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Duchenne muscular dystrophy: overview and future challenges

Dystrofia mięśniowa Duchenne'a: przegląd literatury i wyzwania w przyszłości

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Abstract

Duchenne muscular dystrophy is a muscle disease caused by mutation in the gene that encodes the cytoskeletal protein dystrophin. It is inherited in an X-linked recessive fashion. A number of therapies are continuously being developed to slow down the progression of the disease and increase patients' life expectancy. Steroid use in Duchenne muscular dystrophy is associated with a lower mortality rate (hazard ratio = 0.24; 95% CI = 0.07–0.91; $p = 0.0351$). Although recent studies have concluded that prolonged steroid use is associated with short stature and overweight, a meta-analysis of 12 studies has shown that steroids can increase strength, muscle function, and quality of life. Restoration of dystrophin gene expression is the basis of genetically engineered therapies. Potential therapies of this type include exon skipping, the use of recombinant adeno-associated virus which delivers mini-dystrophin, and surrogate gene transfer. In their development, the common challenges are associated with the size of gene product and the origin of dystrophin gene expression. Stem cells are promising for future therapy. Regardless of the challenges and controversies associated with stem cells, several clinical trials show an increase of muscle strength in patients who have received such therapies.

Keywords: Duchenne muscular dystrophy, therapy, genetic engineering, stem cells

Streszczenie

Dystrofia mięśniowa Duchenne'a jest chorobą dziedziczną w sposób recesywny, sprzężoną z chromosomem X, spowodowaną mutacjami w genie *DMD* kodującym białko dystrofinę. Obecnie opracowywane terapie mają na celu spowolnienie progresji choroby oraz przedłużenie przeżycia pacjentów. Leczenie z użyciem kortykosteroidów wiąże się z mniejszym ryzykiem zgonu (współczynnik ryzyka = 0,24; 95% CI = 0,07–0,91; $p = 0,0351$). Choć ostatnio prowadzone badania wykazały, że długotrwałe stosowanie kortykosteroidów przyczynia się do niskiego wzrostu i nadwagi, w metaanalizie 12 badań stwierdzono ich wpływ na zwiększenie siły mięśni, poprawę ich funkcji i lepszą jakość życia chorych. Odzyskanie ekspresji genu dystrofiny stanowi podstawę terapii genowych, w tym metody pomijania zmutowanego egzonu (tzw. *exon skipping*), zastosowania rekombinowanych wirusów związanych z adenowirusami w celu wprowadzenia minidystrofiny oraz wymiany genu (*gene transfer*). Trudności związane z terapiami genowymi wiążą się z rozmiarem genu oraz pochodzeniem ekspresji dystrofiny. Inną obiecującą terapię stanowią komórki macierzyste. Bez względu na trudności i kontrowersje związane z leczeniem tego typu kilka badań klinicznych wykazało, że poprawia ono siłę mięśniową u osób z chorobą Duchenne'a.

Słowa kluczowe: dystrofia mięśniowa Duchenne'a, terapia, inżynieria genetyczna, komórki macierzyste

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a form of muscular dystrophy in children. Its prevalence in the general population is about 3:100,000 inhabitants. The number of DMD in newborn males is recorded as 1:3,500 (Amato and Brooke, 2012; Passamano et al., 2012). As stated by Mendell, infants are less frequently affected (1:3,802–1:6,291); this particularly concerns male infants (Wein et al., 2015).

DMD is caused by the mutation in the gene that preclude the synthesis of the dystrophin and is related to the gene on the X chromosome (locus Xp 21) (Amato and Brooke, 2012; Angelini and Peterle, 2012; Beytía et al., 2012; Cirak et al., 2012). Dystrophin is one of the proteins connected to muscles and encoded by the dystrophin gene. This gene was identified in 1987 (Mitrpant et al., 2009), and is the largest gene discovered in humans as it accounts for approximately 0.1% of the total human genome (Amato and Brooke, 2012; Beytía et al., 2012; Cirak et al., 2012).

DMD can cause disability, respiratory disorders, cardiac dysfunction, and may lead to mortality (Bendixen et al., 2012; Passamano et al., 2012). Therapies, ranging from pharmacologic agents and genetic engineering to stem cells, are being developed for several reasons. These therapies are designed to improve clinical symptoms, slow down the progression of the disease, and increase patients' life expectancy. This article will provide an overview of DMD and some challenges linked with future therapies.

