Chapter

Duchenne Muscular Dystrophy (DMD) Treatment: Past and Present Perspectives

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Abstract

Duchenne muscular dystrophy (DMD) is one of the fatal X-linked disorders that are characterized by progressive muscle weakness and occur due to mutation in the largest human gene known as the DMD gene which encodes dystrophin protein that is mandatory for keeping the muscles structurally and functionally intact. The disease always affects boys (1 from every ~5000), and in some cases the female carriers are symptomatic. The disease usually leads to impairment in cardiac and pulmonary functions leading to the death of the patients in very young ages. Understanding DMD through precise molecular diagnosis will aid in determining the suitable therapeutic approach for the cases like designing exon-skipping antisense oligonucleotides (AOs) or stem cell-based therapies in conjunction with gene editing techniques (CRISPR/Cas9). Such therapies can correct the genetic defect in the DMD gene and ameliorate the symptoms. In this chapter, we will illustrate the past and current strategies for DMD disease treatment.

Keywords: DMD, exon skipping, CRISPR, cardiosphere, utrophin

1. Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder characterized by skeletal muscle wasting that is resulted from mutations in the dystrophin gene [1]. The disease occurs at a frequency of about 1 in ~5000 newborn males, making it the most common severe neuromuscular disease in humans. Dystrophin is present in normal individuals from fetal life onwards in all skeletal, cardiac, and smooth muscles; the absence of dystrophin protein causes muscle weakness and protein degradation and ultimately causes cell death. Death usually occurs in the third decade of life as the result of respiratory or heart failure [2]. The precise diagnosis for DMD should contain a combination of genetic testing after muscle biopsy and clinical observation of muscle strength and function.

The main current medication so far is corticosteroids, which have been shown to increase muscle strength in many studies. Genetic therapy using mini-/micro-dystrophin vectors, suppression of premature termination codon, exon-skipping antisense oligonucleotides (AOs) which bind with RNA and exclude specific sites of RNA splicing producing a dystrophin that is smaller but functional, and such new emerging drugs are the pass to the new era towards DMD treatment. In the next

section, we will review all available FDA-approved treatments and recent research trials aiming at ameliorating DMD symptoms.

2. Methods for treatment

2.1 Corticosteroids

Corticosteroids were the first line of treatment for DMD; it was first used by Drachman et al. in 1974 [3] when they had promising positive results in their study after using prednisone (anti-inflammatory glucocorticosteroid). Since then, many studies were carried out to test the therapeutic effect of such treatment since it was found to improve muscle performance.

Deflazacart (DFZ), an oxazolidine derivative of prednisone, was used by an Italian group [4] and other groups [5–7], and the drug demonstrated efficiency in disease treatment and preserved lung function. The exact mechanism of DFZ is not yet known; however, it might regulate some signaling cascades. It was found to activate calcineurin/NF-AT pathway [8]. Also, DFZ may act by decreasing necrosis and muscle inflammation and reducing the degree of muscle degeneration. It can also act through modulating dystrophin expression and inducing the myogenesis in addition to having positive effects on muscular tissue mass [9].

Despite the advantages of using steroids, they also had side effects like gaining weight, affecting bone mineral density, which leads to vertebral fractures and behavioral changes. Furthermore, high dosages are required to reach the target effect and to be active at the site inflammation. Also, the drug can be accumulated in other nontargeted areas [10, 11].

In one of their studies, Luhder et al. [12] tried to improve the therapeutic effect of the steroids through developing an 80 nm PEGylated nano-liposome that is conjugated with the steroid prodrug "methylprednisolone hemisuccinate." The results of their study showed that such structure was selectively targeting the diaphragm in vivo (using mdx mouse model) when administered intravenously and the treatment reduced the infiltration with macrophages and serum levels of transforming growth factor beta. Most importantly, the study showed that long-term use of this formulation leads to enhanced mobility and increased muscle strength.

