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Case Report

Stabilization of Disease Progression in a Case of Duchenne Muscular Dystrophy with Cellular Transplantation

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Abstract

Duchenne Muscular Dystrophy (DMD) is an X-linked inherited myopathy where there is progressive muscular degeneration due to dystrophin deficiency. To date, there are no curative treatments for DMD. Cellular therapy is a valuable adjuvant to current palliative treatments. This case report describes an 8 year old boy, with classical signs and symptoms of DMD including difficulty in walking and getting up from the floor, who received cellular therapy. Two doses of autologous bone marrow derived mononuclear cells were injected both intrathecally and intramuscularly in motor points across various large and weakened muscle groups in his body. A wide range of functional improvements were observed in the patient on follow up over a period of 12 months, like increased stamina, faster ambulation, decreased frequency of falls and increased muscle strength. Gower's time decreased from 45 seconds to 15 seconds. Pediatric Berg Balance Scale score and the distance covered in the 6 minute walk test were maintained over a period of one year. No adverse effects were noted after the procedures. Stem cells are capable of differentiating into cells of many lineages and also have the potential to multiply and replenish the depleted pool of resident stem cells. Transplanted cells provide myoprotection through various paracrine mechanisms like stimulating neoangiogenesis, immuno modulation, release of anti-inflammatory cytokines, inhibiting fibrosis and stimulating resident stem cells. Along with neuro rehabilitation, stem cell therapy offers a new alternative to revolutionize the treatment received by patients with DMD.

Keywords: Autologous Bone Marrow Mononuclear cells; Duchenne Muscular Dystrophy; Stem Cell Therapy; Stem Cells

Abbreviations

DMD	:	Duchenne Muscular Dystrophy
BMMNC's	:	Bone Marrow Derived Mononuclear Cells
MNC's	:	Mononuclear Cells
mMRCMMT	:	Modified Medical Research Councils Manual Muscle Testing
rmMRCMMT	:	Revised Modified Medical Research Councils Manual Muscle Testing
PBS	:	Pediatric Balance Scale
6MWT	:	6 Minute Walk Test

Introduction

Duchenne muscular dystrophy (DMD) is a progressive and fatal degenerative muscle disease which has an X-linked recessive inheritance with a mutation of the dystrophin gene in the short arm of chromosome X (locus Xp21.2) [1]. Incidence of DMD ranges

from 10.71 to 27.78 per 100,000 males [2] and X- linked recessive inheritance exclusively restricts the disease to boys. Out of the causative mutations 65% have intragenic deletion, 6-10% duplication, 30-35% point mutation [3]. Also due to the large size of the dystrophin gene, there is a high rate of spontaneous mutation [4]. The function of dystrophin is to form strong mechanical linkages from intracellular cytoskeleton to extracellular matrix [5]. Absence of dystrophin leads to membrane tears in the muscle, increased permeability and eventually chronic inflammation and necrosis. The repeated process of muscle regeneration exhausts the local stem cell pool and eventually leads to muscle degeneration.

Disease onset is in early childhood with difficulties in ambulation and progressive muscular weakness [6]. There is presence of some degree of mental impairment, cognitive and behavioral problems possibly due to altered Dp71 transcripts and deleted Dp 140 DNA chromosomes [7]. The most frequent cause of death is either cardiac or respiratory failure which usually occurs in the second decade of life [8].

Despite extensive research and molecular understanding of the disease, treatment options available to date are limited with palliative treatments like glucocortiocoids [9] and nocturnal ventilation [10]. None of these treatments alter the disease pathology and thus alternative treatments must be explored.

Bone Marrow Derived Mononuclear Stem Cells (BMMNC's) are a valuable alternative. Studies on mdx mouse models have showed the Myogenic Potential of Mononuclear Stem Cells (MNC's) when injected in dystrophic muscle of immuno suppressed mice and their ability to restore sarcolemmal expression of dystrophin [11-14]. BMMNC's can potentially alter the basic disease pathology by replenishing the depleted resident stem cell pool. Autologous stem cells are safe to use in humans and their effectiveness and safety profile has been explored in previous studies. Previous studies however had not measured the disease progression based on overall muscular endurance but had only used muscle strength as a parameter. Therefore we studied the 6 minute walk distance over a period of 1 year.

Case Study

An 8 year old male with complaints of difficulty in getting up from floor, climbing stairs and running since the age of 5, with family history of myopathy on maternal side, was diagnosed as a case of DMD on the basis of clinical signs, Creatine Phosphokinase levels, Musculoskeletal Magnetic Resonance Imaging, electromyography, and muscle biopsy.

