Clinical outcome of the Intrathecal administration of the allogeneic side population Adipose Derived Mesenchymal Stem Cells in Werdnig Hoffman patients: An open-label Phase I clinical trial

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Research

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Abstract

Background

Werdnig Hoffman (WH), a hereditary neurodegenerative disorder of lower motoneurons associated with progressive muscle weakness is the most common genetic cause of infant mortality. There is no effective treatment for WH exists. The field of translational research is active now, and clinical trials or case studies are ongoing. We present a phase 1 clinical trial in patients with WH who received side population adipose-derived mesenchymal stem cells (SPADMSCs).

Methods

The intervention group administered with three intrathecal administrations of escalating doses of SPADMSCs. The safety analysis was assessed by controlling the vital signs and efficacy analysis performed by the Ballard score and EMG test. These tests were performed previous to treatment and at the end of the follow-up.

Results

The treatment well-tolerated, without any adverse event related to the stem cell administration. Patients showed significant improvement in the amplitude response of motor in the tibial nerve (0.56 mV; *p*: 0.029). The weight of patients, ventilation days, and number of hospitalizations were not meaningful parameters in the response of patients in the intervention and control groups. One patient in the intervention group is still alive after 36 months. He gained a normal weight and has a normal growth rate. The patient can breathe without ventilator aid.

Conclusion

The present study for stem cell therapy shows safety and efficacy in WH patients, mainly in the recovery of the tibial nerve, respiratory system, and length of life.

Background

Spinal Muscular Atrophy (SMA) resulting from the degeneration of lower α-motor neurons (MNs) with muscle wasting and consequent paresis, paralysis, respiratory failure, and frequently death in the severe form of childhood SMA [1]. Different types of SMA are classified based on the time of the disease onset during life. Werdnig-Hoffmann (WH) disease or the same SMA Type 1 presents within the first three months of life and is fatal only within two years [2]. It is always caused by the deletion or mutations in the survival motor neuron (*smn*) gene and is inherited in an autosomal recessive way [3]. A homozygous deletion of the exon 7 in the *smn1* gene has been demonstrated in more than 95% of these patients [4]

which present them nonsitters (unable to sit). The main product of the *smn* gene degrades in the cytosol and it causes a 100-fold lower concentration in the spinal cord of WH patients compared with the other people [5]. The centromeric human paralogue *smn* gene (*smn2*) is still present in these patients, but is not able to completely compensate for the lack of *smn1* [6, 7]. In spite of the worldwide efforts, there is only one approved treatment available for SMA patients, a gene therapy, by antisense oligonucleotides with a trading name Spinraza (Nusinersen) [8]. It corrects *smn* splicing, which in turn restore *smn* expression in MNs after intra-cerebroventricular injection, but there are still questions about its cost-effectiveness and its delivery difficulties [9, 10]. In many low-income countries with a prevalence higher than the global average, this has created deep concerns among the neurological society. In Iran, several campaigns launched to dispatch SMA patients to a European center to benefit from the therapeutic course of Spinraza, but due to the severe economic problems, these groups did not succeed in sending patients even as one-handed fingers. So we still in need of new cost-effective therapies that will be available to all SMA patients worldwide even in low-income countries.

As the alternative therapeutic approach, stem cells could be transplanted into the defective nervous system. We should consider that stem cell therapy costs are much less expensive than gene therapy and it is also possible for cell therapy in places with fewer facilities when compared to gene therapies that usually involved in virus production. The potential applications of stem cells in neurological disorders may be classified into several categories. Transplanted stem cells may provide trophic support to host cells [11], slow a degenerative process, or secrete neurotransmitters deficient in the host [12]. In contrast to other neurodegenerative diseases such as stroke or spinal cord injuries, which possess toxic microenvironments with a multifactor pathology that affects more than one target, in Werdnig-Hoffmann patients, the only defect is in spinal MNs; hence, the target for therapy is particularly identified [13, 14]. According to our knowledge, a clinical trial by California Stem Cell Inc. (Irvine, CA, USA) based on intra-spinal injections of high purity human motor neuronal progenitor cells put on hold by the US Food and Drug Administration [15]. But there is the same experience that completed for Amyotrophic Lateral Sclerosis (ALS) patients [16]. Due to the genetic condition of WH disease, allogeneic stem cell therapy could delay the disease advancement, as well as potentially restoring some lost functions [17]. Based on some studies mesenchymal stem cells (MSC) could activate endogenous restorative responses in the injured brain, including angiogenesis [18], neurogenesis [19, 20], synaptogenesis, and reduction of apoptosis in the injury zone [21]. However, so far there are some inexpressive studies using MSCs for WH patients. Previously an Italian group reported their negative findings of intrathecal administration of bone marrow derived MSCs in WH patients [22], but the other group claimed quantifiable improvements in physical function for three patients after intrathecal administration of MSCs [23]. We know that MSCs derived from adipose tissue (ADMSCs) show higher regenerative capacities compared to bone marrow derived MSCs [24]. They are safe for allogeneic use in pediatric patients [25] and show more anti-inflammatory response compared with MSCs derived from other sources [26, 27]. Also, previously we have isolated a side population (SP) of ADMSCs that shows superior characteristics when compared with the main population of fat-derived MSCs [7]. The SP cells are a rare subpopulation found in some adult tissues, identified by their capacity to efflux Hoechst 33342 dye [28]. This property that distinguishes

them from the main population of cells is mediated through the p-glycoprotein multidrug/ATP-binding cassette transporter protein and ATP-binding cassette superfamily G member 2 (ABCG2) on the cell membrane surface [29]. *ABCG2* is a conserved gene among stem cells from a variety of sources, even in embryonic stem cells [30]. It could be express at high levels in primitive stem cells and then downregulated with differentiation. Enforced expression of this gene directly confers the side population phenotype and cause a reduction in maturing progeny both *in vitro* and in transplanted cells [29]. Therefore, we decided to focus more precisely on the more potent side population ADMSCs (SPADMSCs).

While Werdnig-Hoffmann disease certainly is a fatal disease with great unmet therapeutic need, our team conducted this stem cell therapy program as a phase I clinical trial to decide whether this intervention continues in the country for the benefit of the patients. Of course, we tried to not create false hope for the parents whose children suffer from this life-limiting disorder. We also decided not only to do the same work of the earlier groups but upgrade those works by selecting SPADMSCs which have more proliferative capacity and higher differentiation potency in higher doses. Here we report the results of the safety and effectiveness assessment of the dose-escalating SPADMSCs intrathecal administration in Werdnig Hoffman patients.

Materials And Methods

The trend chart of this trial process is mentioned in Fig. 1. The study group consisted of 10 patients (*clinicaltrials.gov* identifier NCT02855112) with genetically confirmed *smn1* complete deletion at the mean age of 9 ± 3 months, from both genders. The mean patient age at the disease onset was 3 ± 1 months. Motor functions were evaluated using the modified Ballard score and electromyography (EMG). Activities included in the scale were rolling, sitting, lifting the head from prone and supine, and propping on arms. Each item scores 0–2 with 0 meaning unable, 1 meaning some adaptation and 2 meaningfully able [31]. Five patients were enrolled in the cell transplantation study, and five others served as the control with only their routine supportive care because we thought that a placebo-controlled trial is unethical (Fig. 1). All the patients included in this study aged under 12 months, with weak muscle tone, weakness in mobility, but with normal brain function and existence of home senses. The patients with the brain abnormality, loss of sensory functions and malignancies excluded from this study. Patient evaluations were performed during V_o visit before the stem cell administration begins. All parents of the patients gave their written informed consent for child participation in this study, and the protocol was approved by the Ethics Committee at the Tehran University of Medical Sciences (TUMS), Tehran, Iran (IRCT2015073023417N1).

