Journal of Musculoskeletal and Neuronal Interactions JMNI

Original Article

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

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Abstract

Objective: Duchenne muscular dystrophy (DMD) patients are often treated with glucocorticoids; yet their precise molecular action remains unknown. **Methods:** We investigated muscle biopsies from nine boys with DMD (aged: 7,6±2,8 yrs.) collected before and after three months of deflazacort treatment and compared them to eight healthy boys (aged: 5,3±2,4 yrs.). mRNA transcripts involved in activation of satellite cells, myogenesis, regeneration, adipogenesis, muscle growth and tissue inflammation were assessed. Serum creatine kinase (CK) levels and muscle protein expression by immunohistochemistry of selected targets were also analysed. **Results:** Transcript levels for *ADIPOQ, CD68, CDH15, FGF2, IGF1R, MYF5, MYF6, MYH8, MYOD, PAX7*, and *TNFa* were significantly different in untreated patients vs. normal muscle ($p<0.05$). Linear tests for trend indicated that the expression levels of treated patients were approaching normal values ($p<0.05$) following treatment (towards an increase; *CDH15, C-MET, DLK1, FGF2, IGF1R, MYF5, MYF6, MYOD, PAX7*; towards a decrease: *CD68, MYH8, TNFa*). Treatment reduced CK levels ($p<0.05$), but we observed no effect on muscle protein expression. **Conclusions:** This study provides insight into the molecular actions of glucocorticoids in DMD at the mRNA level, and we show that multiple regulatory pathways are influenced. This information can be important in the development of new treatments.

Keywords: DMD, Human Skeletal Muscle, Real Time qPCR, MYOD, Glucocorticoids

Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder caused by mutations in the dystrophin gene[1,2] resulting in progressive degeneration of skeletal and cardiac muscle[3]. DMD is the most common childhood neuromuscular disorder and is estimated to affect 1 in 3500 male births world wide[4]. These boys have no or very little functional dystrophin protein[5]. 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 11[6]. The progression in pathology can be alleviated by physiotherapy and ventilation to relieve and postpone respiratory failure[7], but overall effects are small. Although no curative treatment of DMD exists, glucocorticoids have been administered to patients with short-[8] and long-term[9] 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 years[8,10-13]. However, glucocorticoid treatment can have severe side effects such as weight gain, cataracts, osteoporosis, and reduction of height by slowing growth[14] 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,

The authors have no conflict of interest.

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Edited by: F. Rauch
Accepted 6 February 2017

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. [1]Vignos grade for lower extremity functions (1 denotes normal functions and 10 confined to bed). [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.

Id	Age	Gene findings	Deflazacort	Clinical observations	Vignos grade[1]	Brooke grade[2]	Reported effects
		Dystrophin	Dosage (mg/kg/day)	Untreated patients	Untreated patients	Untreated patients	Treated patients
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

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[15], a modulation of the inflammatory response[16] and less formation of connective tissue and fat[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[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-

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 Biosystems) and kept at -20°C. The normal, healthy biopsies (n=8, aged: 5 yrs, 3 month±2 yrs, 4 month) were archived Tissue Tek-embedded frozen skeletal muscle collected from m. vastus lateralis. For RNA analysis, the Tissue Tek was trimmed away and the muscle tissue transferred to lysis solution as described above.

RNA isolation and cDNA synthesis

Total RNA was isolated by homogenizing the muscle samples using an Ultra Turrax T8 (Ika Werke). The homogenized 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, protein 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)[19] 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 Hybridomal Bank) diluted 1:100 and NEONATAL MYOSIN (WB-MHCn, Novocastra) diluted 1:10 in antibody diluent (DAKO). Frozen sections or paraffin embedded sections were used. Paraffin embedded sec-

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	Satellite 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
FKBP51	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
FGF-2	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
IGF1R	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 1	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

tions were deparaffinised in xylene and rehydrated and endogenous peroxidase activity was quenched. To detect NEONATHAL 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 hematoxylin and mounting with AquaTex (Millipore).

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 before and after treatment can be found in Table 1. These results are in accordance with the general acceptance of this treatment having beneficial effects[8]. 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.

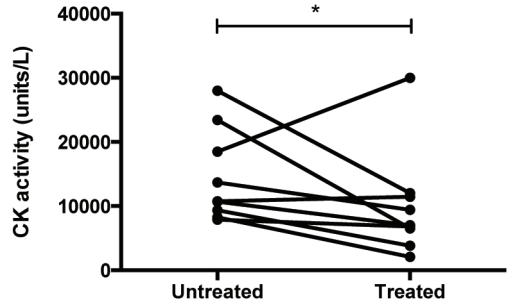

Figure 1. Serum CK levels before and after treatment with glucocorticoids. CK levels were measured in blood samples collected before and after treatment for 3 months. Collectively, the ratio of CK-values decreased following treatment ($p<0.05$).