2.2 Exon skipping

Exon skipping is considered as one of the mutation-based treatments for Duchenne muscular dystrophy [13]. In DMD, some deletions in specific exons lead to the disruption of the reading frame of the dystrophin protein, and consequently such deletions lead to the production of truncated product missing a huge part of the protein (usually missing the rod domain and C-terminal domain).

However, sometimes, deleting additional exons may restore the reading frame and lead to the production of dystrophin protein missing only a portion of the central rod domain while the C-terminal domain remains intact, and hence the protein product in this case is lacking specific regions, but it is semi-functional and can induce Becker-like symptoms instead of the complete loss of the muscular function [14].

The main idea of exon skipping is using the "antisense oligonucleotide" molecules to induce the skipping of a specific exon (other than the already mutated one) and prevent it from being translated to restore the reading frame. As an example, patients with exon 45 deletion could be treated through the skipping of an

additional exon 44. Eteplirsen (Exondys51™) based on phosphorodiamidite morpholino oligomer (PMD) is an FDA-approved antisense treatment to skip exon 51 for patients with mutation ▲49–50 [15]. Also, drisapersen (based on 2′-O-methyl phosphorothioate; 2′-OMePS-modified AOs) is one of the AOs that are designed to treat DMD patients with mutations that can be ameliorated by exon 51 skipping; however it was not approved by the FDA [16, 17].

Various modifications can take place to the sugar of the oligonucleotide or to the backbone of the oligo. This could include phosphorodiamidate morpholino, locked nucleic acid (LNA), or peptide-conjugated oligo. Regarding the morpholinos, the oligonucleotide backbone is replaced with the morpholino backbone which makes the oligonucleotide nontoxic and has high affinity to RNA molecules. The locked nucleic acids are oligonucleotides that have a modified ribose sugar where the 2′ oxygen is connected with the 4′ carbon atom which creates a locked ribose ring. Also, the LNAs are nontoxic with superior affinity to complementary targeted RNA sequences [18].

The main problem in developing such treatments based on the skipping is that it will only fit a small group of patients (a mutation-specific AO should be developed for each group of patients and will not be suitable for other patients); also some patients have deletions in critical parts of the protein, and hence skipping of other exons will not have a therapeutic impact (**Table 1**).

2.3 Induced pluripotent stem cells along with genome editing technique

The sole cause of DMD is the presence of mutation that adversely affects the DMD gene. So, in order to permanently fix such mutations and treat this condition, patients could be provided with muscle cells harboring the normal copy of DMD gene. Since it is hard to get mature muscle fibers from a normal individual to be used as a source of healthy muscle cells with normal DMD gene, also the availability of such source of cells will not guarantee the process of grafting in the patient's muscles since it could be subjected to rejection by the body and can initiate an aggressive immune response. Cell reprogramming and genome editing techniques efficiently aid in solving this puzzling dilemma [25]. The process of cell reprogramming paved the road towards developing normal muscle fibers by starting with patient-specialized adult cells followed by inducing the production of induced pluripotent stem cells (iPSCs) (using the Nobel prize-winning technology of reprogramming using specific transcription factors like Oct4, Sox2, Klf4, and L-Myc) [26]. Also, some microRNAs have the potential to reprogram the adult cells efficiently (like miR-302b, miR-372) [27].

After the reprogramming and the production of stem cells, gene editing technologies should be used to correct the mutation of the gene. CRISPR/Cas 9 is now a leading technology that is presently considered as an avenue for DMD treatment; the RNA-guided DNA endonuclease system allows the correction of the DMD segment which is essential for dystrophin restoration [28, 29].

