Muscular response to the first three months of deflazacort treatment in boys with Duchenne muscular dystrophy

L. Jensen1*, S.J. Petersson1*, N.O. Illum2, H.C. Laugaard-Jacobsen3, T. Thelle4, L.H. Jørgensen1, H.D. Schroder1

1Department of Clinical Pathology, Institute of Clinical Research, University of Southern Denmark and Odense University Hospital, 5000 Odense C, Denmark; 2H.C. Andersen Children’s Hospital, Odense University Hospital, 5000 Odense C, Denmark; 3Pediatric Department, Aalborg University Hospital, 9000 Aalborg, Denmark; 4Pediatric Department, Regional Hospital Central Jutland, 8800 Viborg, Denmark

*Authors contributed equally to the work.

Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder caused by mutations in the dystrophin gene1,2 resulting in progressive degeneration of skeletal and cardiac muscle3. DMD is the most common childhood neuromuscular disorder and is estimated to affect 1 in 3500 male births worldwide4. These boys have no or very little functional dystrophin protein5. DMD presents in early childhood between 3-5 years of age and is rapidly progressive with most boys losing the ability to walk between the age of 9 and 116. The progression in pathology can be alleviated by physiotherapy and ventilation to relieve and postpone respiratory failure7, but overall effects are small. Although no curative treatment of DMD exists, glucocorticoids have been administered to patients with short8 and long-term9 beneficial effects. Clinical trials have documented the positive effects of glucocorticoids on walking speed and ambulation in DMD and conclude that a daily dose of prednisolone (0.75 mg/kg) or deflazacort (0.9 mg/kg) can be used in the treatment of boys between 5 and 15 years old, thereby delaying loss of ambulation with up to 2 years8,10-13. However, glucocorticoid treatment can have severe side effects such as weight gain, cataracts, osteoporosis, and reduction of height by slowing growth14 forcing some patients to withdraw from their treatment regime.

Despite the fact that glucocorticoids have been offered to DMD patients for the past three decades, the underlying molecular mechanisms leading to improved muscle strength,
alleviation of disease pathology and slowing of disease progression are not clear. Improved knowledge of the actions of glucocorticoids in DMD could form a basis for research on chemical compounds that dissociate the beneficial and detrimental effects of glucocorticoids and for the development of less toxic therapeutic approaches. Previous studies have suggested the primary effect to be on skeletal muscle regeneration and the satellite cell in terms of increased proliferation and differentiation\textsuperscript{15}, a modulation of the inflammatory response\textsuperscript{16} and less formation of connective tissue and fat\textsuperscript{17}. These findings suggest that the effect of glucocorticoid therapy in DMD patients is different from what is seen in individuals with normal functioning dystrophin, where glucocorticoids induce muscle atrophy\textsuperscript{18}.

The present study was designed to investigate glucocorticoid-induced changes in transcriptionally regulated signalling pathways in DMD patients before and after their first three months of deflazacort treatment. We hypothesized that the initial response to treatment is key to investigate the primary effects of glucocorticoids in modulating skeletal muscle. Therefore, gene transcript levels and protein expression by immunohistochemistry were investigated in nine DMD patients before and after glucocorticoid treatment as well as compared with normal, healthy subjects.

**Patients and methods**

**Study design and participants**

Nine boys (aged: 7 yrs, 6 months±2 yrs, 8 months), who fulfilled the diagnostic criteria of DMD were ascertained prospectively at the Regional Hospital Central Jutland (Viborg), Aalborg University Hospital or Odense University Hospital in Denmark and included in this open-label national trial. None of the boys received glucocorticoid treatment before inclusion in the study, but initiated administration of 0.9 mg/kg/day of deflazacort (Calcort, Shire Pharmaceuticals) immediately following the initial biopsy. Normal healthy muscle tissue was collected through archived muscle biopsies at the Department of Clinical Pathology, Odense University Hospital, Denmark. These biopsies were collected from m. vastus lateralis of boys suspected to have muscle disease, but ultimately showing no muscle pathology. The study was ap-