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

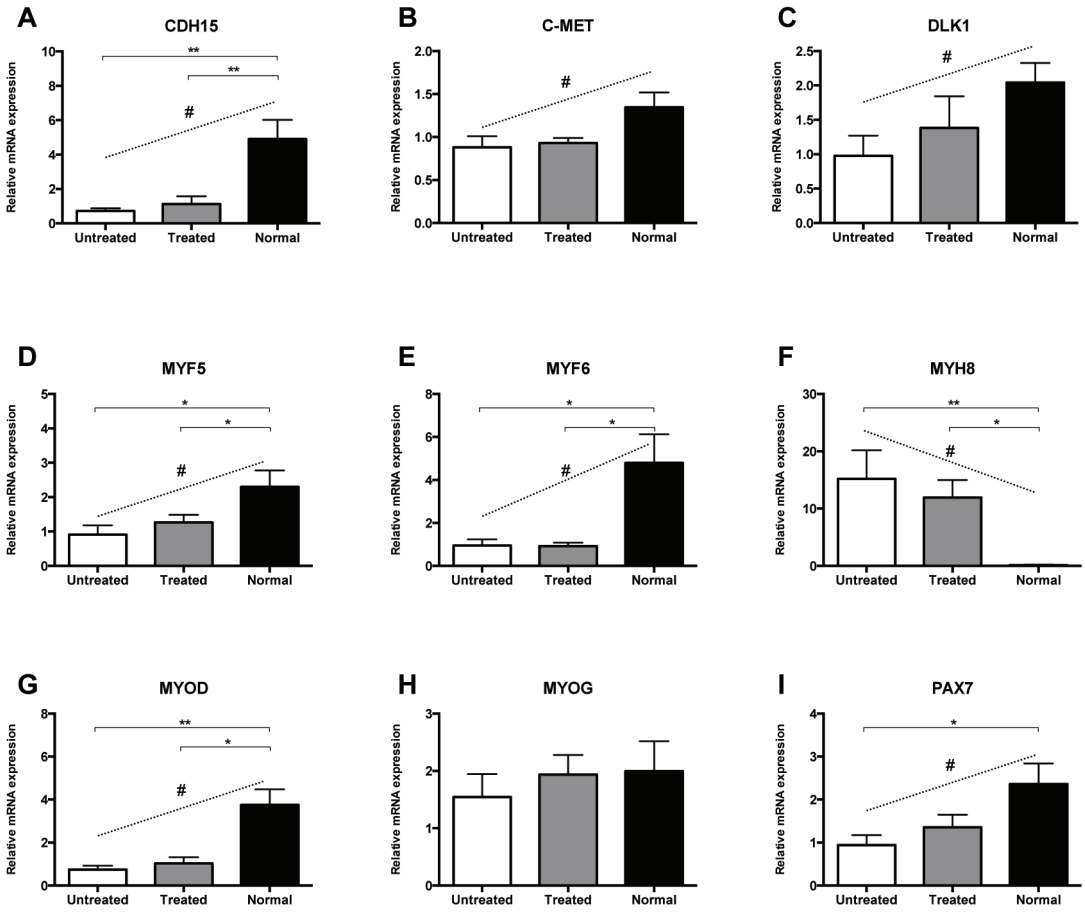

Figure 2. Expression of satellite cell and myogenic factors in response to glucocorticoid treatment. mRNA expression of CDH15, C-MET, DLK1, MYF5, MYF6, MYOD, MYOG, PAX7 and MYH8 in DMD patients before and after treatment compared to normal muscle. mRNA values were normalized to the reference genes B2M and PGK1 and presented as mean±SEM. DMD patient samples (untreated and/or treated as indicated) were significantly different from normal muscle as indicated by: *p<0.05, **p<0.01. All targets were evaluated by linear test for trend and # indicates a significant trend over the groups (untreated > treated > normal), p<0.05.

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, *FKBP5*, 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.