In order to conduct a gene editing experiment with CRISPR/Cas9 system, two important elements should be provided: guide RNA (gRNA) specific for the target gene and Cas9 nuclease (Sp. Cas9 (from *Streptococcus pyogenes*; 4.10 kb) or Sp. Cas9 (*Staphylococcus aureus*; 3.16 kb)) or Cj. Cas9 (*Campylobacter jejuni*; 2.95 kb) that can cleave DNA strands where the guide RNA is bound and in the presence of three- to five-nucleotide proto-spacer adjacent motif (PAM) sequence to be digested. Upon the binding of the gRNA, Cas9 can induce a double-strand break which is then repaired by the cell through the nonhomologous end joining, and this will initiate a repair mechanism in which nucleotides will be added or deleted at the cleaved site which can consequently restore the reading frame of the DMD gene to the normal

Chemistry	Route of administration	The used model	Treatment strategy	Treatment effects	Reference
Phosphorodiamidate morpholino oligomers (Ex6A, Ex6B, Ex8A, and Ex8G)	Intravenous	Neonatal CXMDJ	Exon 6–9 skipping	Dystrophin restoration across skeletal muscles (14% of healthy levels) Reduction of fibrosis and/or necrosis area	[19]
Phosphorodiamidate morpholino oligomer (NS-065/NCNP-01)	Endo-Porter reagent	Fibroblasts from patients with DMD involving deletion of exons 45–52 or exons 48–52 and injected with MYOD for myotube differentiation	Exon 53 skipping	Restored dystrophin protein levels in the cells	[20]
Phosphorodiamidate morpholino oligomer	Intramuscular and intravenous	mdx52 mouse model	Exon 51 skipping	Only the protocol was mentioned	[21]
Phosphorodiamidate morpholino oligomer (NS-065/NCNP-01)	Intravenous	Patients with DMD	Exon 53 skipping	Increased dystrophin/spectrin ratio in 7 of 10 patients in TA muscle biopsies	[22]
Pip6a-PMO; PMOME23, sequence GGCCAAACCTCGGCTT-ACCTGAAAT	Intravenous	Cmah-/-mdx mice	Exon 23 skipping	Dystrophin restoration in the heart Reduction in myocardial fibrosis Reducing maximum pressure and arterial elastance	[23]
Inhibitor of CDC2-like kinase 1 (named TG693)	Oral Lipofectamine reagent	Male Jcl:TCR mice Patient-derived myotubes	Exon 31 skipping	It induces exon skipping and restored dystrophin expression in patient-derived cells. And it modulated splicing in mouse skeletal muscle	[24]
Morpholino AOs targeting DMD exon 51	Endo-Porter transfection Intramuscular	Immortalized DMD muscle cells hDMD/Dmd null mice	Exon 51 skipping	The rescue of dystrophin protein expression	[25]

Table 1.
Studies conducted on treatment of DMD using exon skipping (during 2017–2019).

ORF. In some cases, single (or several) gRNA molecule could be designed to target splicing sites which can lead to the skipping of specific exon leading to the production of functional proteins. Additionally, base editing mediated by CRISPR/Cas9 could be obtained through Cas9 enzymes lacking the nuclease activity, so it can induce only a single-strand break. Such enzymes can catalyze base editing (A:T to G:C) through having a cytidine deaminase activity [30].

Ousterout et al. in their study used another editing protocol (zinc finger nuclease) to delete exon 51 from the transcript from patient-derived myoblasts [31]. Also, Young et al. carried out CRISPR/Cas9 experiment utilizing a single pair of guide RNAs to delete exons 45–55 in iPSC, and such deletion leads to the expression of stable dystrophin protein with improved membrane stability in derived skeletal myotubes and cardiomyocytes [32]. Another study by Duchene et al. utilized a single guide RNA to produce a hybrid exon which led to the production of functional dystrophin protein with completely normal structure [33]. The main advantage of this reprogramming protocol is that it allows performing an autologous grafting of the muscle cells to patients.

For the expression of the specific gRNA molecules inside the muscle cells, adeno-associated virus (AAV) vectors will be used. Sometimes, the expression of the gRNAs can lead to off-target effect due to the incorrect binding with another similar DNA sequence inside the host cell. In order to avoid this damaging effect, AAV vectors expressing multiple gRNA molecules could be used.