| Id | Age   | Gene findings                           | Deflazacort Dosage (mg/kg/day) | Clinical observations | Vignos grade\textsuperscript{1} | Brooke grade\textsuperscript{2} | Reported effects |
|----|-------|-----------------------------------------|-------------------------------|-----------------------|-------------------------------|-------------------------------|------------------|
| 1  | 7.11  | Hemizygosity for c.6955C>T, p.Q2319X     | 0.82                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre | 2 | 1 | Muscular strength unchanged but better endurance |
| 2  | 9.01  | Hemizygosity for c.6955C>T, p.Q2319X     | 0.80                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre | 2 | 1 | Walking endurance better. More independent |
| 3  | 5.06  | No duplication or deletion detected (analysed 2005) | 1.02                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre | 1 | 1 | Difficulty rising from floor without help unchanged |
| 4  | 6.10  | Deletion exon 51                         | 0.98                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre | 2 | 1 | Muscular strength better. Less need for pause in athletic activities |
| 5  | 9.02  | Deletion exon 12-15                      | 0.86                          | Pseudohypertrophy of calves. No Gower’s sign observed | 1 | 1 | Walked same distance but more agile |
| 6  | 13.09 | Deletion exon 10-44                      | 0.83                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre | 2 | 1 | Able to bike faster and longer |
| 7  | 4.11  | Out of frame deletion of exon 45         | 0.95                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre occasionally | 1 | 1 | More agile but unchanged running distances |
| 8  | 5.07  | Deletion of exon 45                      | 0.86                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre occasionally | 2 | 1 | Better endurance when running |
| 9  | 5.05  | Deletion exon 36-43                      | 0.91                          | Pseudohypertrophy of calves. Used Gower’s manoeuvre occasionally | 2 | 1 | Physical activities and endurance unchanged |

Table 1. Treatment regime and functional capability of the patients. Patients were evaluated immediately before and after three months of deflazacort treatment. Mutations identified in the dystrophin gene are described. \textsuperscript{1}Vignos grade for lower extremity functions (1 denotes normal functions and 10 confined to bed). \textsuperscript{2}Brooke grade for upper extremity function (1 denotes normal function and 6 denotes no arm and hand function). Effects following three months of treatment as reported by the patients and/or parents.
proved by the Ethical committee of the Region of Southern Denmark (S-VF-20050166), was performed in accordance with the Helsinki Declaration and registered at ClinicalTrials.gov (identifier: VF-20050166).

Patient evaluation

Patient assessment included evaluation of age of onset, ambulation status, use of Gower’s manoeuvre, and general muscle function (Vignos scale and Brooke scale). Patient/parent reported effect of the treatment is also presented (Table 1). Serum creatine kinase (CK) levels as well as selected mRNA and protein targets were determined before and after glucocorticoid treatment.

Genetic confirmation

The patients included in the study presented with mutations in the dystrophin gene and/or absent/reduced dystrophin expression on a muscle biopsy. This was evaluated by standard clinical genetic analysis of genomic DNA performed at the Department of Clinical Genetics, Odense University Hospital, Denmark, and the results are included in Table 1. Genetic variants are submitted to the DMD database under LOVD (http://grenada.lumc.nl/LSDB_list/lsdbs/DMD). To support the genetic findings dystrophin protein expression was evaluated in all patients by standard diagnostic immunohistochemical methods performed at the Department of Clinical Pathology, Odense University Hospital, Denmark. For details of methods see section 2.8. In one patient (patient 3) no genetic defects could be detected, however immunohistochemistry confirmed reduced expression of the dystrophin protein (data not shown).

Sample collection

All samples were obtained after informed and written parental consent and obtained under institutionally approved protocols. Blood samples and muscle biopsies from m. vastus lateralis were collected from all patients before and after three months of deflazacort treatment. CK levels were evaluated using standard clinical procedures. Muscle biopsies were performed through an incision in the skin using 2 mm Tru-cut needles. The muscle biopsy was divided in two parts. One was used for histological investigations, and the other was transferred to lysis solution (Applied Biosysos) and kept at -20°C. The normal, healthy biopsies (n=8, aged: 5 yrs, 3 months±2 yrs, 4 month) were archived Tissue Tek-embedded sections were used. Paraffin embedded sections using an Ultra Turrax T8 (Ika Werke). The homogenized muscle samples were incubated with proteinase K for 1h at room temperature followed by 30 min. incubation on ice. Total RNA was prepared from 500 µl of homogenate using the ABI PRISM™ 6100 Nucleic Acid PrepStation with the Total RNA Chemistry kit (Applied Biosystems) including a DNase wash step to remove any contaminating gDNA according to the manufacturer’s instructions. Total RNA from muscle biopsies from a subset of the patients was extracted using standard Trizol (Gibco) extraction. Briefly, biopsies were transferred to 1mL Trizol, homogenized, centrifuged and the supernatant transferred to a new tube and incubated for 5 min. Chloroform was added and the RNA extracted, followed by precipitation with isopropanol and natriumacetate. The pellet was then washed with cooled ethanol (75%), dried and resuspended in molecular biology grade water. Quantity and purity of all RNA samples were determined using a NanoDrop device (Thermo Scientific). Due to inadequate amount and quality of RNA (260/280 ratio below 1.5, 260/230 ratio below 1.5, and/or <150 ng total RNA) from two patients, these had to be excluded from the remaining transcript analyses.