PATHOPHYSIOLOGY

A mutation of dystrophin synthesis is the basic pathophysiology of DMD (Angelini and Peterle, 2012). The open reading frame of the dystrophin gene may include deletions, duplications, point mutations, or other smaller rearrangements (Cirak et al., 2012). Most deletions occur between exons 44 and 55. When these mutations cause interference in the reading frame of dystrophin ("out of frame-mutation"), this dystrophin protein formation is truncated, resulting in no dystrophin production and the development of DMD (Beytía et al., 2012).

Dystrophin connects the actin cytoskeleton with the extracellular matrix through dystrophin-associated protein complex (DAPC) (Consalvi et al., 2011; Johnson et al., 2012). DAPC contains sub-complexes, namely sarcoglycan sub-complex, syntrophin, nNOS (neuronal nitric oxide synthase) and dystrobrevins sub-complex, as well as β -dystroglycan and α -dystroglycans sub-complex (Cirak et al., 2012).

Dystrophin is located at the cytoplasmic side of the sarcolemma. It contributes to signal delivery (Cirak et al., 2012) and maintains structural integrity of the skeletal muscles and the heart (Moorwood et al., 2011). It transduces by the power of the contractile apparatus with the extracellular matrix (Beytía et al., 2012), and functions as a shock

absorber that protects the muscle fibres from necrosis associated with muscle contraction (Beytía et al., 2012; Mitrpant et al., 2009).

In the absence of dystrophin, the entire DAPC is lost from the sarcolemma. Consequently, muscles are unable to withstand the stress of normal muscle contractions (Koo et al., 2012; Moorwood et al., 2011). This damage leads to influx of extracellular calcium, followed by the activation of proteases in the cell (Rosenkranz, 2004; Spurney, 2012). Next, a cascade of deleterious processes takes place (Beytía et al., 2012; Consalvi et al., 2011). Protease activity induces myocyte apoptosis, inflammation, and fibrosis (Rosenkranz, 2004; Spurney, 2012), resulting in failure of regeneration and replacement of muscle fibres with fat and connective tissues (Koo et al., 2012; Moorwood et al., 2011).

The absence of dystrophin in the cardiac muscle increases intracellular calcium levels, leading to the activation of calpains, myocardial cell degeneration or apoptosis and fibrosis. Excessive fibrosis conduces cardiomyopathy. Cardiac function decline stimulates the renin–angiotensin system and releases angiotensin II (AT II). AT II increases the transforming growth factor- β (TGF- β) expressions (particularly TGF- β 1) through the activation of angiotensin receptor type 1 in cardiac myocytes and fibroblasts. TGF- β induces cardiac fibroblast proliferation, deposition of extracellular matrix proteins, and development of cardiomyocyte hypertrophy (Rosenkranz, 2004; Spurney, 2012).

In the brain, the absence of dystrophin results in the disruption of the synapse integrity and interneuron transmission. Cognitive impairment is suspected due to a decrease in glucose metabolism in the cerebellum, i.e. the area associated with cognitive ability (Nardes et al., 2012).

CLINICAL SYMPTOMS

Patients appear normal at birth and begin to show signs of clumsiness at the age of 3–5 years (Mitrpant et al., 2009). The diagnosis is often made at the age of 4, when proximal muscle weakness is seen (Gowers' sign). Patients experience difficulty in rising from the floor between the age of 7–9 years, followed by inability to walk by the age of 13 years (Merlini et al., 2012). Scoliosis occurs after the patient loses the walking ability (Nardes et al., 2012).

Patients are in the plateau phase if they do not experience motor development any longer (ranging from the age of 4 to 8 years). Plateau phase is followed by declination phases which are characterised by the loss of motor skills, a decrease in physical endurance, and more frequent falls (Bushby et al., 2010).

Cardiac abnormalities are discovered after the age of 10 years (Spurney, 2012). The severity and onset of cardiomyopathy vary and are unrelated to the individual dystrophin gene mutation (Strehle and Straub, 2015). Respiratory muscles run into fibrosis, which results in respiratory insufficiency. Delay in language acquisition, cognitive impairment, and mental retardation are also observed (Nardes

et al., 2012). Death occurs due to cardiac or respiratory failure (Passamano et al., 2012).

