 Mesodermal differentiation ability of hUC-MSCs

The hUC-MSCs were grown in 6 well plate till reach 70- 80 % of cell confluency in MSC complete medium and then the cells were induced to differentiate into osteocytes and chondrocytes by supplementing the respective differentiation medium (STEMPRO®). After differentiation, the osteocytes and chondrocytes were fixed with 4 % formalin and stained with alizarin red and alcian blue respectively. Cell image was captured under confocal microscope at 100X magnification.

2.7**Karyotyping for hUC-MSCs**

Cytogenetic analyses were performed on passage 3 hUC-MSCs. Cells were seeded in T25 culture flask and allowed to grow until reach the confluence of 70 -80%. After that the culture flasks (duplicate) has been sent to NUH Referral Laboratories Pte Ltd, Singapore for chromosomal stability (G-band) testing.

2.8 Stemness of hUC-MSCs

The cells were washed with cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (Merck Millipore, Germany) containing protease-phosphatase inhibitor cocktail (Roche and Sigma-Aldrich). Cell lysates were centrifuged at 10,000 rpm/min for 10 min and supernatant was collected. Protein quantification was done by using Bradford method. Protein lysates were mixed with 3x loading dye containing 5% β-mercaptoethanol followed by boiling for 3 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation. The separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk in 1x TBST-T for 1 h. After washing, the membranes were then probed with respective primary antibodies (REX1 and Notch1 (Abcam) at dilution from 1:1000 to 1:2500 in 1x TBST buffer containing 2–3% bovine serum albumin for overnight at 4°C. Following washes, membranes were incubated with horseradish peroxidase conjugated anti-rabbit (1:10,000) antibodies for 1 h at room temperature. Finally, membranes were subjected to chemiluminescence detection using ECL kit (Western Bright, USA). The intensity of the signals was visualized by bio-illuminator (FluorChem 5500, CA).

2.9 Transplantation of hUC-MSCs

Once achieved the therapeutic number of cells, cells were diluted in injectable saline with human albumin solution in appropriate cell numbers. The study objective was planned to transplant the hUC-MSCs through intravenous (i.v) and intramuscular (i.m) mode and thus, cells were diluted at different concentration. Cells were diluted one million per mL and five million per mL for intravenous and intramuscular, respectively. Patients were received one single dose ($50x10^6$via i.v and $50x10^6$ via multi site of i.m) of hUC-MSC and followed up for 12 months. Patient's peripheral blood was drawn on 3, 6, and 12 months for analysis the plasma CK level. Data was compiled and analyzed for therapeutic outcome of hUC-MSCs in patients with DMD.

2.10 Statistical analysis

Data analyzed from each group were expressed as mean and standard error (SE) of at least two to three separate experiments performed. Statistical comparison between groups was analyzed using Student's t test. A value of $*P<0.05$ was considered to be statistically significant.

III. RESULTS

3.1 Isolation and characterization of human Umbilical Cord derived Mesenchymal Stem Cells (hUC-MSCs)

The hUC-MSCs were isolated from the umbilical cord through tissue expansion method and the primary cells were attached to the culture flasks over the period of 6 to 7 days. Such adhered primary cells exhibited different morphologies, for instance spindle-shaped fibroblast-like morphology (>75%) and a polygonal morphology (~25%). After serial passage, the polygonal morphology was decreased and achieved uniform spindle shaped fibroblast like morphology at passage 3 (Figure 1A). Furthermore, our western blot analysis demonstrated that hUC-MSCs express unique pluripotent markers Rex1 and Notch1 (Figure 1B). Next, we evaluate whether hUC-MSCs met the ISCT qualifying criteria of MSCs and thus MSC specific cell surface markers (CD44, CD90, CD105 and CD73) were analysed. Our flow cytometry analysis showed that hUC-MSCs (at passage 3) were positive for CD44, CD90, CD105 and CD73 (Figure 1C) and were negative for CD34 and HLA-DR (data not shown). Other essential characteristic of MSC is their ability to differentiate into chondrogenic and osteogenic mesenchymal lineages. Calcium mineralization was observed in hUC-MSCs after 3 weeks of osteogenic induction by using positive alizarin red staining. Similarly, hUC-MSCs were subjected to 21 days chondrogenic induction and were stained with alcian blue (Figure 2A). Finally, we performed the cytogenetic analyses for chromosomal stability of hUC-MSC at passage 3 and/or before clinical application. Our Karyotyping (G-band) analysis revealed a normal diploid karyotype with 46 chromosomes and no major chromosomal abnormalities were detected. Overall, these results conclude that hUC-MSCs maintain the stemness, mesodermal lineage differentiation and stabilize the immunophenotype cum chromosomal structure, which provides strong evidence that umbilical cord derived MSC is safe and can be used as therapeutic tools for life-threatening diseases, including muscular dystrophy (MD).