Real time RT-PCR

Five micrograms of total RNA were reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Evaluation of mRNA targets and reference genes were carried out using a custom-designed 384-well TaqMan® Array (Applied Biosystems). All target assays have a FAM™ reporter dye at the 5’ end of the MGB probe and a non-fluorescent quencher at the 3’ end. Five hundred ng cDNA mixed with TaqMan Universal Master Mix containing uracil-N-glycosylase (P/N 4304437) was loaded into the TaqMan® Array card, followed by centrifugation and sealing. All real-time RT-PCR reactions were performed on an ABI Prism 7900HT Sequence Detection system (Applied Biosystems). Table 2 lists the mRNA targets investigated in this study (Gene symbol, gene name and function, and assay ID).

Raw data was extracted using the software SDS2.1.1 (Applied Biosystems) by applying automatic detection of Ct-values followed by export to the qBase software program (Biogazelle)9 for semi-quantitative analysis. In the qBase program is an in-built software, geNorm, which we used to determine the references genes that were most stable between all samples analysed. The criteria for selection of reference genes in geNorm were an M<1.5 and V<0.15, and the geNorm algorithm for finding the most stable reference genes is based on the geometric mean of the selected genes used. Based on the geNorm results we chose B2M and PGK1 as reference genes for all targets analysed.

Immunohistochemistry

Immunoreactions were developed using the Envision+ peroxidase detection system (DAKO) using antibodies specific to CD68 (KP1, DAKO) diluted 1:2000, PAX7 (Developmental Studies Hybridomral Bank) diluted 1:100 and NEONATAL MYOSIN (WB-MHCn, Novostra) diluted 1:10 in antibody diluent (DAKO). Frozen sections or paraffin embedded sections were used. Paraffin embedded sec-
tions were deparaffinised in xylene and rehydrated and endogenous peroxidase activity was quenched. To detect NEONATAL MYOSIN sections were then incubated in 0.002% protease type XIV at 20°C for 8 min. (P5147, Sigma). Heat-induced antigen retrieval in TEG (10mM Tris, 0.5mM EGTA, pH 9.0) was performed before incubating with primary antibodies for 1 h. Frozen sections were fixed in 4% NBF before incubating with primary antibodies for 1 h. Sections were washed and incubated with HRP-labelled secondary antibody for 30 min (Dako), before development with DAB+ (DAKO), counterstaining with Mayer’s haematoxylin and mounting with AquaTex (Millipore).

Table 2. Overview of mRNA targets. Gene symbol, protein names and function, and assay ID of the mRNA targets included in the real-time qPCR analysis.

| Gene symbol | Protein | Function | Reference | Assay ID |
|-------------|---------|----------|-----------|----------|
| **Reference genes** | | | | |
| B2M | BETA-2-MICROGLOBULIN | House-keeping gene | | Hs99999907_m1 |
| PGK1 | PHOSPHOGLYCERATE KINASE 1 | House-keeping gene | | Hs99999906_m1 |
| **Target genes** | | | | |
| ADIPOQ | ADIPONECTIN | Hormone secreted by adipose tissue that regulates metabolic processes involving glucose and fatty acids. | 20 | Hs00605917_m1 |
| ANXA1 | ANNEXIN A1 | Putative mediator of the anti-inflammatory actions of glucocorticoids | 20,21 | Hs00167549_m1 |
| CD68 | CLUSTER OF DIFFERENTIATION 68 | Marker of macrophages | 22 | Hs00154355_m1 |
| CDH15 | M-CADHERIN | Marker of activated satellite cells (proliferating myogenic precursors) | 23 | Hs00170504_m1 |
| C-MET | MET/HEPATOCYTE GROWTH FACTOR RECEPTOR | Satelite cell marker, delamination/migration and proliferation of myogenic cells in myogenesis | 24,25 | Hs00179845_m1 |
| DLK1 | DELTA-LIKE HOMOLOG 1 | TGF-beta/activin effector | 26 | Hs00171584_m1 |
| FKB51 | FK506 BINDING PROTEIN 51 | A regulator of steroid hormone receptor signaling including hormone binding and translocation to the nucleus. | 27-29 | Hs00188025_m1 |
| FGFR1 | FIBROBLAST GROWTH FACTOR RECEPTOR 1 | Receptor involved in late myogenesis | 30-32 | Hs00241111_m1 |
| FGFR2 | FIBROBLAST GROWTH FACTOR 2 | Growth factor, late myogenesis | 30-32 | Hs00266645_m1 |
| GDF8 | GROWTH DIFFERENTIATION FACTOR 8/MYOSTATIN | Member of the TGF beta superfamily, negative regulator of skeletal muscle growth, inhibits satellite cell differentiation. | 33,34 | Hs00193363_m1 |
| IGFR1 | INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR | IGF is an anabolic growth catalyst in muscle | 35-38 | Hs00609566_m1 |
| MYF5 | MYOGENIC FACTOR 5 | Myogenic regulatory factor (MRF), myogenic precursor cell determination/proliferation of skeletal myoblasts | 37 | Hs00271574_m1 |
| MYF6 (MFR4) | MYOGENIC FACTOR 6 | Myogenic regulatory factor, terminal differentiation marker | 37 | Hs00231165_m1 |
| MYH8 | MYOSIN-8/NEONATAL MYOSIN | Marker of regenerating muscle fibers | 39 | Hs00267293_m1 |
| MYOD1 | MYOGENIC DIFFERENTIATION 1 | Determination of satellite cell/skeletal myoblast proliferation/differentiation | 37 | Hs00159528_m1 |
| MYOG | MYOGENIN/MYOGENIC FACTOR 4 | Muscle regeneration, terminal differentiation marker | 37,40 | Hs00231167_m1 |
| NR3C1 | GLUCOCORTICOID RECEPTOR | Glucocorticoid receptor – mediates downstream events | 38,41 | Hs00230818_m1 |
| PAX7 | PAIRED BOX 7 | Marker of satellite cells, commits pluripotent stem cells to the myogenic lineage | 42,43 | Hs00242962_m1 |
| PTGS2/COX-2 | PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE 2/CYCLOOXYGENASE 2 | Inflammatory cells, peroxidase activity, inhibited by corticosteroids | 44,45 | Hs00153133_m1 |
| TNFa | TUMOR NECROSIS FACTOR ALPHA | Proinflammatory cytokine | 46 | Hs00174128_m1 |
Image acquisition, analysis and presentation