Stem cell Donors

A total of three healthy adult donors who volunteered to donate their adipose tissue after their cosmetic liposuction surgeries were entered into the study. The subcutaneous adipose tissue samples were collected from each donor under local sedation. The study was conducted in compliance with current Good Clinical Practice (GCP) standards and in accordance with the Declaration of Helsinki. Institutional

review board approval of the TUMS for the study protocol was obtained before starting enrollment. All donors entering the program agreed to and signed an institutional review board-approved statement of informed consent.

Stem cell isolation, Culture and Characterization

The liposuction aspirates were obtained from the para-umbilical subcutaneous fat tissue site of the donors and immediately transferred to the GMP qualified clean rooms in the Iranian Blood Transfusion Organization (IBTO) central laboratory building. Isolation of stromal vascular fraction (SVF) and mesenchymal stromal cell cultures was prepared as described before [32]. Briefly, adipose tissue was washed five times with phosphate-buffered saline (PBS; Gibco, Paisley, UK) and suspended in an equal volume of HANKS balanced salt solution (HBSS; Gibco, Paisley, UK) supplemented with 0.05% Celase (Celase; Cytori, California, US). The tissue placed in a shaking 37 °C incubator with continuous agitation for 30 min and centrifuged for 10 min at 300 *g* at room temperature. Then the pellet (SVF) subjected to the culture in Dulbecco Modified Eagle Medium (DMEM; Gibco, Paisley, UK) with StemPro (Gibco, Paisley, UK) supplemented with 10% human umbilical cord-derived serum plus 1 µg/ml Gentamicin (Sigma; Saint-Quentin Fallavier, France), with 10 ng/mL vascular endothelial growth factor (VEGF; Sigma, St. Louis, US), 10 ng/mL epidermal growth factor (EGF; Sigma, St. Louis, US), and 2 mM L-glutamine (Gibco; Paisley, UK). After 6 h in fibronectin-coated culture flask, with 10% CO_2 and 95% humidity, the media was changed and non-adherent cells were removed. After 24 h only colony forming units were removed from the culture flask by a cell scraper under the stereomicroscope and transferred into sterile 2 mL micro-tubes and side population cells were isolated as previously described [30, 33, 34]. Isolated SPADMSCs were cultured on the new fibronectin pre-coated flasks. After 24 h a portion of the obtained cells was used in CFU assays to estimate a frequency of colony-forming units in the cell population. The remaining cells were plated in T175 flasks (Corning, NY, US) in the culture medium at a density of about 100,000/cm^2 of the surface area for expansion and culture. The media were changed every day and once 80% confluence had been reached, adherent cells were harvested by 0.25% TrypLE (Gibco; Dublin, Ireland), and then re-plated. Besides this way, the conventional method also was used to obtain a hetero-population of ADMSCs as described previously [7].

Quality control of final SPADMSCs product

For release testing, SPADMSCs were assessed for cell appearance, viability, identification, purity, and content. The minimum criteria for release was 90% cell viability. In addition, the cells were screened for contamination with adventitious agents, mycoplasma and other bacteria, fungi, and viruses such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-lymphotropic virus-1 (HTLV-1) and cytomegalovirus (CMV). We also monitored the SPADMSCs karyotype stability all throughout passaging in culture. Chromosome G-banding was used in this study to show the normal, 46 XX or 46 XY karyotype profile all throughout culturing. The endotoxin amount was evaluated by the gel-clot technique Limulus Amebocyte Lysate (LAL test; GenScripts, Leiden, The Netherlands) as described previously [35].

SPADMSCs characterization

To analyze immunophenotypical characteristics of the SPADMSCs, passage three stem cells were analyzed by flow cytometry for hematopoietic lineage cell markers CD34, CD19, and CD45 (Abcam, Cambridge, UK) and for stromal cell markers CD105, CD90, CD73, CD146 (Abcam, Cambridge, UK) and for pluripotency markers SSEA-1 and Nanog (Abcam, Cambridge, UK) by using directly conjugated fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC) antibodies (Becton-Dickinson Biosciences; NJ, US) on a BD FACS Calibur using CELLQuest acquisition software.

SPADMSCs CFU & Proliferation assay

The same number of ADMSCs and SPADMSCs were respectively seeded in T-25 flasks. The medium was changed every 24 h. After three days in culture, the flasks were washed twice, fixed with absolute methanol, and stained with 3% Crystal violet. Three replicates were used for the experiments to obtain a mean value. Cell clusters consisting of at least 50 cells were scored as a colony forming unit (CFU). In the proliferation test, the isolated cells were respectively seeded in triplicate in 24-well plates. Three replicas were performed for each cell population. Twenty microliters of sterile cell counting kit-8 (Beyotime; Jiangsu, China) that allows sensitive colorimetric assays for the determination of cell viability were added to each well and incubated for 3 h at 37℃. The viable cell numbers were determined every day for one week. The optical density values were determined at a wavelength of 450 nm and a reference wavelength of 630 nm.

SPADMSCs in vitro differentiation

Each source of stem cells was plated at a density of 10^4 cells in the 6 well plates; culture medium was then replaced with the specific differentiation media, which was changed twice a week for the full induction period. For the Adipogenic differentiation, confluent stem cells at passage 4 were differentiated in a chemically defined serum-free media containing 2 nM triiodothyronine, 100 nM human insulin, 100 nM dexamethasone, and 1 mM rosiglitazone, as previously reported [36]. Osteogenic differentiation was induced as previously described [37]. Briefly osteogenic medium containing DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 ng/mL L-thyroxine (Sigma, US), 20 mM β-glycerol phosphate, (Sigma, US), 100 nM dexamethasone (Sigma, US) and 50 μM ascorbic acid (Sigma, US). The medium was changed every 3 days. After 15 days, cells were fixed in 9% paraformaldehyde and stained with 10% Alizarin Red (Sigma, US). Differentiation into chondrocytes was induced by Chondrogenesis Differentiation Medium, according to the previous study [38]. The stem cells were incubated in 500 μl of complete chondrogenic medium containing DMEM, 100 nM dexamethasone, 50 μg/mL ascorbic acid, 40 μg/mL L-proline (Sigma, US), 1 mM sodium pyruvate (Sigma; US), 10 ng/mL TGFβ-3 (Sigma, US) and 100 ng/mL BMP-2 (Sigma, US). The medium was changed three times a week. After 21 days the pellets were harvested, fixed and stained with Alician blue. The neurogenic differentiation of the stem cells was induced, as described previously [39], with some modifications [7]. All the differentiation genes were evaluated using RT-PCR.

SPADMSCs administration

The neurologists of the staff visited all the eligible patients and summoned them for filing their documents at the recruitment center. All clinical procedures were performed at the Children Medical Center (CMC) hospital in Tehran, Iran. Before transplantation, cells were counted and suspended in phosphate buffered saline (PBS; Gibco, Paisley, UK) at the concentration of 2×10^4 cell per µL. The cells administered by a 21-gauge needle via the intrathecal route in the lumbar puncture. Patients received three doses of 10^6, 2×10^6 and 5×10^6 cells/kg into the lumbar spinal region every 15 days. After injection, the patients admitted to the Intensive Care Unit for 1 h. They would be discharged if did not have any sign of allergic reactions, fever, and malaise.