Disease onset was at the age of 3 with the first symptom being pain in the lower limbs. Thereafter, lower limb weakness progressed and the condition worsened with the patient having difficulty in walking, easy fatigability, decreased balance leading to 3-4 falls a week and difficulties in transfers. Patient had undergone extensive regular physiotherapy since 2 years prior to the intervention.

On examination, patient was hypotonic and normoreflexic with bilateral pseudo hypertrophy of calf muscles. Manual muscle testing was done using the revised modified Medical Research Councils Manual Muscle Testing (rmMRCMMT) scale to grade the strength of the muscles. Since the modified Medical Research Councils Manual Muscle Testing (mMRCMMT) scale cannot measure the minute differences in the strength that we observe in our patients, we further modified the scales to detect the subtle changes in the muscular strength (Appendix 1).

mMRC-MMT grade	Description	rmMRC-MMT grade	Description
0	No Movement	0	No movement
1	A flicker of movement is seen or felt in the muscle	1	A flicker of movement is seen or felt in the muscle
		1+	Muscle moves the joint through up to 1/3rd of the range of motion (ROM) when gravity is eliminated
		1++	Muscle moves the joint more than 1/3rd less than 2/3rd of the ROM when gravity is eliminated
2	Muscle moves the joint when gravity is eliminated	2–	Muscle moves the joint more than 2/3rd but less than the full ROM
		2	Muscle moves the joint through complete ROM when gravity is eliminated
		2+	Muscle moves the joint up to 1/3rd ROM against gravity
		2++	Muscle moves the joint >1/3rd, <2/3rd of ROM against gravity
3–	Muscle moves the joint against gravity, but not through full mechanical range of motion	3–	Muscle moves the joint more than 2/3rd but less than complete ROM

3	Muscle cannot hold the joint against resistance but moved the joint fully against gravity	3	Muscle moves the joint through complete ROM against gravity
3+	Muscle moves the joint fully against gravity and is capable of transient resistance, but collapses abruptly	3+	Muscle moves the joint against gravity and moderate resistance up to 1/3rd of ROM
		3++	Muscle moves the joint against gravity and moderate resistance from 1/3rd to 2/3rd of ROM
4–	Same as grade 4, but muscle holds the joint only against minimal resistance	4–	Muscle moves the joint more than 2/3rd but less than complete ROM against gravity and moderate resistance
4	Muscle holds the joint against a combination of gravity and moderate resistance	4	Muscle moves the joint against gravity and moderate resistance though complete ROM
4+	Same as grade 4 but muscle holds the joints against moderate to maximal resistance	4+	Muscle moves the joint against gravity and moderate to maximal resistance up to 1/3rd of ROM
5–	Barely detectable weakness	4++	Muscle moves the joint against gravity and moderate to maximal resistance from 1/3rd to 2/3rd of ROM (barely detectable weakness)
5	Normal strength	5	Muscle moves the joint against gravity and moderate to maximal resistance though complete ROM (normal strength)

Appendix 1: Comparison of the grades of the modified Medical Research Council-Manual Muscle Testing (mMRC-MMT) and revised modified Research Council-Manual Muscle Testing (rmMRC-MMT) scales.

Proximal muscles were found to be weaker than distal. Both the upper limbs were at grade 4 while lower limbs and trunk muscles were below functional level. Sitting posture was kyphotic and on standing there was hyperlordosis of lumbar spine. He was independent in bed mobility and partially dependent for transfers. Patient was ambulatory with an abnormal gait which included walking with bilateral knee hyperextension, internal rotation, foot drop and hyperlordosis of lumbar spine. Gower's sign was present. Functional Independence Measure score was 111. Brooke-Vignos score was Brooke: 2 Vignos: 2. On Pediatric Balance Scale (PBS), he rated 42/56. On North Star Ambulatory Assessment, he scored 14/34. The distance covered in the 6 Minute Walk Test (6MWT) was 198m and the maximum inspiratory capacity was 350ml.

On investigation, Creatine Phosphokinase levels were raised (1897.4 IU/L). Muscle biopsy from the right calf muscle showed mild myopathic features. Musculoskeletal Magnetic Resonance Imaging reports showed early fatty replacement with minimal muscular atrophy involving muscles of both thighs and legs and bilateral upper limbs with predominant lower limb affliction. There was also mild bilateral pseudo hypertrophy of gastrocnemius muscles in upper and mid calf consistent with muscular dystrophy. Electromyography showed increased incidence of polyphasic potentials, decrease in amplitude and duration of motor unit action potential's and signs of rapid recruitment in relation to the strength of contraction which was suggestive of myopathic pattern. Genetic testing was performed using Multiplex Ligation Dependent Probe Analysis (MLPA) for 79 exons of Dystrophin gene. The MLPA showed no deletions or duplications in any of the 79 exons. Since MLPA is limited and cannot detect point mutations, in view of the clinical features and findings from MRI-MSK and EMG, probability of a point mutation was more likely.