Clinical features and increased plasma creatinine kinase are important in establishing the diagnosis (Wein et al., 2015). The values of plasma creatinine kinase generally exceed 1,000 IU/L and can reach 30,000 IU/L (Strehle and Straub, 2015). A muscle biopsy is indicated in patients without a detectable mutation. Molecular genetic testing has replaced the role of muscle biopsy in many diagnostic centres (Strehle and Straub, 2015).

STEROID AGENTS

Corticosteroids are the standard treatment (McAdam et al., 2012). The mechanisms of action of corticosteroids are: (1) reduction of muscle necrosis and inflammation; (2) modulation of cell response to inflammation; (3) increase in muscle regeneration and growth due to anabolic effects; (4) reduction of the rate of muscle breakdown; (5) action as a direct transcriptional modifier to increase dystrophin expression in muscle fibres; and (6) increase in synergistic molecules (Angelini and Peterle, 2012). Corticosteroids can slow the decline in function and muscle strength, prolong independent ambulation, improve lung function, delay the onset of cardiomyopathy, and reduce the incidence of scoliosis (Beytía et al., 2012; McAdam et al., 2012; Wilton and Fletcher, 2011). Corticosteroid therapy is associated with a lower mortality rate (hazard ratio = 0.24; 95% CI = 0.07–0.91; $p = 0.0351$) and a lower incidence of cardiomyopathy (hazard ratio = 0.38; 95% CI = 0.16–0.90; $p = 0.0270$) (Schram et al., 2013).

Initiation of corticosteroid therapy is not recommended for patients whose motor skills are still developing, especially patients under the age of 2 years. Corticosteroid therapy should be started in the plateau phase. The requirements for scheduled national immunisation should be met before starting this therapy (Angelini and Peterle, 2012; Beytía et al., 2012; Bushby et al., 2010).

The most frequent adverse effect of corticosteroids is a reduction in the patient's height and weight gain. Other adverse effects include cataracts, vertebral fractures, cushingoid facies, acne, hirsutism, arterial hypertension, behaviour disorders, delayed puberty, immunosuppression, and gastrointestinal problems (Beytía et al., 2012). Recent studies on the corticosteroid use in DMD have concluded that prolonged steroid use is associated with short stature and heavier weight (Lamb et al., 2016).

Prednisone dosage is 0.75 mg/kg/day (Amato and Brooke, 2012; Beytía et al., 2012; Bushby et al., 2010; Moxley et al., 2005). The effect is expected to end at least in 3 years (Amato and Brooke, 2012). Meta-analyses of 12 studies showed that 0.75 mg/kg/day prednisone or prednisolone in DMD improved muscle strength in 6 months and quality of life in 12 months (Matthews et al., 2016).

Deflazacort is a synthetic steroid (Amato and Brooke, 2012). It possesses anti-inflammatory and

immunosuppressive properties (Angelini and Peterle, 2012). Deflazacort 0.9 mg/kg/day is equivalent to 0.75 mg/kg/day of prednisone (Angelini and Peterle, 2012; Beytía et al., 2012; Moxley et al., 2005). This synthetic steroid has fewer side effects, but causes more cataracts than prednisone (Beytía et al., 2012; McAdam et al., 2012). Corticosteroids can be administered every other day, on the weekends in high doses and occasionally (Tab. 1).

GENETIC ENGINEERING

Restoration of gene expression addressed to the loss of gene product or protein defect is the foundation of gene replacement therapy. Challenges of gene therapy derive from the size of gene product and/or origin of gene expression (Mitrpant et al., 2009).

Myostatin is a part of a protein that forms the TGF- β group which regulates muscle size. Myostatin inhibition can be a potential therapy (Beytía et al., 2012; Malerba et al., 2012; Wilton and Fletcher, 2011). Several ways to inhibit myostatin are: administering follistatin, a myostatin receptor blocker, and destructive exon skipping for the myostatin gene. Follistatin inhibits the TGF- β member during muscle growth. The disturbance of myostatin signalling pathway from excessive expression of follistatin-related gene (FLRG), a serum protein associated with growth and differentiation factors (GDF), and myostatin propeptide, results in substantially improved muscle function and muscle mass in both normal and dystrophic tissue (Malerba et al., 2012; Wilton and Fletcher, 2011).

Utrophin ("ubiquitous dystrophin") is an autosomal homolog of dystrophin. It binds to proteins in the protein complex attached to dystrophins. Dystrophin and utrophin share 74% of features at the amino acid level and possess very similar domain structures (Lovering et al., 2005; Moorwood et al., 2011; Wilton and Fletcher, 2011). Nabumetone, a cyclooxygenase inhibitor, activates utrophin and has anti-inflammatory activity (Beytía et al., 2012; Moorwood et al., 2011).