Microscopy was carried out using a Leica DM LB2 microscope and images acquired using a digital camera Leica DFC 300F and Leica FireCam software or a digital camera Leica DFC 290 connected to a Leica DMR microscope equipped with the Leica Application Suite software (all from Leica). Images were assembled using Adobe CS6 software (Adobe Systems Incorporated). The protein expression pattern of NEONATAL MYOSIN, PAX7, and CD68 in untreated and treated biopsies was evaluated microscopically by an experienced pathologist (blinded to the patient identification).

Statistical analysis

The sample population was assumed to be collected from a normally distributed population. Differences between the patient samples and the normal samples were tested using student’s t-test (untreated vs. normal and treated vs. normal), while the effect of treatment was tested with a paired t-test (untreated vs. treated). A linear test for trend was subsequently performed to examine whether the patient samples approached the values in the normal, healthy subjects after treatment. To perform a linear test for trend, the groups were categorized in the following order; untreated > treated > normal. CK-values were evaluated by a ratio paired t-test. All statistical analyses were carried out using Prism 6 (GraphPad Software Inc.).

Results

Glucocorticoid treatment affects CK-values and patient/parent reported motor skills

In our small cohort of DMD patients serum CK levels decreased following treatment with deflazacort for 3 months: Untreated: 14500±2405 units/L vs. treated: 9004±2735 units/L, mean±SEM (p<0.05), even though one patient showed a large increase in CK (Figure 1). In line with the general improvement in CK-value, the patients improved in their motor function, muscle strength or muscular endurance after treatment based on reports from the patients or their parents. A description of patient skills and muscle function after treatment can be found in Table 1. These results are in accordance with the general acceptance of this treatment having beneficial effects8. However, to fully support these findings functional tests e.g. a 6-minute walk test could have been performed.

Differences in baseline mRNA levels between patient and normal muscle

A set of 20 selected genes was semi-quantitatively assessed in untreated, treated and normal biopsies. The genes were selected on the basis of their involvement in key regulatory pathways controlling such diverse processes as myogenesis, regeneration, satellite cell activation, inflammation and adipogenesis as well as specific glucocorticoid receptor analysis and factors involved in the response to glucocorticoids (Table 2). Compared to normal muscle, untreated patients showed reduced expression of the satellite cell markers/regulators CDH15 (M-CADHERIN), and PAX7, the myogenic regulatory factors MYF5, MYF6, MYOD, and the growth factors/late myogenic regulators FGF2 and IGF1R. The expression of MYOG was not different from normal muscle. The expression of the pro-inflammatory cytokine TNFα, as well as the macrophage marker CD68 and the marker for immature, newly formed myotubes, MYH8, were all increased in untreated patients compared to normal muscle (p<0.05) (Figure 2 and 4). These results indicate that in dystrophic muscle the myogenic program mediated by satellite cells and myogenic regulators could be partly suppressed, whereas a pro-inflammatory environment might be favoured. Increased expression of MYH8 however, indicates that regeneration is occurring in the patients.