Intervention

Selection of this patient for the treatment was based on the World Medical Association Revised Declaration of Helsinki. Ethical approval was obtained from the Institutional Committee for Stem Cell Research and Therapy (IC-SCRT). Parents of the pa¬tient provided written informed consent for the procedure and sub¬sequent reporting. An audiovisual recording of the consent was also taken.

Preoperative fitness was assessed by serological and biochemical blood tests, chest X-ray, electrocardiogram, and 2-D echocardiography a week before stem cell transplantation. 300 mcg of granulocyte colony stimulating factor was injected subcutaneously 72 hours and 24 hours prior to the cellular transplantation so as to enhance mobilization of MNCs [15]. 28 motor points, were identified and marked by an experienced physiotherapist using external electrical stimulation. A motor point is a point at which motor nerve enters the muscle and there is maximum density of motor endplates and myoneural synapses at this point. Therefore this area of the muscle is the most sensitive to electrical stimulation. Criteria for selecting motor points included only those muscle groups that were of functional importance or had a grade less than 3 on Manual Muscle testing.

Bone marrow was aspirated from the anterior superior iliac

spine under short general anaesthesia and local anaesthesia. 100 ml bone marrow was collected in heparinized tubes and transported to the stem cell laboratory. In the laboratory, MNC's were separated by density gradient centrifugation method. Fluorescence-activated cell sorting analysis showed a $CD34^+$ count of 700cells/µL. The cell viability was assessed using trypan blue and TALI machine and was found to be 98%. The total cell count was $1.2x10^8$. These cells were divided into two equal fractions. The first fraction of cells was injected intrathecally at the level between L4 and L5. The remaining half was then diluted in the patient's own cerebrospinal flu¬id, as evidence suggests that cerebrospinal fluid can promote proliferation, increase viability and stimulate migration speed of stem cells [16]. These cells were then injected intramuscularly into the specific motor points of triceps, glutei, quadriceps, tibialis anterior, peronei, back extensors, and abdominals. Methyl prednisolone (600mg) in 500 ml of Isolyte P was admin¬istered intravenously to reduce the immediate inflammation.

Cellular transplantation was supplemented with extensive rehabilitation which was undertaken by a physiotherapist, an occupational therapist, a speech therapist, aquatic therapist and a psychologist. After 4 days of in-hospital rehabilitation, the patient was discharged and was given a detailed program to be followed under professional guidance. Patient was called for follow up assessment 3 months and 6 months after therapy.

Nine months later, in view of improvements seen with first dose of cell transplantation, patient underwent cellular therapy for the second time. The procedure fundamentally remained the same as before. The numbers of cells injected were 1.1×10^8 with a viability of 98%. The $CD34^+$ absolute viable counts were 282 cells/µL. These cells were divided into 2 equal fractions and injected both intrathecally and intramuscularly in 26 motor points which included the glutei, quadriceps, tibialis anterior, peronei, abdominals and back extensor muscle groups. Triceps were not included as they remained at grade 4. Patient was followed up at 3 months after the second therapy.

Results

On follow up assessment 3 months after the first stem cell therapy, Gower's time was 45 seconds. Frequency of falls had reduced to once a month. Scores were maintained on all the objective scales. Another follow up was done 6 months after therapy. Standing balance, chest expansion and maximum inspiratory volume (350ml to 500ml) had improved. Frequency of falls had further reduced. Gower's time decreased from 45 sec to 15 sec. PBS score increased from 42 to 47. There were also improvements in forward reach, backward reach, left reach, right reach score (increased from 7 to 14cm, 5 to 11cm, 6 to 20cm, 6 to 14cm respectively). Stamina had also increased. Muscle strength increased in certain muscle groups as seen in rmMRCMMT (Table 1). This table grades the weak muscles and the improvement seen in them. Only 2 muscle groups (tibialis posterior and plantar flexors) showed decline over the course of one year. Neck, shoulder, arm, forearm, wrist and hand muscles were maintained at grade 4. The distance covered in the 6MWT increased from 198 meters to 224 meters.

In light of these improvements, a second dose of cellular therapy was scheduled 9 months after the first cell transplantation. At 3 months follow up post second cell transplantation, previous improvements were maintained. North Star Ambulatory Assessment score was 13/34 and PBS score was 42/56. Six minute walk test was again noted to be 198 meters. Forward reach, backward reach, left reach, right reach score were maintained at 13cm, 10cm, 21cm and 14cm respectively. Maximum inspiratory volume remained at 500ml. No adverse effects were noted after the procedures and in subsequent follow ups.