After the completion of the gene editing process, the edited cells would be treated with myogenic factors to convert the edited stem cells again to myoblasts for the myogenic differentiation (**Table 2**).

2.4 Gene therapy

Gene therapy is one of the most appealing techniques that are used to deliver a normal copy of the DMD gene to express the fully functional dystrophin protein. This method implies injecting the patients with plasmids carrying normal dystrophin cDNA (~12 kb).

In 2002, the first phase 1 trial of DMD gene therapy took place using full-length dystrophin [52]. After that, adeno-associated viral vectors carrying mini forms of dystrophin cDNA were used for gene therapy, and this was better regarding the packaging size of the plasmids, and it is much easier to transfer/deliver mini forms of DMD gene [53, 54].

However, such therapeutic approach faced another problem which is the distribution of the plasmids across all affected muscular tissue that is spreading all over the body, and that is why microdystrophin plasmids and systemic AAV delivery were developed and improved to solve such problem. Evidence from many trials using animal models revealed that gene therapy can lead to long-term expression of functional protein [55–57].

In 2017, Le Guiner et al. studied the effect of the delivery of rAAV2/8 vector expressing a canine microdystrophin (cMD1) in golden retriever muscular dystrophy (GRMD) dogs in the absence of immunosuppression. Such treatment affected the deterioration of the muscular activity, and the gene expression was maintained over a long period [56]. Recently in 2020, Genthon and Sarepta contracted Yposkesi for manufacturing the AAV microdystrophin vector on a large scale.

2.5 Dystrophin-expressing chimeric cells

As previously mentioned, the absence of dystrophin is the main cause of DMD disease and the aggressive symptoms including muscle weakness and degeneration

Plasmids (source of Cas9 and guide RNAs)	Route of administration	The used model	Treatment strategy	Reference
Adeno-associated viral vectors of serotype 9 carrying an intein-split Cas9 A pair of guide RNAs targeting sequences flanking exon 51 (AAV9-Cas9-gE51)	Intramuscular injection	DMDΔ52 pigs	Excision of exon 51	[34]
SaCas9 expression plasmid Two gRNA expression cassettes driven by the human U6 pol. III promoter (AAV8 and AAV9)	Locally in the TA muscles	C57BL/10ScSn-Dmdmdx/J	Excision of exon 23	[35]
pSpCas9 expression plasmid AAV TRISPR-sgRNA-CK8e-GFP plasmid contained three sgRNAs driven by the U6, H1, or 7SK promoter and green florescent protein (GFP) driven by the CK8e regulatory cassette	Transfection reagent Locally in the TA muscles	Human DMD-derived iPSCs ▲Exon 44 DMD mice	Excision of exons 43 and 45	[36]
Streptococcus pyogenes Cas9 Single guide RNA (sgRNA-51) (AAV9-Cas9 and AAV9-sgRNA-51)	Locally in the cranial tibialis muscles	▲Exon 50 canine model	Excision of exon 51	[37]
spCas9 and crDMDint2.1 and int2.6 gRNAs	Transfection reagent (linear polyethylenimine derivative)	Immortalized myoblasts from DMD patient	Excision of duplicated exon 2	[38]
Lenti-V2-Ugi-nCas9-AIDx or Lenti-V2-AIDx-nSaCas9 (KKH)-Ugi (2.5 μg) and pCDNA3 Ugi	Transfection reagent (lipid-based)	▲51-iPSCs of a male DMD patient	Excision of exon 50	[39]
CRISPR-Cas9 variant (D10A Cas9 nickase (nCas9) or catalytically deficient D10A/H840A Cas9 (dCas9) from *S. pyogenes*) and a deaminase protein from various sources sgRNA (gX20) under the control of the U6 promoter (pAAV-ITR-ABE-NT-sgRNA)	Micromanipulator	Mouse zygote from DMD knockout mouse	Base editing of exon 20	[40]
Plasmids containing regulatory cassettes for expression of Cas9 or gRNAs flanked by AAV serotype 2 inverted terminal repeats (ITRs)	Electroporation Intramuscular	Fibroblasts isolated from male mdx4cv mice Male mdx4cv mice	Excision of exons 52 and 53	[41]