Effect of glucocorticoids on transcriptional signalling pathways involved in myogenesis and regeneration

Several genes implicated in myogenesis and regeneration were analysed (Figure 2 A-I and Table 2). The linear test for trend showed that the mRNA values after treatment of CDH15, c-MET, DLK1, MYF5, MYF6, MYOD, MYH8 and PAX7 all significantly approached values of normal muscle (p<0.05) (Figure 2). This suggests that the gene expression response to glucocorticoids in the muscle approaches normalization of the regenerative and myogenic pathways.

To further investigate these changes in myogenic regulatory factors we performed immunohistochemical analyses of selected proteins involved in myogenesis.

We stained for the satellite cell regulator PAX7, but there was no observable difference in the presence of PAX7 positive cells in untreated and treated patient biopsies (Figure 3). Since we observed a reduction in MYH8 mRNA following treatment, which points towards a reduction in active repair, we stained for NEONATAL MYOSIN, but again we did not detect differences in the presence of NEONATAL MYOSIN positive muscle fibres.
in untreated vs. treated biopsies (Figure 3). The pattern was similar in all patients suggesting that the changes on mRNA level at this early time point in the treatment is not reflected on protein level as measured by immunohistochemistry.

**Effect of treatment on glucocorticoid signalling pathways and growth factors**

The effect of glucocorticoids was also evaluated on genes involved in downstream glucocorticoid signalling and control of glucocorticoid signalling by analysing ANXA1, a mediator of the inhibitory actions of glucocorticoids, FKBPS, a direct steroid hormone receptor regulator, PTGS2/COX-2, an inflammatory mediator inhibited by glucocorticoids as well as the glucocorticoid receptor, NR3C1. None of the transcripts were significantly changed between untreated and treated biopsies, and there was no significant difference between normal biopsies and untreated patient samples (Figure 4A-D).

These results suggest that the glucocorticoid signalling pathways are not altered on gene expression level in response to the dystrophic pathology and appear not to be affected by treatment as well. Thus the positive action of the glucocorticoids in these patients probably do not relate to the glucocorticoid signalling in itself.

When we analysed growth factors with known actions in muscle; FGF2, FGFR1, IGF1R and GDF8 (Figure 4E-H), we observed that mRNA levels of FGF2 and IGF1R were expressed at lower levels in the DMD patients compared to normal muscle in both untreated and treated samples (p<0.05), and the linear test for trend showed that values of both IGF1R and FGF2 approached normal values after treatment (p<0.05), suggesting a normalization of the expression level in response to treatment. Expression of FGFR1 and GDF8/myostatin remained unchanged following treatment, suggesting that these pathways are not implicated in the response to deflazacort treatment.
Adipogenic and inflammatory actions of glucocorticoids

In DMD there is a replacement of muscle tissue for fat. We therefore analysed the mRNA expression of ADIPONECTIN (ADIPOQ), which is a hormone exclusively secreted by adipose tissue and thus a marker for adipogenesis. Expression of ADIPOQ was increased in untreated samples compared to normal muscle (p<0.05) and this expression was decreased following treatment. However, the expression level in treated samples was still significantly higher compared to normal muscle (p<0.05) (Figure 4I). Even though the level of ADIPOQ approached the normal level following treatment, this was not statistically significant when analysed with the linear test for trend.

In DMD patients the destruction of muscle tissue results in increased inflammation, which was supported by our observation of a significantly increased mRNA expression of both the macrophage marker CD68 and the pro-inflammatory cytokine TNFα in untreated samples compared to normal muscle (Figure 4L and 4K). Following treatment with deflazacort, which acts anti-inflammatory, we did observe a reduction in expression of both CD68 and TNFα mRNA. Linear test for trend showed that the expression level of the treated samples approached normal values (p<0.05) for both inflammatory markers. However, when analysing the presence of macrophages in tissue sections (Figure 3E and 3F), there was no clear change in the presence of CD68-positive cells between untreated and treated biopsies. So, even though the mRNA results suggest that deflazacort acts to reduce inflammation, this is not at this time reflected in the presence of macrophages in the affected muscle tissue.