Muscle Groups		rmMRCMMT Grade Before 1st SCT		rmMRCMMT Grade on Follow up at 6 Months		rmMRCMMT Grade Before 2nd SCT		rmMRCMMT Grade on Follow up at 12 months	
		R	L	R	L	R	L	R	L
Hip	Flexors	1+	2+	1+	2+	2+	2++	2+	2++
	Extensors	2+	2+	2+	2++	3-	2++	3-	3-
	Abductors	3	3+	3	3+	3++	3++	3++	3++
	External Rotators	1++	1++	1++	1++	1++	1++	1++	2+
Knee	Extensors	1	1+	1	1+	1+	1+	1+	1+
Ankle and Foot	Tibialis posterior	2++	1	2++	1	1+	1	1+	1
	Peroneus tertius	0	0	0	0	1+	1+	1+	0
	Plantar Flexor	2++	2++	2++	2++	3+	3+	1+	1++
	Extensor Hallucis Longus	1+	1+	1+	1+	3	2+	3-	3-
	Extensor Digitorum Longus	2+	2++	2+	2++	3	3-	3-	3-

Trunk	Abdominals upper	1	1	1+	1+
	Abdominals lower	1	1	2+	2-
	Back Extensors	1+	1+	1++	2

Table 1: Changes in the grades of muscle strength over 12 months as measured by rmMRCMMT.

Discussion

Dystrophin is a protein product of duchenne locus Xp21.2, which forms strong mechanical links between cytoskeletal and membrane elements of muscle. The consequence of dystrophin deficiency is increased membrane fragility. The stress of contraction can lead to tears in the membrane which causes increase in membrane permeability which in turn allows soluble enzymes like creatine kinase to leak out of the cell and ions such as Ca2+ to enter the cell. Increase membrane permeability also cause massive infiltration of immune cells leading to inflammation, necrosis, replacement of muscle fibers by fat cells and fibrotic tissue and eventually severe muscle degeneration [17,18]. Infact the proliferation of connective tissue is not just a compensatory replacement of atrophied muscle but an increase in endomysial tissue can be seen before there is any apparent muscle degeneration [19]. Although initiated by dystrophin deficiency, Sacco et al. came to the conclusion that DMD is ultimately a stem cell disease [20]. Growth and repair of skeletal muscles are mediated by local stem cells with myogenic potential called satellite cells which surround the muscle fibers and are located between the sarcolemma and the basal lamina of the muscle fiber [13,21]. Following injury to the muscle, due to a continuous cycle of degeneration and regeneration, these satellite cells are exhausted.

There is no known cure for DMD at present. Thus current treatment aims to control symptoms to improve quality of life, delaying loss of ambulation, providing respiratory assistance, make patient functionally independent and prolong survival. Corticosteroids are the only drug that effectively delays the progression of DMD, prolongs ambulation and improves cardiopulmonary function [9]. Anti-fibrotic therapy and nocturnal ventilation may also serve as additions to current treatment [10,22]. All these interventions only delay the progression of muscular degeneration which is why alternative forms of treatments are needed.

New advances in the treatment of DMD are Exon Skipping, Gene Therapy and Cellular Therapy. Gene therapy is not yet a standard therapy and this approach is still in the process of clinical human trials. There are still issues regarding its safety and efficacy. Some of the challenges to gene therapy are the inability to deliver the vectors at high doses safely without eliciting an immune response [23]. Also, the viral vectors needed to introduce the normal genes, are not large enough to be able to carry a nucleic acid polymer to replace the defective dmd gene (which is one of the largest known genes at 2.2 megabase pairs) in order to encode the dystrophin protein [24]. Eteplirsen, a disease modifying drug for the treatment of DMD, which has recently been granted accelerated approval by the FDA, induces exon skipping by selectively binding to exon 51 of dystrophin pre-mRNA. Since the patient had no duplications or deletions in the exons and had a suspected point mutation, eteplirsen triggered skipping of exon 51 would have no effect on the disease progression in this patient [25].

Cellular therapy is one of the most promising treatments available. In animals, several encouraging studies have been conducted using the mdx mouse model showing restoration of dystrophin expression and myogenic differentiation after stem cell transplantation [12-14].