3.2 Growth kinetics and doubling time of hUC-MSCs

We certainly observed that a hUC-MSC growth pattern was varied between the passages. Particularly, we have measured the growth kinetics of passage 3 hUC-MSCs at appropriate/ optimized culture condition. The cell number of hUC-MSC was progressively increased from day 1 to day 5. Cell count was performed every 24 h interval using trypan blue dye exclusive method. Growth kinetic analysis revealed that followed by lag phase, continuous exponential log phase was observed throughout study period (Figure 3A). Furthermore, the population doubling time of hUC-MSCs was measured using MTT assay. Doubling time of passage 3 hUC-MSCs was achieved after 72 h (Figure 3B).

3.3 Therapeutic outcome of single dose of hUC-MSCs in patient with muscular dystrophy

Clinical outcome of muscular dystrophy (DMD) was evaluated by measure the Creatinine Phosphokinase (CK) level in plasma. Age 24 (male) and 27 (female) years old DMD patients were administered (intravenous [$50X10^6$/50mL] + intramuscular [$50X10^6$/10mL]) one single dose of hUC-MSCs and followed up for 12 months. Plasma CK levels were measured on 3rd month, 6th month and 12th month in both patients. The CK levels were significantly dropped in both patients after 3rd month of hUC-MSC treatment. Followed by 6 months follow-up period, the CK levels were further decreased in both patients as compared to 3 months post-treatment. However, the level of CK was considerably increased in both patients on 12th month post-treatment as compared to 6th month follow-up period, however the level of CK was still significantly lower than the basal level (before therapy). These results suggested that repeated dose of hUC-MSCs may give a better solution or remedy to patient with DMD.

IV. DISCUSSION

DMD is described by devastating progressive muscle weakness diseases. This can be enlightened in cellular level as muscle necrosis that exceeds the regenerative potency of the muscles. Dystrophin plays a role in muscle contraction that connects cytoskeletal muscle fibers to surrounding extracellular matrix through cell membrane protein complexes. Consequently, the abnormality of dystrophin gene and absence of dystrophin protein leads to cell necrosis in DMD [16, 17]. Therefore, the regeneration of necrotic cells is replaced by local stem cell or satellite cell. However, they are not enough and competent to repair the damaged cells due to cell number and microenvironment of the necrotic cells [18].

To finding out for an effective therapy (true-cure) for DMD and unfortunately there was no such effective treatment for DMD so far and alternatively some occupational therapy may helping patients for locomotion and/or slowing in the declining course of the disease [19].Thus, new therapeutic approaches are needed for patients with DMD. In the present study, we proposed to use allogeneic hUC-MSCs for patients with DMD as an alternative therapeutic approach. On privilege of immunosuppressive properties of MSCs, allogeneic hUC-MSC can be successfully adopted or engrafted in the host tissue [20] hUC-MSCs were successfully produced in GMP compliance laboratory and characterized the properties at passage 3. The Cultured hUC-MSCs showed fibroblast like spindle shape morphology and has faster doubling time between 48 and 72 h [15]. As these cells are derived from pre and peri natal tissues which have unshorten telomere length [21]. Additionally, produced hUC-MSCs expressed identical levels of surface antigens such as CD44, CD73, CD90, and CD105. Finally, the mesodermal lineages differentiation of hUC-MSCs supporting to this therapy that umbilical cord derived MSCs have the ability to differentiated into other mesodermal lineage cells [22,23]. Throughout the culture period hUC-MSCs maintain the stemness pluripotency (Rex1 and Notch1) without altering the chromosomal structure or genetic pattern [15].