Discussion

Despite decades of usage in the clinical setting, the precise actions of glucocorticoids in dystrophic muscle are still not clear. Here we describe the very early muscular responses and adaptations to deflazacort treatment of patients. Specifically, levels of mRNA transcripts important for myogenesis, muscle regeneration and muscle maturation were increased after three months of treatment and were approaching the expression levels of normal muscle. Furthermore, treatment affected mRNA levels of CD68 and TNFα, supporting a role for glucocorticoids in regulation of tissue inflammation, and increased mRNA levels of FGF2 and IGF1R point towards adaptations in pathways of cellular growth. Overall, our data indicates a wide range of actions of glucocorticoids involved in modulating skeletal muscle and possibly affecting disease progression and clinical performance in patients, and the
most pronounced effects seem to be on muscle regeneration. When healthy individuals are treated with glucocorticoids, the result is often muscle atrophy\(^{18}\), while glucocorticoid treatment leads to increased muscle strength and improved muscle function in DMD patients\(^{21-23}\). Our findings are consistent with previous studies implicating a wide range of glucocorticoid actions - at least in the initial treatment phase\(^{24}\). With the linear test for trend, we find that mRNA levels of the satellite cell markers \(\text{CDH15, C-MET, and PAX7}\) in the treated patients are approaching normal levels indicating increased satellite cell activation after three months of treatment. This is in line with previous findings of an increased number of satellite cells in DMD patients after six months of treatment\(^{15}\). In adult muscle \(\text{C-MET}\) is important for myoblast motility and efficient myoblast fusion\(^{25}\), while \(\text{PAX7}\) is essential for regulating the expansion and differentiation of satellite cells during myogenesis\(^{26}\). We observed a normalization of both factors, which is an important finding, as this indicate potential increase in the regenerative capacity. However, in our study the protein expression analysis did not support the finding the on mRNA level, as we did not detect differences in the presence of \(\text{PAX7}\) positive nuclei between untreated and treated biopsies at the three months time point. Our results therefore suggest that the very early effects are not yet observable on protein level or the method of immunohistochemistry is not sensitive enough to find the small differences that may exist.

The myogenic factors, consisting of \(\text{MYF5, MYF6, MYOG, and MYOD}\) play essential roles in myogenic specification, differentiation, and maintenance during muscle development but also during regeneration\(^{27-30}\). We find that mRNA expression of these factors approached the normal level after three months of glucocorticoid treatment suggesting improved regeneration and fibre maturation. In further support of this, the neonatal form of \(\text{MYOSIN, MYH8}\) is highly increased in dystrophin-deficient muscle compared to normal muscle reflecting the on-going cycles of degeneration and regeneration\(^{31}\), but is decreased after treatment indicating that the maturation process might be improved and there is less damage to be repaired. The immunohistochemical analysis of \(\text{NEONATAL MYOSIN}\) in patient biopsies did not show any obvious differences in the presence of positive myofibers. Thus the change in gene expression of \(\text{MYH8}\) is not observed.
activating actions (direct binding of the glucocorticoid receptor) and, in contrast, the trans-repressing actions (interference with activation of transcription factors). Flanigan and coworkers identified the presence of CAC3, while others have found that this early after treatment using histological evaluation.

Dystrophic muscle is characterized by chronic inflammation, and numerous studies indicate that glucocorticoid treatment plays a role in modulating the inflammatory response. Flanigan and colleagues identified the presence of anti-dystrophin T cell immunity in some DMD patients, and that glucocorticoid treatment decreased this risk compared with no treatment, suggesting that some beneficial effects may derive through modulation of the T cell response. Glucocorticoid treatment also improves dystrophic muscle pathology by activation of the NFAT pathway by up-regulating the activity of CALCINEURIN, while others have found that this part of the beneficial effect can be attributed to a reduction in Ca²⁺ influx and of the size of Ca²⁺ pools in dystrophic muscle fibres. Since CD68-positive macrophages are involved in the inflammatory process and development of tissue necrosis, and necrotic muscle fibres undergoing phagocytosis display strong expression of TNFα, our findings of reduced levels of CD68 and TNFα mRNA post treatment indicate a reduced tissue invasion with a subsequent diminished necrosis, which is consistent with previous findings of decreased formation of connective tissue and fat following glucocorticoid treatment. However, the immunohistochemical analysis of CD68 did not show a clear reduction in invasion of macrophages following the three months of treatment, thus the initial changes in mRNA expression is not immediately reflected on protein level or actual presence of macrophages in the affected tissue.

The present study investigated the role of a number of growth factors, as six to eight weeks of glucocorticoid treatment is associated with an increase in muscle mass, which however, is proposed to be mediated by inhibition of muscle proteolysis rather than stimulation of muscle protein synthesis. Contrary to this, the muscle wasting pathways controlled by the ubiquitin ligase ATROGIN-1 or the MYOSTATIN/ AKT/mTOR were not induced during any stage of DMD disease progression. We find a significant linear trend indicating that post values of FGF2 and IGF1R are approaching normal values, however it should be noted that the increases are small. Likewise, we find a non-significant decrease in GDF8 perhaps supporting an activation of pathways inducing protein synthesis.