SPADMSCs Safety assessment

The primary outcomes evaluated the safety of SPADMSCs, which consisted of assessing the reported side effects after lumbar puncture until discharge from hospital and lifetime follow-up of patients. Every 2 weeks, a full history was taken from the patients. The safety of the stem cells was evaluated based on the WHO toxicity scale, where the severity of adverse events is divided into four grades, namely mild, moderate, severe, and life-threatening. Based on this scale cardiovascular, respiratory, digestive, neurological, skin, and systemic (infection and allergic reactions) evaluations were done for all patients. Potential, delayed adverse events included intra-spinal tumor formation, and persistent sensory loss or paralysis not related to the progression of the disease was assessed as well.

SPADMSCs Effectiveness Outcome assessment

The secondary outcome measures were the difference in functional outcomes measured by the electromyography (EMG) test. The Neurophysiologic test was performed by an electromyographer. The Nihon Kohden electrodiagnostic instrument (Neuropack S3 electrodiagnostic (EDX) system; Irvine, US) was used to perform the test. For the nerve conduction velocity, we used adhesive surface electrodes and children's specific stimulator electrode and EMG was performed using concentric single-use needle. Before starting the neurophysiological tests, the temperature of the organs was checked and, if the temperature was low, the organs were heated using an infrared lamp or a hot pack. The tests that were evaluated included: 1) NCV: A) motor responses: recording of tibial and peroneal nerves in the lower extremities, median and ulnar nerves in the upper extremities and B) Sensory responses: Record of Median nerve in the upper extremities, Plantar medial nerve in the lower extremities; 2) EMG: A) Evaluation of distal and proximal muscles in upper and lower extremities and cranial muscles.

Statistical analysis

Statistical analysis was performed using the Prism 5.04 (GraphPad, USA) software. Relationships between the variables were confirmed using non-paired *t*-test and Mann–Whitney U test. The *p*-value of ≤ 0.05 was considered statistically significant.

Results

Flowcytometry analysis

To find the antigen expression profile of the established SPADMSC, immunophenotypical analysis using fluorescently labeled antibodies against a panel of markers including CD34, CD19, CD45, CD90, CD146, CD73, and CD105, which are surface markers associated with hematopoietic and MSCs that found in adipose tissue was performed (Fig. 2A). Also, we found the expression of SSEA-1 and Nanog, the embryonic stem cell (ESC) markers, on SPADMSCs (Fig. 2A). The flow-cytometry results showed that the main population ADMSCs culture after the first passage still reacted with anti-CD34 (2.20 ± 1.0%), CD19 (1.0 ± 0.3%) and CD45 (2.50 ± 0.6%) monoclonal Abs (Fig. 2B). In contrast, SPADMSCs were negative for these markers (0.50 ± 0.2%, 0.32 ± 0.1% and 0.64 ± 0.1% respectively) (Fig. 2A). Furthermore, the percentages of cells positive for CD146 (94.11 ± 1.2%) and CD105 (97.23 ± 1.62%) in SPADMSCs were higher than the control group ($p \leq 0.05$) (Fig. 2B). The important part of the marker expression returns to the SSEA1 and Nanog expression of SPADMSCs (66.42 ± 1.50% and 58.15 ± 1.22%) was much higher than conventional cultured main population ADMSCs (Fig. 2B).

Clonogenicity assays

Clonogenicity results showed that SPADMSCs displayed significantly more colony forming units (CFU) than the control group *in vitro* (Fig. 2C). The side population cells yielded a significantly higher number of CFU colonies (74.25 ± 2.55%) than the main population ADMSCs (36.65 ± 1.40%) ($p \leq 0.05$) (Fig. 2C). In addition, SP cells had a higher proliferation rate compared to other cells in 3, 6 and 10 days ($p \leq 0.05$). However, these cells had a very small size when compared to the main population ADMSCs (Fig. 2D).

Differentiation assays

The adipogenic culture was stained for the presence of intracellular lipid droplets. Oil Red staining of cultures for intracellular lipid vacuoles revealed these droplets while there was no sign of adipogenic differentiation in control cells (Fig. 3A). Under chondrogenic differentiation conditions, the SPADMSCs formed regions rich in sulfated proteoglycans as detected using Alcian blue staining (Fig. 3A). These regions are indicative of the presence of sulfated proteoglycans within the matrix, where no positive staining was observed in the control cultures. Under osteogenic differentiation conditions, the SPADMSCs formed regions rich in osteocalcin that revealed apparently with alizarin red staining (Fig. 3A). The neurogenesis of SPADMSCs also can be seen under a fluorescent microscope (Fig. 3A) by the expression of nestin as a pre-neural and β-III-tubulin as neural cell markers. RT-PCR analysis showed that adipocyte-related transcripts, including lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor γ2 (PPARγ2), were induced in differentiated SPADMSCs (Fig. 3B). The differentiated cultures showed marked induction of mRNAs from chondrogenic genes, including *sox9*, *col2A1* and *col11A1* genes, and osteogenic expressed genes including *osteocalcin* and *alp* genes that significantly increased (Fig. 3B).

Chromosome stability

An aliquot of the same stem cells that were injected into the patients' cerebrospinal fluid was fixed the day of the intervention and tested for karyotype stability. All of the SPADMSCs used in this study showed a normal chromosomal asset (Fig. 3C).

Patients outcome

A total of ten pediatric patients with confirmed 5q13 deletion of the *SMN1* gene entered into this study. In both groups, there were 3 male and 2 female patients, and there was no difference in gender between the two groups (Fig. 4A). Electro-diagnosis showed decreased compound motor unit action potentials. Five of them received stem cell intervention. The principal characteristics of the recruited patients are described in Fig. 4. Their median age was 9 months (Range: 6–12). The median duration of the disease from the onset of symptoms to recruitment was 5 months (Range: 3–7). The treatment caused no severe adverse events in the intervention group. All patients were discharged without problems in the hospital and showed no immediate, post-injection complications. The most common adverse event was pain and it was confined to the injection site. Various items including the weight, number of admissions and the number of days under ventilation were assessed and compared with the control group. The weight of all patients in the intervention and control groups after 3 injections was evaluated (Fig. 4B). The weights of patients in the stem cell treated group were 7.6 kg, whereas, in the control group, the mean weight was 5.6 kg (p: 0.48) (Fig. 4B). Patients in both groups were evaluated for the number of hospitalizations times. In the intervention group, one patient was intubated and hospitalized from the beginning of the study to the end of her life. Among other patients in this group, the average number of admissions was 3.4 days Vs 4 days in the control group (Fig. 4C). There were no differences between the two groups of cell therapy and control in terms of the number of hospitalizations. In other words, transplantation does not seem to play a role in the development of various complications of the disease and the resulting hospitalizations (p: 0.182) (Fig. 4C). Both groups were also evaluated for the number of days that were under ventilation (Fig. 4D). In the intervention group, one of the patients had a tracheostomy, which was not included in this study. Another patient in this group was intubated twice. At the first time, she extubated immediately after cellular therapy due to the stability of the patient's general condition. For this reason, the first occurrence of ventilation in calculations was not considered. Generally, in the cell therapy group, patients had an average of 46.75 days of ventilation, while in the control group, they were on average 17.3 days under ventilation (Fig. 4D). No difference was observed between the two groups of cell therapy and control in terms of the number of days under ventilation (p: 0.56) (Fig. 4D). Clinical assessments ranging from 3 to 28 months after SPADMSCs transplantation (in survived patients) showed no acceleration in the course of progression of the SMA due to the treatment. Out of 10 patients in this study, one of those who have been in the intervention group is still alive, and the rest of the patients, in both groups have died as described in Fig. 5A. Most of the patients died due to the progression of respiratory failure related to the natural course of the disease. The mean life expectancy of the intervention group was 11.17 months and the mean lifetime of the control group was 8.52 months (Fig. 5A).