Exon skipping is a potential therapy since the discovery of gene expression manipulation using antisense oligonucleotides (Hoffman et al., 2011; Wilton and Fletcher, 2011). Antisense oligonucleotides are designed to produce a transcription messenger of ribonucleic acid (mRNA) that produces several levels of truncated but functional dystrophin (Koo et al., 2012; Lovering et al., 2005). Antisense oligonucleotides are short nucleic acid sequences designed to selectively bind to the mRNA sequences or specific pre-mRNA to inflict a small double-helical region on the target mRNA. Through binding with this region and forming double helices at the target location, the mutated exon will be skipped. In this process, another pre-mRNA is re-edited properly within the framework, despite its shorter size (Lovering et al., 2005). They also mediate exon skipping during the pre-mRNA process for the excision of the exons that carry the termination codons in DMD (Fletcher et al., 2012).

In the intra-exonic mutations (exons that are located within the frame shift), excision of two or more exons are required to maintain the reading frame (Adkin et al., 2012).

To date, three different clinical trials have used deoxyribonucleic acid oligomers on phosphorothioate backbone (ODN), an analogue, such as RNA with 2'-O-modified bases on phosphorothioate backbone (2-OMeAO), and phosphorodiamidate morpholino oligomers (PMOs) (Wilton and Fletcher, 2011). PMOs give consistent exon skipping effects in *in vivo* mdx animal models and in human muscle (Koo et al., 2012). The strategy of combining the restoration of dystrophins and myostatin inhibition through dual exon skipping has been reported using 2'-O-methyl-phosphorothioate RNA antisense oligonucleotides (Malerba et al., 2012).

Exon 51 is chosen as the target because, by contrast with other exons, skipping at this exon can correct the reading frames in several patients (Mitrpant et al., 2009). Exon 51 has a potential value to be applied in about 1 out of 10 patients with DMD (Fletcher et al., 2012). The skipping process of dystrophin exon 51 in patients with relevant deletions repairs the open reading frames and induces the expression of dystrophin proteins after an intramuscular injection (Cirak et al., 2012).

Drisapersen is an antisense oligonucleotide that causes skipping of exon 51. Triple-blind randomised placebo-controlled studies have revealed significant improvement of walking distance in patients receiving continuous subcutaneous drisapersen 6 mg/kg weekly. Eteplirsen is a morpholino preparation that leads to removal of exon 51 during the RNA splicing process (excision of introns and joining of exons). Studies in patients receiving 30 mg/kg and 50 mg/kg intravenous eteplirsen weekly showed an

increasing production of dystrophin by 40–50% on biopsy and improvement of walking ability (Strehle and Straub, 2015; Wein et al., 2015).

Recombinant adeno-associated virus vectors (rAAV) carrying a miniaturised functional dystrophin gene (mini-dystrophin), have the potential to reduce the speed of muscle failure or to repair it (Bowles et al., 2012). The transfer of mini-dystrophin expressed by AAV is characterised by low efficiency and triggering of an immune response (Lovering et al., 2005).

One alternative approach is through a surrogate gene transfer, in which overexpression of a different protein allows for a functional correction of muscle pathologic abnormality or dysfunction. One of the examples is overexpression of the $\alpha 7$ -integrin gene. It encodes a protein that directly links the extracellular matrix to the actin cytoskeleton (Wein et al., 2015).

STEM CELLS

In the skeletal muscle, in addition to the satellite cell populations (myogenic mononuclear precursor cells), other progenitor cell populations that hold myogenic potential are also observed. They include mesangioblasts, MDSCs (muscle-derived stem cells), muscle-derived CD133+ progenitors, mesenchymal stem cells, and PW1 interstitial cells (Koo et al., 2012; Meregalli et al., 2013). Dystrophin expression also occurs in non-ambulatory patients after endometrial regenerative cell therapy (Ichim et al., 2010).