Plasmids (source of Cas9 and guide RNAs)	Route of administration	The used model	Treatment strategy	Reference
pX601-AAV CMV::NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA (PX601)	Transfection reagent (lipid-based)	Myoblasts	Excision of exons 47 and 48	[33]
		DMD iPSC	Excision of exons 44–55	[32]
The gRNA cassettes gI43, gI52, gI53, and gI54.2 (targeting different introns) controlled by human U6 RNA polymerase III promoter Plasmids AU53_pAd Shu.gI52.gI53.PGK.Cas9.SV40pA, plasmids pLV.gI52 and pLV.gI53	Transduction (by gelatin)	DMD myoblasts Δ48–50 and Δ45–52	Excision of exon 53	[42]
pAAV-ITR-CjCas9-sgRNA, pAAV2/9 encoding for AAV2rep and AAV9cap, and helper plasmid	Intramuscular in the TA muscle	Knockout mice	Excision of exon 23	[43]
pSpCas9(BB)-2A-GFP (PX458)	Transfection reagent	DMD hiPSCs, hiPSC-derived cardiac muscle cells	Excision of exon 51, introns 47, 50, 54	[44]
pSpCas9(BB)-2A-GFP (PX458)	Intramuscular in the TA muscle	Mice ▲50	Excision of exon 51	[45]
Purified Cas9 protein and in vitro transcribed gRNA	Gold nanoparticles	Primary myoblasts C57BL/10ScSn-Dmdmdx/J (mdx) mice	Excision of exon 23	[46]
pSpCas9(BB)-2A-GFP (PX458)	Nucleofection	Induced pluripotent stem cells (iPSCs)	Deleting exons 3–9, 6–9, or 7–11	[47]
Nuclease-expressing plasmids (TALENs, left and right; CRISPR, Cas9 and sgRNA)	Electroporation	DMD fibroblasts were derived from a DMD patient lacking exon 44	Excision of exon 45	[48]
Cas9 mixed with 44C1, 44C2, 45C2, and 45C3 gRNAs produced via in vitro transcription	Electroporation	hDMD (Tg(DMD)72Thoen/J, 018900), C57BL/10 mdx (001801), and mdxD2 (D1.B10-Dmdmdx/J, 013141)	Excision of exons 45–55	[49]

Plasmids (source of Cas9 and guide RNAs)	Route of administration	The used model	Treatment strategy	Reference
AdV-Cas9-RFP AdG-gRNA-Donor	Transfection reagent (lipid based)	Skeletal muscle cell culture derived from C57BL/10ScSn-Dmd mdx/J	Excision of exon 23	[50]
Cpf1 gRNAs targeting the human DMD or the mouse Dmd locus (subcloned into pLbCpf1-2A-GFP and pAsCpf1-2A-GFP)	Nucleofection	DMD iPSC cells	Excision of exon 51	[51]

Table 2.
Studies conducted on treatment of DMD using gene editing techniques (CRISPR/Cas9) (during 2017–2020).

of muscle fibers. Such defect in the DMD gene can be edited using gene editing technology; however such technology can lead to off-target mutations which consequently can have damaging effects, and that is why more therapies had to be developed to address the disadvantages of such techniques.

Chimeric cell therapy is an alternative therapeutic approach that is usually done through the fusion of normal myoblasts and dystrophin-deficient myoblasts using polyethylene glycol (PEG). The success of this process could be tested using immunophenotyping (flow cytometry) and dystrophin immunostaining. This fusion will be followed by transplantation of chimeric cells in the defected muscle. The chimeric cells always show behavior like the donor cells regarding dystrophin expression and myogenic differentiation, and this dramatically improves the muscle function after being transplanted [58].