The Ballard Scoring System was used to evaluate the clinical changes. After each injection, the Ballard score of the patients in the injection group was calculated and then the score of the control group was measured at the same interval (every two weeks). The mean Ballard score of the patient was 10.6 after the first injection and in this time the mean score in the control group was 9.2 (p: 0.58) (Fig. 5B). The

mean score after the second injection in the transplant group was 11 and in the control group was 9.6 (*p*: 0.32) (Fig. 5C). Also, the mean scores after the third injection in the transplant group was 11.6 and in the control group, was 9.6 (*p*: 0.42) (Fig. 5D).

In this study, patients were subjected to neurophysiologic tests three times. The first test before injection was used to confirm the diagnosis and to obtain the baseline data. The second test was performed one month after the first injection and the third test was performed one month after the last injection (Table). The mean amplitude of the median nerve motor response was 0.71 mV in the intervention group (SD: 0.2) while this mean amplitude was equal to 0.31 mV (SD: 0.19) in the control group. In the case of the ulnar nerve, the mean amplitude response of the motor in the cell therapy group was 0.38 mV with a standard deviation of 0.13 and in this interval, the mean ulnar nerve amplitude in the control group was 0.28 mV (SD: 0.24). In the lower extremities in the Tibial nerve, the mean of the amplitude response of the motor in the cell therapy group was 0.54 mV and with a standard deviation of 0.19 while in the control group, this mean was 0.28 mV (SD: 0.19). In the peroneal nerve, the mean amplitude of the motor response was reported 0.81 mV in the intervention group (SD: 0.13) and in the control group the mean amplitude response was 0.96 (SD: 0.13). After the third stem cell administration, only the tibial nerve was significantly different (*p*: 0.029) between the patients in the intervention group and the control group (Table). As mentioned earlier, one patient is alive after follow-up for up to 28 months and he had an improvement in chest muscle movements so his breathing takes place with the chest muscle contraction and relaxation. He has an ordinary growth pattern and he wants no more intubation.

Discussion

Werdnig Hoffman is an untreatable neuromuscular disorder caused by reduced expression of the SMN protein, leading to the loss of MNs in the spinal cord. Conducting clinical trials in WH patients is extremely difficult due to the medical fragility of these patients and the frequent development of respiratory illnesses among these children. Stem cell therapy is a potential therapeutic approach for spinal muscular atrophies, as it results in the activation of molecular and cellular mechanisms that support endogenous neuronal activities and protects against neurodegeneration.

As with all clinical trials in the first phase, what is important is the safety of the intervention. Our study showed that allogeneic side population adipose-derived mesenchymal stem cells in patients with Werdnig Hoffman is safe. In this clinical trial, the cell expansion process did not involve any alteration to the genome of the cells in any of the cases. In 2015 Villanova and Bach reported the safety of allogeneic mesenchymal stem cells from different sources and protocols in three patients with WH [23]. Selection of the proper cell type and administration of the appropriate amounts of cells is very important. The cells must exhibit the characteristics of neuroprotection while avoiding the potential for tumor formation [40]. Also, issues regarding cell graft survival must be examined within the transplanted microenvironments, as well as immune rejection potential. Many studies show us that while MSCs move into areas of damage, they only survive for a short period and cannot be found in the CNS parenchyma a few weeks

after administration [41]. However, when MSCs directly injected into the sciatic nerve following a crush injury prevented denervation of neuromuscular junctions and improved motor performance [42].

Recent reports using bone marrow stem cells in the clinical trial of ALS has shown preliminary hopeful results in the motor performance of transplanted patients [43–45]. This indicates that trophic factors could recover and stimulate the function of MNs, or rescue neurons in reversible phases of cell death. Surely we had no expectation of the differentiation of SPADMSCs into motoneurons. The confined differentiation of stem cells in the spinal cord has been previously reported using neural stem cells [46, 47], while, these stem cells were able to differentiate in the brain [48, 49]. Probably the spinal cord has not an inductive capacity to develop a neural phenotype from undifferentiated stem cells. However, It should be noted that the beneficial effects due to neurotrophic factors were first described in the 80's [50].

For the first time, stem cells transplantation was used in a SMA model as a putative therapeutic pathway in 2008 [51]. In this clinical trial, we have chosen a population of the MSCs because of their immunomodulatory capacity to neutralize neurotoxins and exert their neuroprotective potential by producing bioactive neurotrophins. Also, we counted on the possibility of stimulating local progenitor cells to replace SMN1 protein. Motor neurons are unlikely to be the only cellular population of the nervous system that is affected in SMA. Disrupted sensory pathways have been described in severe SMA patients [52]. Sometimes the disruption of functional connectivity between sensory and motor neurons in the spinal cord occurs that can worsen the patients' disease phenotype. So not only protecting the motoneurons but also preventing the destruction of these sensory neurons in the spinal cord may play an important role in improving the phenotype of WH patients.

In 2012, Carrozzi *et al.*, reported no benefits from bone marrow MSC infusions for five WH patients [22]. They have reported the outcome in less than one month of cell administration and subsequently other investigators have denied the validity of earlier positive outcomes of cell therapy for WH patients on the basis of Carrozzi report [53–55].

Based on the frustrating results obtained so far, WH patients currently have little choice for effective treatments, which is restricted to expensive gene therapy, so clinicians have been limited to only treat the secondary complications arising from the disease especially pulmonary complications that lead the patients to death. This study on five patients with WH shows that transplantation of SPADMSCs into the lumbar spinal cord appears to be a safe procedure that causes no major, short- or medium-term, deleterious effects. No patients suffered side effects from SPADMSC treatment except for mild fever in the two patients under the intervention. All patients in the control group died within an average of nine months (Fig. 5A), but in the cell therapy group, one patient is alive after follow-up for up to 24 months and he has improved chest muscle movement. This patient, who was also weightier than the other patients, received the highest SPADMSCs dose (on his weight) than others (Fig. 4B). Since the adipose tissue donors in this project were all female, therefore, the donor gender cannot be considered as one of the influencing factors in the differences between mesenchymal stem cell function.

Our findings emerge from the similar clinical protocols that are comparable to these studies, but we, in turn, employed a more potent side population cells and much higher cell dosage. The first founded result is that the CSF cell injection in human appears to be a safe, reproducible and reliable procedure. Also, the perspective improvements were made here, particularly concerning the number of administered cells, that in our study was five times higher than in previous approaches – a maximum of 5×10^6 cells/kg used here versus the earlier 1×10^6 cells [23]. It is possible to administer this amount of cells in a volume of only 3 ml using SPADMSCs whereas, it is impossible to use this dose of MSCs if it was prepared by the conventional methods.

Careful attention must be paid to the technical aspects of cell delivery within the nervous system. As MSCs could pass the blood-brain barrier we did chose intrathecal delivery of the cells to get the most effect of injected cells on the desired site. In many studies, the stem cells were injected intravenously or in combination with intrathecal route [23]. In the intravenous (IV) delivery route MSCs mostly distribute in the lungs, and then in the spleen, liver, bone marrow, thymus and kidney they will spread [56]. Other studies showed that the total body count of labeled MSCs by bioluminescence method revealed a decrease from the intensity measured at fifteen minutes post IV infusion to about 60% after 24 hours and less than 10% after 72 hours [57].