In line of homing (chemo-attractive mechanisms) and engraftment properties of MSCs, patients were received 50% of hUC-MSCs systematically which probably mobilized and home to the site of injury [24,25]. The remaining 50% of hUC-MSCs were directly delivered through intramuscular mode which facilitates stem cell to reach the site of injury without any obstruction. Both patients were highly respond to hUC-MSCs (intramuscular followed by intravenous mode) after 3

months of post-therapy and the effect was sustained until 6 months, thereafter the clinical outcome (CK level) was slowdown on the 12 month of follow-up period. Recent reports also demonstrated that administration of MSCs can dynamically regenerate the dystrophin expressing muscle cell in animal model [26,27]. In conclusion, our study finding that one single dose (intramuscular followed by intravenous) of hUC-MSCs potentially improves DMD and this study also suggested that multi dose of hUC-MSCs may provide a better and long-term clinical outcome in patients with DMD. Further studies are needed to prove this hypothesis.

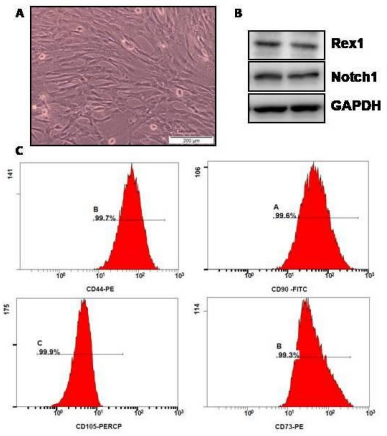

Figure 1. Identification and characterization of human umbilical cord derived Mesenchymal Stem Cells (MSCs). A) Morphological image of umbilical cord derived MSCs at passage 3. B) Western blot image of stemness and potency specific markers. C) Flow cytometry analysis of MSC specific surface antigen.

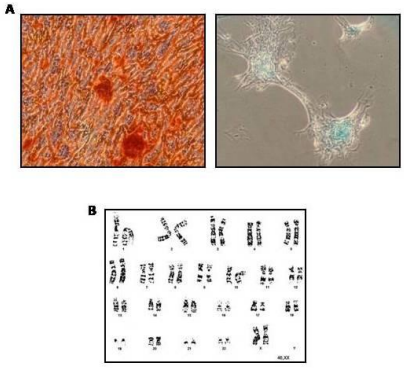

Figure 2. Analysis of Mesodermal differentiation and chromosomal stability of hUC-MSCs. A) Morphological image of osteogenic and chondrogenic differentiation of hUC-MSCs B) Karyotyping analysis (G-band) of chromosomal abnormality of hUC-MSCs

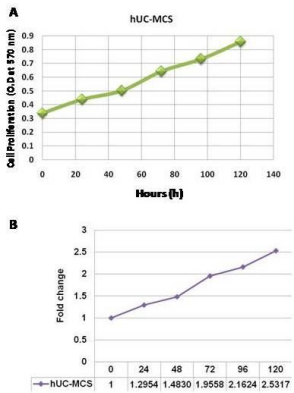

Figure 3. Growth kinetics of hUC-MSC at passage 3. A) hUC-MSCs proliferation rate at different time periods. B) Doubling time analysis of hUC-MSCs at passage 3.

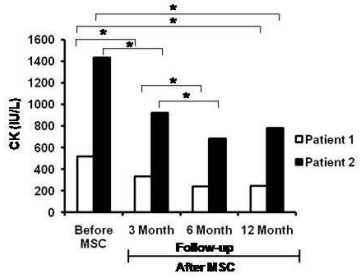

Figure 4. Therapeutic effect of hUC-MSC in Muscular Dystrophy (MD) patients by measure the plasma Creatinine Phosphokinase (CK). Patients were administered (i.m followed by i.v) one dose of hUC-MSCs and followed up for 12 months. Plasma levels of CK was measured on 3 months, 6 months and 12 months and the CK levels were significantly dropped after hUC-MSC treatment throughout the following periods.

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