2.6 Cardiosphere-derived cells (CDCs)

Cardiosphere-derived cells are a type of cells that are retrieved from cardiac explants that can be cultured in vitro. Such cells have anti-inflammatory, antioxidant, and antibiotic activities. Several studies tested the ability of CDCs to alter the pathophysiology of DMD after the injection of these cells directly into the cardiac muscle.

Recently it was found that using CDCs greatly enhanced the phenotypic status of cardiac and skeletal muscles. The therapeutic effects of CDCs are usually attributed to the secretion of specific exosomes carrying specific genetic material to distal cells to exert its biological effect. Such CDCs along with their secreted exosomes can be injected intravenously, and it was found that they can greatly enhance the skeletal and cardiac muscle functions and boost the muscle generation process [59, 60].

2.7 Stop codon read-through therapy

In some of the mutations affecting the DMD gene, a premature stop codon is produced that can significantly disturb the reading frame and gives a truncated abnormal protein that cannot maintain the structural and functional properties of the muscle fibers.

A class of antibiotics called aminoglycosides was found to bind to rRNA at their decoding sites, preventing the stop codons from being read by binding to the A site (acceptor site) in the ribosomes and forcing the cell to prevent reading the stop codon, hence leading to the production of fully functional proteins.

PTC124 (ataluren; trade name, Translarna™) is one of the drugs with the read-through properties that are used for the treatment of DMD. Clinical trials showed that this drug when administered orally induced the expression of the dystrophin protein.

However, this treatment can only be used in ~15% of the cases who have a stop signal resulted from point mutation in the DMD gene. Also, it must be administered in increasing doses; beside it has many side effects such as renal toxicity. That is why there is a need to develop other alternatives with other structures to be safer with chronic usage.

2.8 Utrophin modulation

Recently, DMD symptoms were found to be managed after the administration of utrophin protein expression enhancers (utrophin is a dystrophin homolog; 395 KDa in size) to DMD patients delays the need of wheelchair and efficiently substitutes non-functional dystrophin.

Like dystrophin, the utrophin is present in the sarcolemma in the first developmental stages, and then replacement with dystrophin took place during muscular maturation. However, utrophin was found to be present in the myotendinous junction in adults. Interestingly, the expression of utrophin becomes elevated as a normal repair mechanism to compensate the absence of functional dystrophin in regenerated muscles.

SMTC-1100 is one of the chemical molecules that showed a great potential to increase the expression of DMD transcript and protein as well. This drug can be administered orally, if it was found to be safe and well tolerated in volunteers. However further studies are still required to detect if high dosage of this drug is safe or not.

Recently, ASA or adenylo-succinic acid improved the status of the TA muscles in mdx mice after administration of this compound in the drinking water. This molecule regulated the expression of the utrophin protein and hence greatly reduced the damaged area [61].

Another study group designed AAV vector carrying mini forms of the utrophin protein (μUtro). Their results showed that expression of this functional copy of utrophin protein (dystrophin analogue) after administration of the utrophin vector in DMD animal models completely reduced the muscle necrosis and regeneration [62].

3. Conclusion

Many medications have been used for DMD treatment and for preventing further deterioration of the cases. Corticosteroids were the first line of effective therapy of DMD; however, it does not modify the genetic mutations of the gene and does not affect the expression levels of dystrophin protein. Consequently, other treatments were developed including read-through stop codon, gene therapies, and exon skipping AOs which modulate and upregulate the levels of functional dystrophin transcript and protein in the muscles. Genome editing technology is also a powerful tool that can treat DMD permanently through the correction of the mutated sequence of DMD gene through the administration of sequence-specific guide RNA strands to bind selectively in the sequence to be edited. Also upregulating utrophin can help in the management of the cases. In addition, dystrophin-expressing chimeric cells and cardiosphere-derived cells are two emerging techniques that have the potential to treat DMD. Other medications will be developed to treat all DMD patients with different mutations with minimum side effects and maximum improvement in the status of the muscular system.

DOI: http://dx.doi.org/10.5772/intechopen.92765

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