Electrophysiological outcomes include the estimation of the numbers of motor units and motor action potential amplitude. The increase in motor action potential amplitude captures both an increase in the number of motor units and the presence of collateral sprouting of axons from adjacent surviving motor neurons [58]. In WH patients spontaneous rhythmic firing of motor units have been recorded and it shows some short and low amplitude potentials in addition to long duration, high amplitude potentials [59]. In our study, there were no significant differences in the amplitude of motor responses in any of the upper and lower extremity nerves between the two groups after the first injection. We found that in the tibial nerve, the mean of the amplitude response of the motor in the cell therapy group was 0.54 mV with a significant difference to the control group after the third injection (Table). This may have occurred through the release of neurotrophic and growth factors, cytokines and immunomodulatory molecules that diffuse into the pathological tissue, thereby eliciting neuroprotective effects. Also, there are pieces of evidence now exist that neural stem cells, which integrate and survive in the brain parenchyma, exert their immunomodulation ability through secretion of extracellular membrane vesicles or exosomes, influencing the microenvironment through the traffic of bioactive molecules [60, 61]. Xin and colleagues showed that intravenous injection of MSC-derived exosomes induces neurogenesis, neuronal repair, and angiogenesis after stroke [62]. In addition, mesenchymal stem cell-derived exosomes induce axonal growth through the transfer of microRNAs to the neuronal cell body, which may be a novel approach in the treatment of progressive neurodegenerative diseases [63].

We have been able to reproducibly expand SPADMSCs *ex-vivo*. Methodological varieties in the isolation, processing, delivery, and the assessments between those referred to in the letter and those in our trial could have played an important role in the difference in patients' treatment outcome. This trial is actually based on the use of only three stem cell donors. The use of such a limited number of donors inherently

reduces the inter-treatment variability that may arise from the implantation of different stem cell lines into different patients.

Very few studies in the scientific literature report the results of clinical trials with stem cell transplantation for Werdnig-Hoffman patients. These results are not definitive and no trial has been replicated in multiple centers. Therefore, we believe that instead of preventing these kinds of clinical trials, it is better to learn from the previous trials, develop more efficient methods of safe cell therapy for these patients and to investigate them as a multi-center study.

Conclusion

The results from this trial describe a cell therapy platform that will allow broadening the number and reproducibility of cell therapy clinical trials for SMA and, other neurological disorders. These investigations can now be carried out under standardized conditions and will be based on a more homogenous repertoire of clinical-grade, allogeneic side population mesenchymal stem cells, which also lacking any ethical concerns. One case in this study demonstrated objective and quantifiable improvements in physical function and suggest that allogeneic SPADMSC infusions may be an effective treatment. Although it seems that the use of stem cells leads to results that are not comparable to the ones obtained with molecular therapy, but our conclusion is that stem cells and gene therapy could be joined to reach the cost-effective and best therapeutic effects.

Abbreviations

Adipose Derived Mesenchymal Stem Cells (ADMSCs); Allo-phycocyanin (APC); Amyotrophic Lateral Sclerosis (ALS); ATP-binding cassette superfamily G member 2 (ABCG2); Colony Forming Unit (CFU); Cytomegalovirus (CMV); Central Nervous System (CNS); Dulbecco Modified Eagle Medium (DMEM); Electromyography (EMG); Fluorescent Activated Cell Sorter (FACS); Fluorescein isothiocyanate (FITC); Good Clinical Practice (GCP); Good Manufacturing Practice (GMP); HANKS balanced salt solution (HBSS); Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human immunodeficiency virus (HIV); Human T-lymphotropic virus-1 (HTLV-1); Iranian Blood Transfusion Organization (IBTO); Intravenous (IV); Lipoprotein Lipase (LPL); Motor neurons (MNs); Mesenchymal Stem Cells (MSC); Nerve Conduction Velocity (NCV); Peroxisome Proliferator-Activated Receptor γ2 (PPARγ2); Phosphate-Buffered Saline (PBS); Phycoerythrin (PE); Real time polymerase chain reaction (RT-PCR); Spinal Muscular Atrophy (SMA); Survival Motor Neuron (*smn*); Stromal Vascular Fraction (SVF); Side population adipose-derived mesenchymal stem cells (SPADMSCs); Werdnig Hoffman (WH); World Health Organization (WHO).

Declarations

Consent for publication

All the consent forms are signed by the parents of the patients or their legal representatives. A copy of the signed consent forms is available in the supplementary file section.

Availability of data and material

All of the data and material is available in the Pediatrics Cell Therapy Research Center of the Tehran University of Medical Sciences. The data will be available on the ClinicalTrials.gov with the Identifier no: NCT02855112.

Funding

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Competing interest

All authors declare that: There is no conflict of interest.

Acknowledgment

The authors thank the anonymous referees for their time.

Authors Contribution

RM carried out cell harvest, culture and characterization, AH participated in the design and conduction of the study, AS participated in quality control test and final approval of the stem cell products, MG carried out EMG and NCV, AM selected the patients according to the criteria, MM visited all patients, JA wrote the manuscript draft, MN carried out immunophenotyping, RS participated in visiting patients, HM participated in stem cell administration to patients, MRA participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethical Approval

This study approved by the ethical committee of the Tehran University of Medical Sciences and the Iranian Ministry of Health and Medical Education (MOHME) Ethics board, Tehran, Iran.

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the institutional animal care guidelines of Tehran University of Medical Sciences and approved by the administration committee of Experimental Animals, Tehran, Iran.

Statement of Informed Consent

The informed consent forms were filled by the legal guardian (parents) of the all participants in our intervention. All donors entering the program agreed to and signed an institutional review board-approved statement of informed consent.

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Tables

Table. Relationship between the amplitude motor responses in the EMG between the two groups after the third SPADMSC administration.

Nerve	***Median***	***Ulnar***	***Tibial***	***Peroneal***
Intervention group	0.71 mV	0.38	0.54	0.81
Control group	0.31 mV	0.28	0.28	0.96
p Value	0.118	0.360	0.029*	0.182