Ideal stem cells used to treat DMD should fulfil several criteria: be expandable *in vitro* without losing stem cell properties; be immune-privileged; differentiate into muscle fibres either to repair damaged fibres or to replace fibres

Mode of administration	Prednisone dosage	Deflazacort dosage	Comment	When side effects are found
Alternating	0.75–1.25 mg/kg, every other day	2 mg/kg, every other day	Less effective, but can be considered in the situation when administering daily doses causes side effects that cannot be overcome. According to Merlini et al. (2012), this method gives prolonged results of motor function but cannot replace the lost motor function	Dosage adjustments should be made when side effects cannot be tolerated
High dosage on weekends	5 mg/kg, every Friday and Saturday	Experiments have not been done	Lack of data to make comparisons with daily dosage. Can be considered as an alternative if there are problems with weight gain and behavioural change. Escolar et al. (2011) concluded that prednisone given on weekends (dosage 10 mg/kg/week and divided in 2 days) has similar positive effects with prednisone given daily	Dosage adjustments should be made when side effects cannot be tolerated
Intermittent	0.75 mg/kg for 10 days, followed by 10–20 days without medication	0.6 mg/kg on the 1–20 days and not given on the remaining days of the month	Less effective, but has fewer side effects. Considered to have the least therapeutic effectiveness, but is thought as a regimen that can be tolerated before the stoppage of steroid therapy. Intermittent therapy is thought to provide a safer profile in terms of side effects. According to a cohort study on deflazacort therapy, intermittence gives prolonged results in ambulated patients	Dosage adjustments should be made when side effects cannot be tolerated

Sources: Beytia et al., 2012; Bushby et al., 2010; Escolar et al., 2011; Merlini et al., 2012.

Tab. 1. Alternative strategies of corticosteroid administration

that have already been lost; reconstitute the satellite cell pool with functional stem cells, and lead to the improvement in muscle strength (Sienkiewicz et al., 2015).

Isolated stem cells can be transduced *in vitro* by retroviral or lentiviral vectors. Genetically modified cells or healthy foreign donor cells are developed *ex vivo* and injected systemically into the target muscle. Transplantation of autologous gene stem cell modification has the advantage of preventing side effects of immune response and cell rejection (Koo et al., 2012).

At present, mesenchymal stem cells are defined as undeveloped biological cells, capable of proliferation, cell renewal, and tissue repair (Mafi et al., 2011). Several clinical trials have shown an increase in muscle strength that was functional in patients receiving stem cell therapy, which was also verified in electromyography (Hogrel et al., 2013; Périé et al., 2014; Sharma et al., 2013, 2014; Sienkiewicz et al., 2015).

The therapeutic effect of mesenchymal stem cells is presumably not only gained through direct differentiation towards the injured tissue, but also through the production of paracrine factors inhibiting apoptosis. Other effects include stimulating proliferation within endogenous cells, and/or activating stem cells housed in the injured tissue. Local intramuscular administration of mesenchymal stem cells is possible with the addition of chemotactic/chemotropic support in the form of intravenous CD34 (Ichim et al., 2010).

At present, two approaches to cell transfer are carried out for the systemic transport from regenerative transplant cells to the skeletal muscle. They are also known as mesangioblast stem cell system and CD133+. The CD133+ cells can be isolated from peripheral blood or skeletal muscle tissue, and differentiate into muscle cells, haematopoietic cells and endothelial cells (Koo et al., 2012).

A myoblast is a muscle precursor cell that can proliferate and produce thousands of daughter cells. It can be obtained from biopsies and grown *in vitro* (Lovering et al., 2005). Intramuscular transplantation of these cells produces dystrophin-expressed muscle fibres at the level of 20–30% after more than 18 months of transplantation (Koo et al., 2012).

Limitation of the clinical efficacy of myoblast transfer therapy is mainly due to poor cell survival post-transplantation (Koo et al., 2012; Vella et al., 2011). The challenges of intramuscular myoblast transfer are associated with cell migration, systemic transport to the entire body, administering hundreds of intramuscular injections, overcoming immunological rejection, and high cost (Koo et al., 2012; Lovering et al., 2005).

CONCLUSION

Various therapies have been developed in treating DMD. Corticosteroids, with all their benefits and side effects, have been the standard treatment. The potential genetically

engineered therapies include exon skipping, use of recombinant adeno-associated virus carrying mini-dystrophin, and surrogate gene transfer. The challenges encountered in the development of these therapies refer to the product gene size, origin of dystrophin gene expression, transport effectiveness, and immunological rejection. Stem cell therapy is the latest, controversial form of treatment. Its challenges include transport, immunological rejection, and high cost.

Conflict of interest

The authors declare no conflict of interest.

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