We transplanted autologous bone marrow derived mononuclear cells into several motor points in various muscles. Autologous BMMNC's were selected because of their safety profile and lack of adverse effects as shown in previous studies [26-28]. Although DMD is often referred to as a muscle disease, it actually affects multiple organ systems. Central nerve system involvement is also highly relevant to the health of DMD patients. Dystrophin gene is transcribed from different promoters in neuronal and glial cells. Also Dp116, which is a dystrophin isoform, is a component of the submembranous cytoskeletal system that anchors the Dystroglycan Complex (DGC) in schwann cells [29-31]. So the absence of dystrophin leads to the disruption of myelin structure and reduced myelin stability. There is a common association between patients with DMD and mental retardation [32]. Histological evidence, such as the presence of group atrophy in biopsy specimens of muscular dystrophy, has also linked neurogenic factors with DMD [33]. Many pathways of neurotransmission involve DGC and impaired DGC can lead to abnormal neurotransmission, abnormal synaptic activity and problems in the formation of neuromuscular junction. The abnormalities of synaptic activities and muscle recruitment has been postulated to be one of the mechanism of muscle wasting in DMD [34,35]. Thus half the stem cells were also administered intrathecally to address the neurogenic component of DMD. In this case, 2 doses were given over 9 months which could have helped replenish the depleted stem cell pool.

While the original theory explaining the action of stem cell regenerative therapy was based on functional recovery as a consequence of stem cell differentiation, there are also other mechanisms of action. Biomolecules such as growth factors and cytokines that

are secreted by stem cells are just as important as the differentiating cells. These secreted molecules act on the neighboring cells, the process of which is known as the paracrine mechanism of action. In addition, these cells secrete angiogenic factors, antifibrotic factors and anti-inflammatory or immuno modulatory factors. Stem cells can also protect other cells from damaging oxygen free radicals through the production of antioxidants and anti-apoptotic molecules [36,37]. Mesenchymal stem cell derived exosomes have been shown to accelerate skeletal muscle regeneration and angiogenesis thereby promoting skeletal muscle repair. These exosomes also contribute to the paracrine effect on muscle repair during stem cell transplantation along with the various growth factors and cytokines [38].

Cellular therapy was supplemented with extensive neurorehabilitation. Exercise and endurance training has been shown to improve the fitness and strength in patients of muscular dystrophy [39]. Exercise, in moderation, has also been shown to reduce oxidative stress in mdx mice [40]. Studies also show a marked increase in endothelial progenitor cells and angiogenic growth factors in peripheral circulation after a single session of exercise. This exercise induced stem cell mobilization may assist in the repair process [41].

Improvements seen in our patient were increased muscle strength during manual muscle testing in various muscle groups such as hip extensors, flexors, abductors, external rotators, knee extensors, extensor hallucis longus, extensor digitorum longus, upper and lower abdominals, and back extensors. 6MWT values increased from 198 m to 224 m and then reverted back to 198 m. In 1 year follow up, a 43 meter decline in distance walked in 6 minutes is expected in patients treated with conventional therapies [42]. The distance covered in the 6MWT remained at 198m over 1 year. Gower's time also improved from 45 seconds to 15 seconds showing an increase in lower limb muscle strength. The effect of stem cell transplantation played a vital role in halting the disease progression since the patient was undergoing regular rehabilitation for 2 years prior to intervention and was still deteriorating functionally. The stability seen in these parameters highlights the significant alteration seen in the progression of the disease.

A case of allogenic umbilical cord blood transplantation showed definite engraftment of donor dystrophin however this did not alter the disease phenotype and there was no significant functional outcomes [43]. Previous case report has suggested the ability of bone marrow derived cells to be engrafted in the muscle fibers [44]. In case reports of autologous cell transplantation, similar improvements in muscle strength and functional activities have been reported in DMD as well a milder phenotype of DMD, Becker's muscular dystrophy [45-48]. A case series of autologous BMMNCs intrathecal and intramuscular transplantation in patients with Limb girdle muscular dystrophy showed improved muscle strength, functional recovery and slowing down of disease progression [49]. There is some evidence of efficacy of cellular therapy in muscular dystrophy however evidence specific to DMD is still preliminary and limited. Our case report concurs with the findings of previous case reports and in addition we report maintenance in 6 minute walk distance.

Despite the functional and objective improvements seen in this patient, the results of this study cannot be generalized to a larger population. The inclusion of imaging, biomarker analysis and a longer follow up period would give a more detailed outlook of the disease and its progression and may be incorporated in future studies.

Conclusion

The administration of autologous BMMNC's promises a new path to recovery for patients of Duchenne's muscular dystrophy. A singular case study is not sufficient to establish new protocols of treatment. But stabilization of the disease progression after cellular therapy in this case study warrants further investigation in the form of large scale clinical trials.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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