Figures

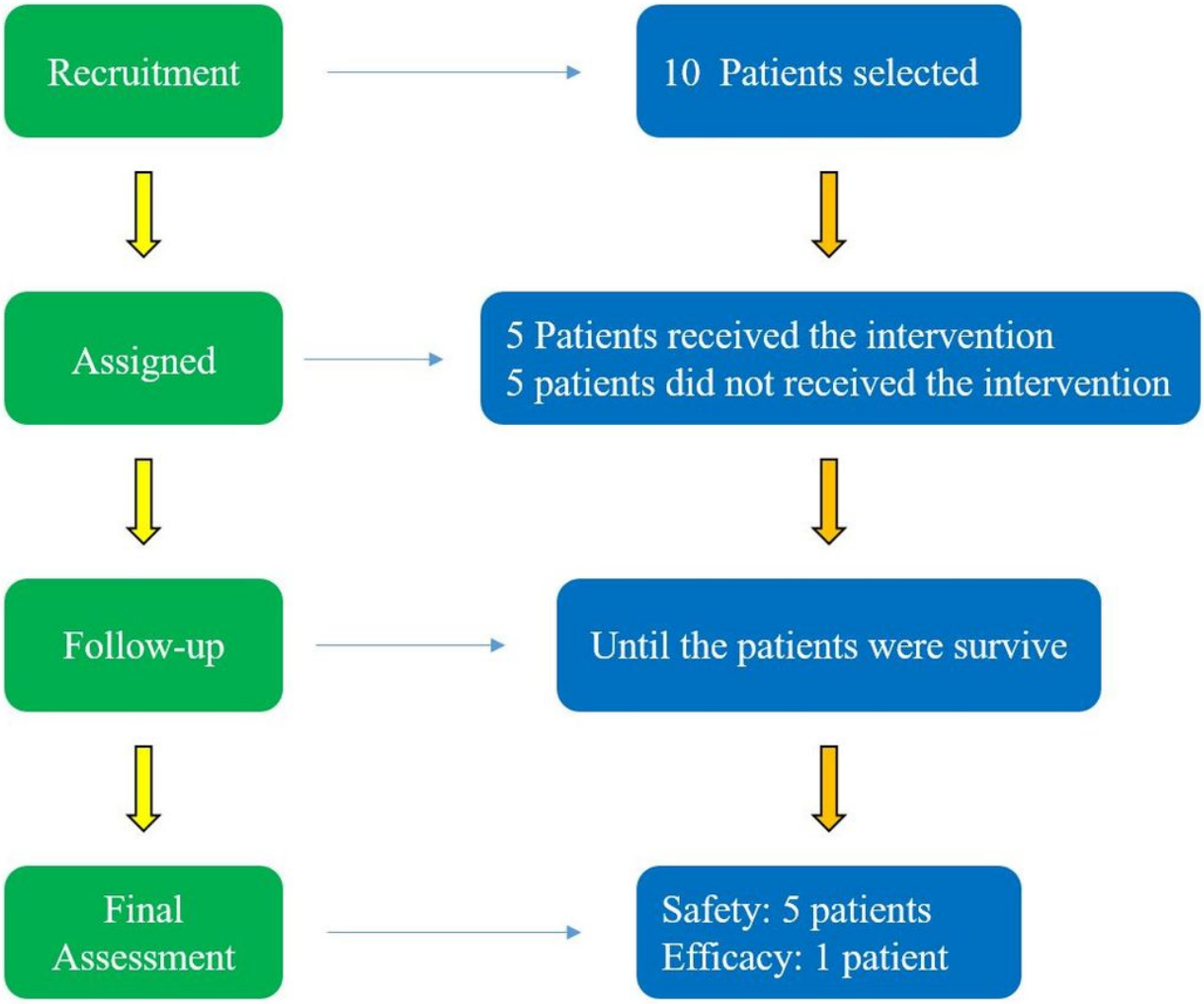

Figure 1

Trend chart of the passed process in this trial.

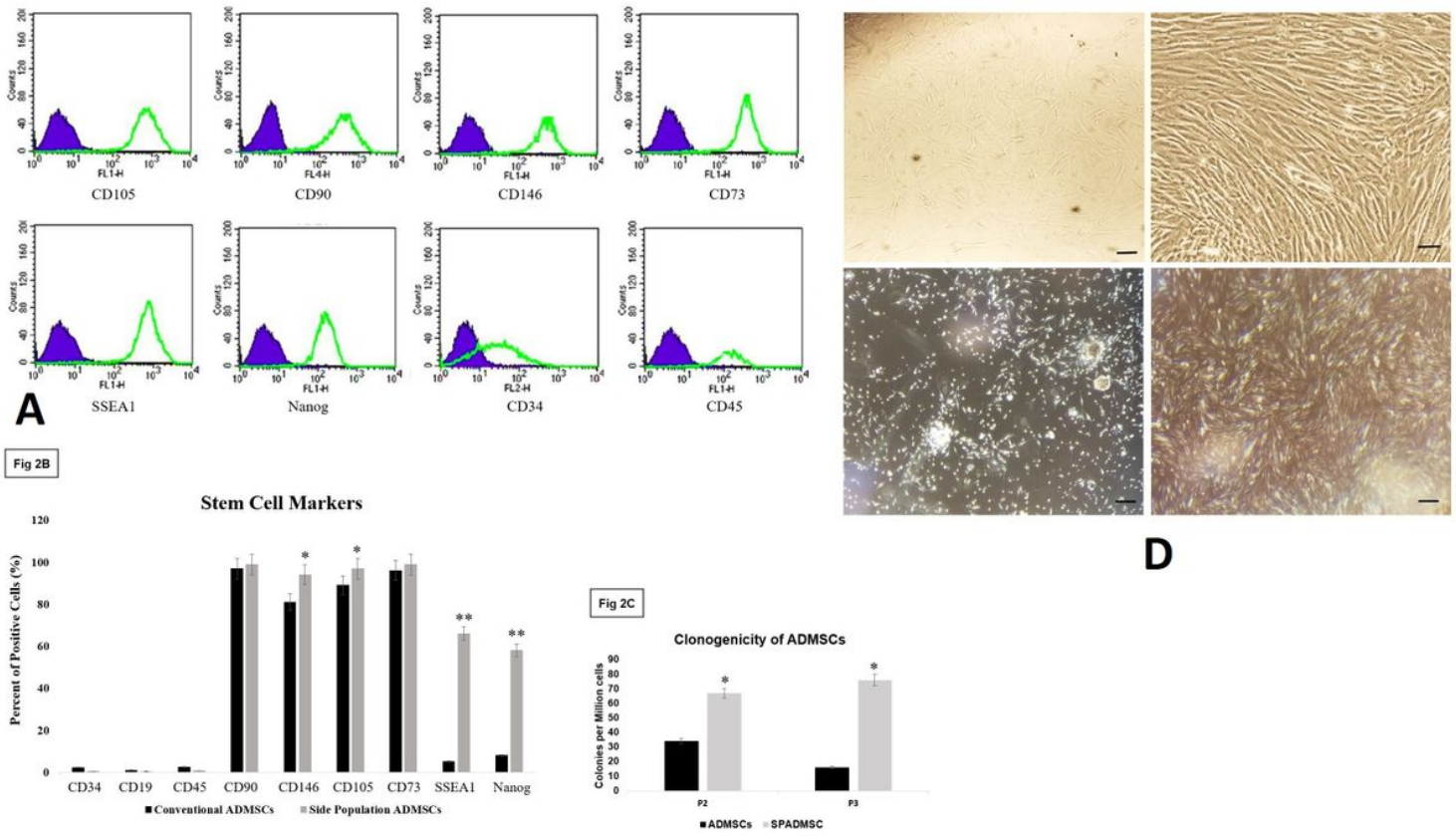

Figure 2

(A) Flow-cytometric analysis of SPADMSCs. The flow-cytometric analysis of CD34, CD45, CD90, CD146, CD105, CD73, SSEA1 and Nanog marker expres¬sion on side population ADMSCs. =Data represented mean ± SD *p ≤ 0.05 (n=3). (B) Comparison of the marker expression. The diagram shows the differences in the marker expression between SPADMSCs and conventionally isolated ADMSCs. Data represented mean ± SD *p ≤ 0.05; **p ≤ 0.001 (n=3). (C) Clonogenicity of SPADMSCs. CFU frequencies among SPADMSCs versus ADMSCs at passages 2 and 3. Data are the mean ± SD of at least three independent experiments. *p < 0.05. (D) Morphology of ADMSCs and SPADMSCs. The top row in this image shows the bright field microscopy of isolated and unstained primary population of ADMSCs in the second (left) and third passages (right) and we can see the SPADMSCs (bottom row) which formed multi colonies in the second passage (left) and filled the flask so fast at the third passage (right) when compared to the conventional ADMSCs cultures