Recent studies have attempted to separate the positive effects of glucocorticoids from the unwanted side effects by parting the chemical properties and designing new drugs. Separating the trans-repressing actions (interference with e.g. pro-inflammatory transcription factors) from the trans-activating actions (direct binding of the glucocorticoid response elements of specific genes) might reduce the unwanted side effects. With the present data in mind, revealing effects of glucocorticoid treatment on a range of parameters in the patients and stimulates the muscle transcript profile to approach that of normal muscle. This knowledge can be important in the development of modified corticosteroids and treatment regimes with less severe side effects.

Conclusion

In conclusion, this study demonstrates a very early response to glucocorticoid treatment, and confirms that multiple pathways are involved in implementing the actions of glucocorticoid treatment. It is likely that it is the combination of these effects that induces improved muscle function in the patients and stimulates the muscle transcript profile to approach that of normal muscle. This knowledge can be important in the development of modified corticosteroids and treatment regimes with less severe side effects.

Acknowledgements

This work was supported by grants from Danish Stem Cell Consortium and The Research Council of the Region of Southern Denmark.

Author contributions

SJP, NOI and HDS conceived the idea and designed the project; SJP, LJ, NOI, HCLJ, TT and HDS performed the experiments; LJ, SJP, LHJ and HDS interpreted the results of the experiments; LJ, SJP and LHJ drafted the manuscript; LJ, SJP, NOI, LHJ and HDS edited and revised the manuscript; LJ, SJP, NOI, HCLJ, TT, LHJ and HDS approved the final version of the manuscript.