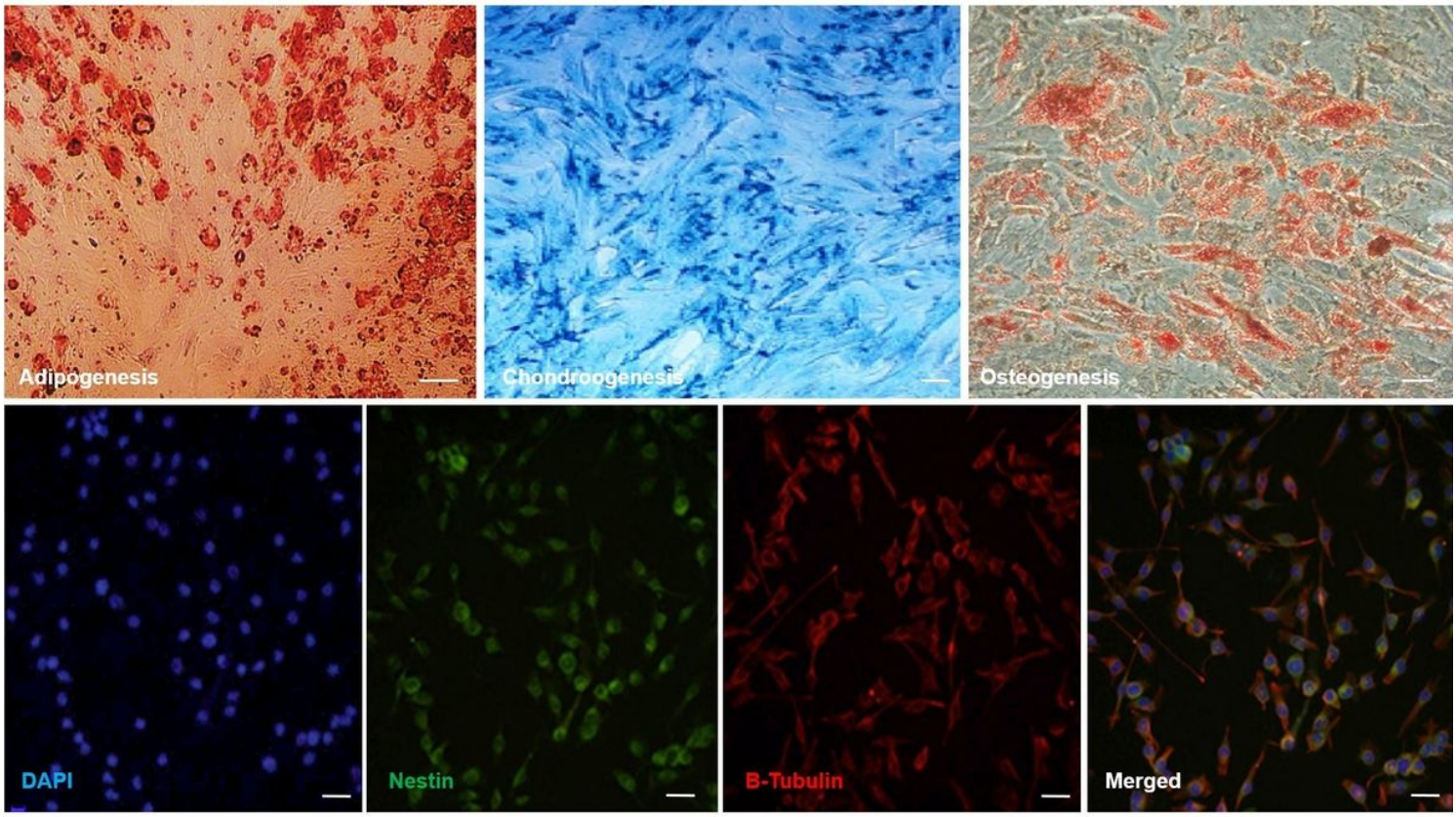

Fig 3B

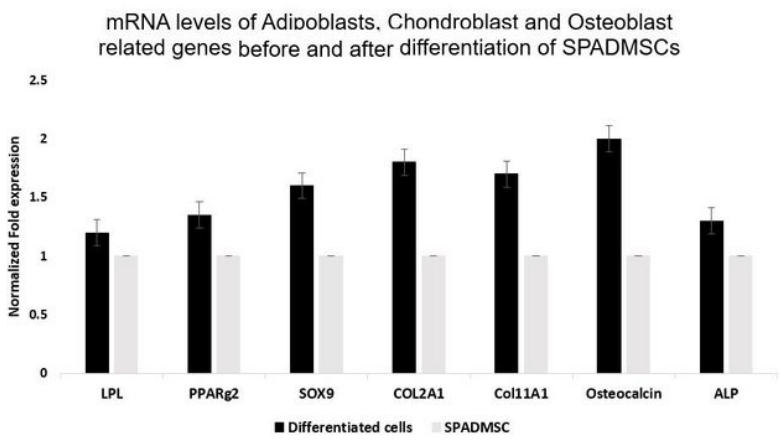

Fig 3C

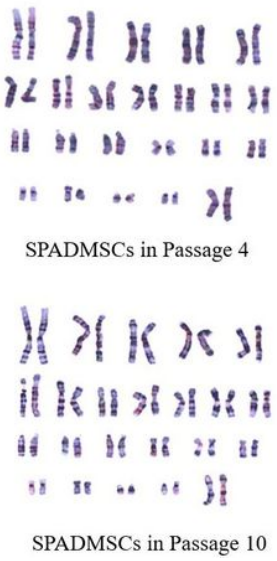

Figure 3

(A) Microscopic detection of the SPADMSCs Differentiation. The multilineage differentiation ability of SPADMSCs detected in cultures. Increasing in neutral lipid accumulation following adipogenic differentiation which stained with Oil Red. Chondroblast differentiation confirmed with Alcian Blue staining and Osteogenesis was pointed out with Alizarin Red staining. Neurogenic differentiation confirmed by immunofluorescence staining of pre-neural and neural cell markers by monoclonal antibodies towards nestin and β-III-tubulin (Barr: 20 μm). (B) Molecular detection of the SPADMSCs Differentiation. RT-PCR analysis revealed lipoprotein lipase (LPL) and peroxisome proliferator-activated

receptor γ2 (PPARγ2) over-expression after adipogenic differentiation. Induction of SOX9, COL2A1 and COL11A1 genes were obvious after chondrogenesis, and osteocalcin and ALP genes were expressed after osteogenic induction of SPADMSCs. (C) Karyotype analysis. Representative karyotype analysis of SPADMSCs at culture passages 4 and 8 evidencing stability and no alteration in chromosomes in these cultures.