References

1. Hoffman EP, Brown RH Jr, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919-28.
2. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 1987;50:509-17.
3. Shin J, Tajrishi MM, Ogura Y, Kumar A. Wasting mechanisms in muscular dystrophy. Int J Biochem Cell Biol 2013;45:2266-79.
4. Emery AE. Population frequencies of inherited neuromuscular diseases - a world survey. Neuromuscul Disord 1991;1:19-29.
5. Hoffman EP, Kunkel LM, Angelini C, Clarke A, Johnson M, Harris JB. Improved diagnosis of Becker muscular dystrophy by dystrophin testing. Neurology 1989; 39:1011-7.
6. King WM, Ruttencutter R, Nagaraja HN, et al. Orthopedic outcomes of long-term daily corticosteroid treatment in Duchenne muscular dystrophy. Neurology 2007; 68:1607-13.
7. Jeppesen J, Green A, Steffensen BF, Rahbek J. The Duchenne muscular dystrophy population in Denmark.
1977-2001: prevalence, incidence and survival in relation to the introduction of ventilator use. Neuromuscul Disord 2003;13:804-12.
8. Manzur AY, Kuntzer T, Pike M, Swan A. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev 2008:CD003725.
9. Takeuchi F, Yonemoto N, Nakamura H, et al. Prednisolone improves walking in Japanese Duchenne muscular dystrophy patients. J Neurol 2013;260:3023-9.
10. Mayhew AG, Cano SJ, Scott E, et al. Detecting meaningful change using the North Star Ambulatory Assessment in Duchenne muscular dystrophy. Dev Med Child Neurol 2013;55:1046-52.
11. Griggs RC, Moxley RT, 3rd, Mendell JR, et al. Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). Neurology 1993;43:520-7.
12. Bushby K, Muntoni F, Urtizberea A, Hughes R, Griggs R. Report on the 124th ENMC International Workshop. Treatment of Duchenne muscular dystrophy: defining the gold standards of management in the use of corticosteroids. 2-4 April 2004, Naarden, The Netherlands. Neuromuscul Disord 2004;14:526-34.
13. Campbell C, Jacob P. Deflazacort for the treatment of Duchenne Dystrophy: a systematic review. BMC Neurol 2003;3:7.
14. Ricotti V, Ridout DA, Scott E, et al. Long-term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne muscular dystrophy. J Neurol Neurosurg Psychiatry 2000;69:203-6.
15. Hussein MR, Abu-Dief EE, Kamel NF, Mostafa MG. Steroid therapy is associated with decreased numbers of dendritic cells and fibroblasts, and increased numbers of satellite cells, in the dystrophic skeletal muscle. J Pathol 2010;221:145-53.
16. Flanigan KM, Campbell K, Viollet L, et al. Anti-dystrophin T cell responses in Duchenne muscular dystrophy: prevalence and a glucocorticoid treatment effect. Hum Gene Ther 2013;24:797-806.
17. Arpan I, Willcocks RJ, Forbes SC, et al. Examination of effects of corticosteroids on skeletal muscles of boys with DMD using MRI and MRS. Neurology 2014;83:974-80.
18. Schakman O, Kalista S, Barbe C, Loumaye A, Thissen JP. Glucocorticoid-induced skeletal muscle atrophy. Int J Biochem Cell Biol 2013;45:2163-72.
19. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:RESEARCH0034.
20. Fisher I, Abraham D, Boui K, Hoffman EP, Muntoni F, Morgan J. Prednisolone-induced changes in dystrophic skeletal muscle. FASEB J 2005;19:834-6.
21. Moxley RT 3rd, Pandya S, Ciafaloni E, Fox DJ, Campbell K. Change in natural history of Duchenne muscular dystrophy with long-term corticosteroid treatment: implications for management. J Child Neurol 2010;25:1116-29.
22. Hoffman EP, Reeves E, Damsker J, et al. Novel approaches to corticosteroid treatment in Duchenne muscular dystrophy. Phys Med Rehabil Clin N Am 2012;23:821-8.
23. Angelini C, Peterle E. Old and new therapeutic developments in steroid treatment in Duchenne muscular dystrophy. Acta Myol 2012;31:9-15.
24. Pescatori M, Broccoli A, Minetti C, et al. Gene expression profiling in the early phases of DMD: a constant molecular signature characterizes DMD muscle from early postnatal life throughout disease progression. FASEB J 2007;21:1210-26.
25. Webster MT, Fan CM. c-MET regulates myoblast motility and myocyte fusion during adult skeletal muscle regeneration. PLoS One 2013;8:e81757.
26. van Maltzahn J, Jones AE, Parks RJ, Rudnicki MA, Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc Natl Acad Sci U S A 2013;110:16474-9.
27. Tapskott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development 2005;132:2685-95.
28. Gayraud-Morel B, Chrétién F, Flamant P, Gomès D, Zammit PS, Tajbakhsh S. A role for the myogenic determination gene Myf5 in adult regenerative myogenesis. Dev Biol 2007;312:13-28.
29. Jin Y, Murakami N, Saito Y, Goto Y, Koishi K, Nonaka I. Expression of MyoD and myogenin in dystrophic mice, mdx and dy, during regeneration. Acta Neuropathol 2000;99:619-27.
30. Gunther S, Kim J, Kostin S, Lepper C, Fan CM, Braun T. Myf5-positive satellite cells contribute to Pax7-dependent long-term maintenance of adult muscle stem cells. Cell Stem Cell 2013;13:590-601.
31. Chen YW, Zhao P, Borup R, Hoffman EP. Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. J Cell Biol 2000;151:1321-36.
32. De Paepe B, De Bleecker JL. Cytokines and chemokines as regulators of skeletal muscle inflammation: presenting the case of Duchenne muscular dystrophy. Mediators Inflamm 2013;2013:540370.
33. St-Pierre SJ, Chakkalakal JV, Kolodziejczyk SM, Knudson JC, Jasmin BJ, Megeney LA. Glucocorticoid treatment alleviates dystrophic myofiber pathology by activation of the calcineurin/NF-AT pathway. FASEB J 2004;18:1937-9.
34. Metzinger L, Passaquin AC, Leijendekker WJ, Pindron P, Ruegg UT. Modulation by prednisolone of calcium handling in skeletal muscle cells. Br J Pharmacol 1995;116:2811-6.
35. De Paepe B, Creus KK, Martin JJ, De Bleecker JL. Up-regulation of chemokines and their receptors in Duchenne muscular dystrophy: potential for attenuation of myofiber necrosis. Muscle Nerve 2012;46:917-25.
36. Tews DS. Tumour necrosis factor-mediated cell death pathways do not contribute to muscle fibre death in dystrophinopathies. Acta Neuropathol 2005;109:217-25.
37. Rifai Z, Welle S, Moxley RT 3rd, Lorenson M, Griggs RC. Effect of prednisone on protein metabolism in Duchenne dystrophy. Am J Physiol 1995;268:E67-74.
38. Chen YW, Nagaraju K, Bakay M, et al. Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. Neurology 2005;65:826-34.
39. Huynh T, Uaesoontrachoon K, Quinn JL, et al. Selective modulation through the glucocorticoid receptor ameliorates muscle pathology in mdx mice. J Pathol 2013;231:223-35.
40. Morrison-Nozik A, Anand P, Zhu H, et al. Glucocorticoids enhance muscle endurance and ameliorate Duchenne muscular dystrophy through a defined metabolic program. Proc Natl Acad Sci U S A 2015;112:E6780-9.