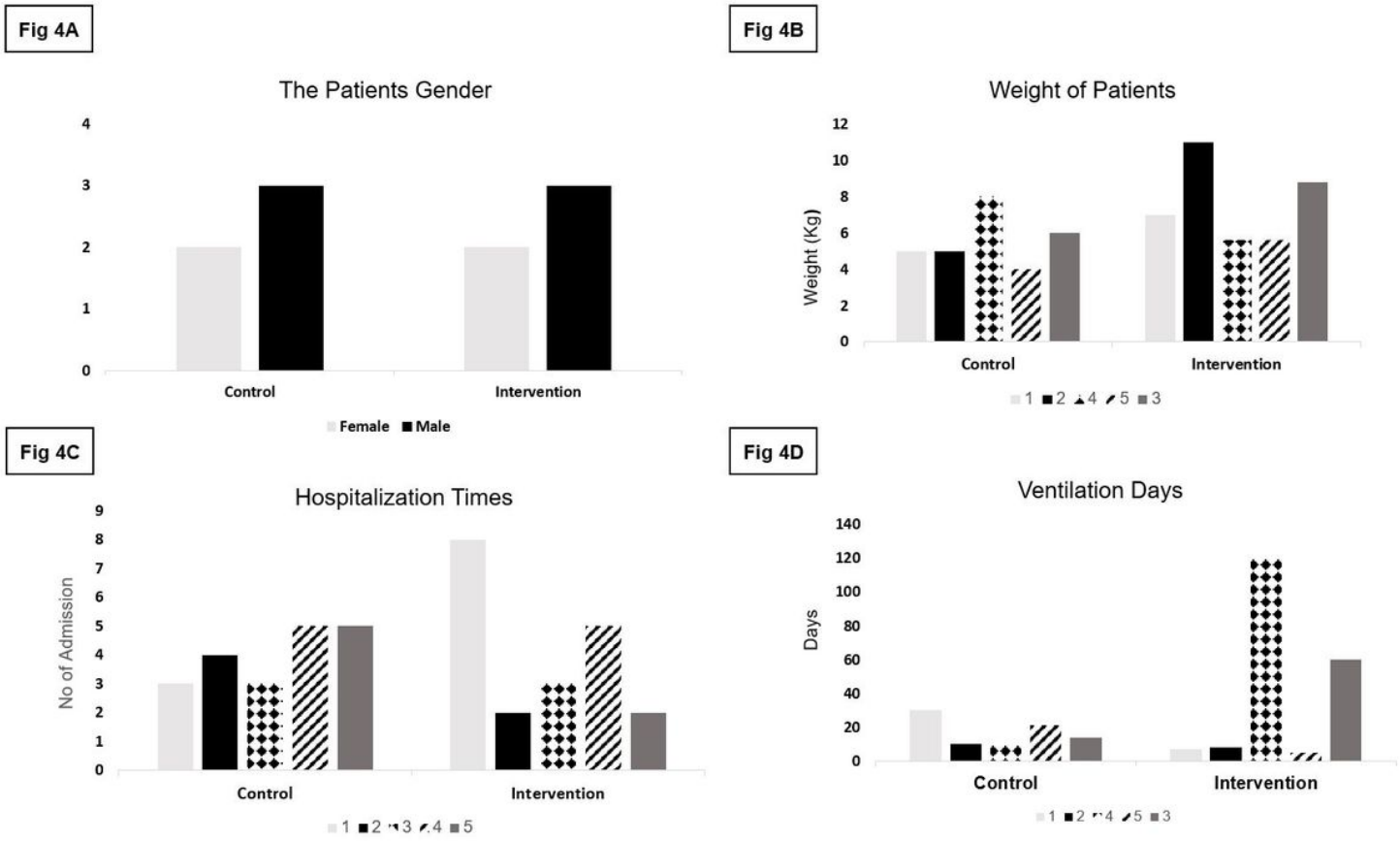

Figure 4

(A) Gender of patients. Of the 10 patients entered into the clinical trial, 5 patients were enrolled. The progress of disease and symptoms was also examined in 5 other patients as controls. In both groups, the gender of patients was the same. (B) Weight of patients. The weights of individuals in the intervention group were 7.6 kg, whereas, in the control group, the mean weight was 5.6 kg. There was no difference in the weight factor between the two groups in this study (p: 0.48). (C) Number of Hospitalizations. The number of admissions in hospital for each study subject (case 4 in the intervention group was admitted in the hospital to the end of her life). (D) Ventilation days. The number of days in which the patients in the intervention and control groups were under ventilation due to the respiratory problems (Patient 2 in the intervention group had tracheostomy from the beginning).

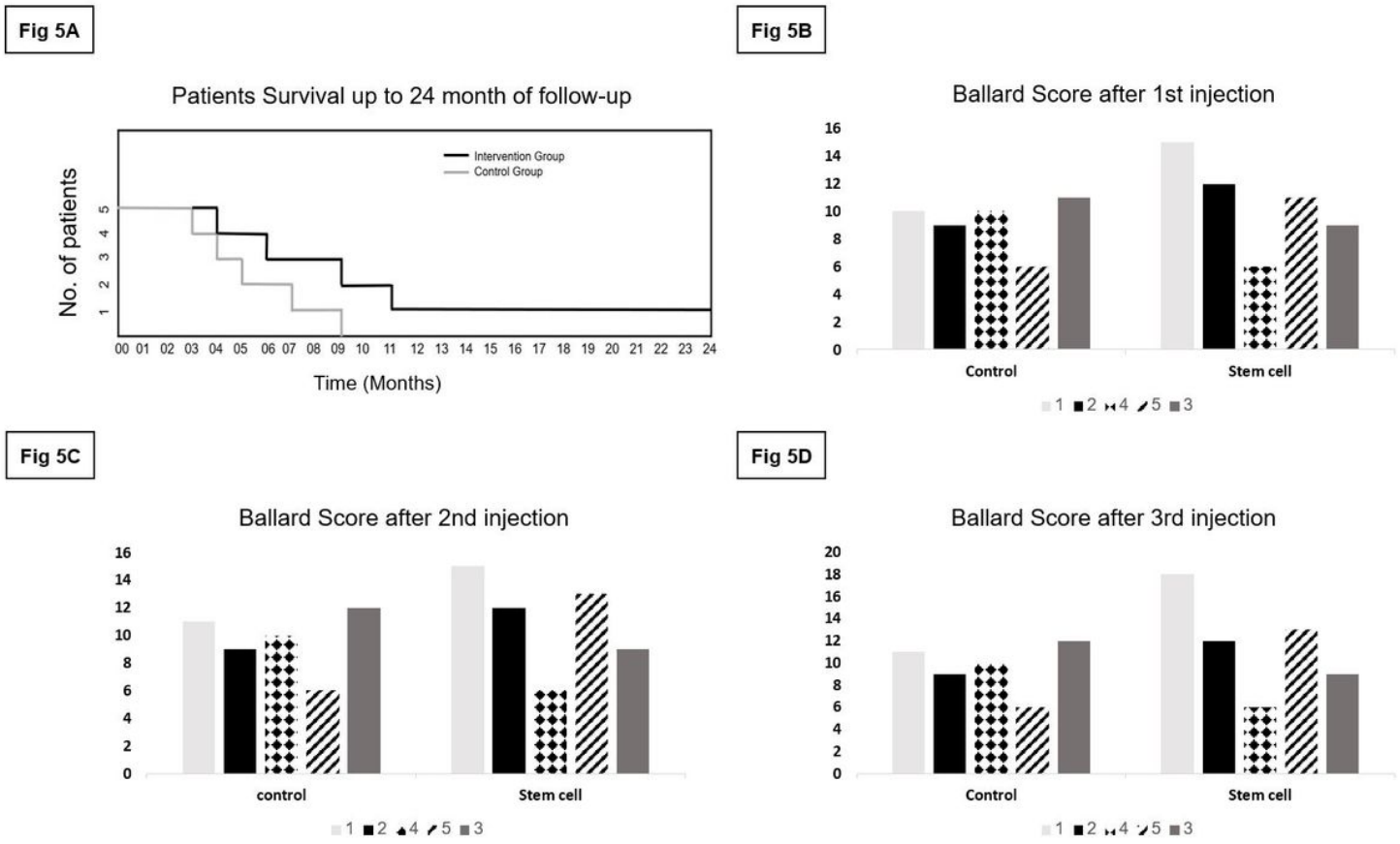

Figure 5

(A) Survival of patients. The mean lifetime of the intervention group for 4 patients was 11.17 months and the mean lifetime of the control group was 8.52 months. One of the patients in the intervention group has been alive until now (more than 28 months). (B) Ballard score after the 1st stem cell administration. The mean score of the patients in the intervention group after the first injection was 10.6 post 1st injection and in this time the mean score in the control group was 9.2 (p: 0.58). (C) Ballard score after the 2nd stem cell administration. The mean score after the 2nd injection in the intervention group was 11 and in the control group was 9.6 (p: 0.32). (D) Ballard score after the 3rd stem cell administration. The mean scores after the 3rd injection in the intervention group was 11.6 and in the control group, it was 9.6 (p: 0.42).