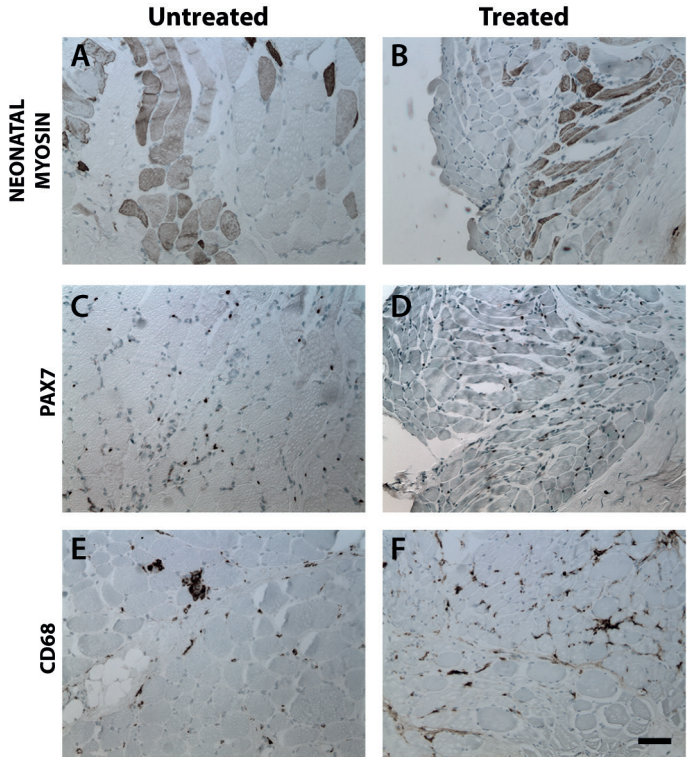

Figure 3. Expression pattern of protein targets in skeletal muscle in response to glucocorticoid treatment. Immunohistochemical analyses of NEONATAL MYOSIN (A, B), PAX7 (C, D) and CD68 (E, F) protein expression in untreated (left column) and treated (right column) patient biopsies. Representative images from patient 7 is shown. Scalebar= 100 m.

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[20]. 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

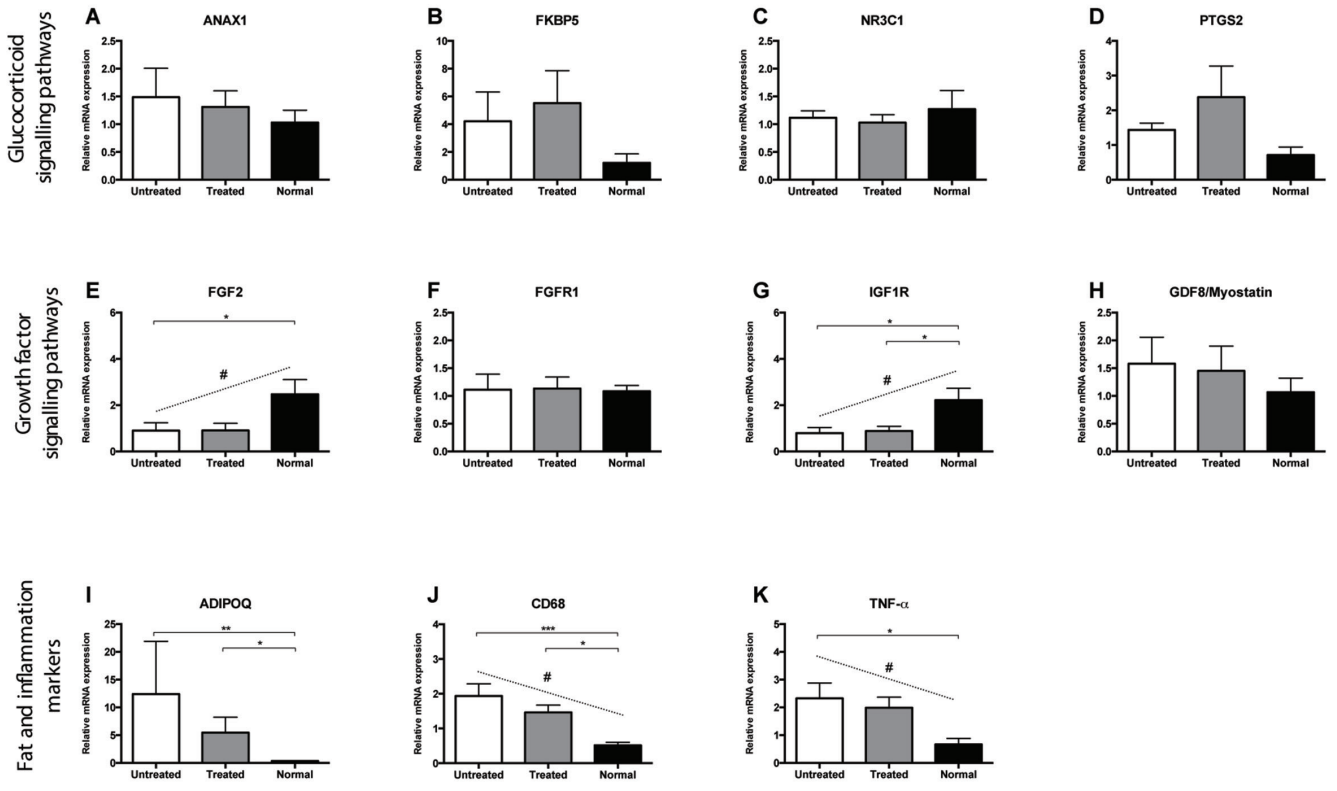

Figure 4. Expression of genes involved in glucocorticoid signalling pathways, regulating growth and markers of inflammation and adipogenesis. The expression levels of mRNA transcripts in the DMD patients before and after treatment and in normal muscle are shown for ANAX1, FKBP5, NR3C1 and PTGS2 in A-D. FGF2, IGF1R, FGFR1 and GDF8/Myostatin mRNA levels are shown in E-H, and the mRNA levels of ADIPOQ, CD68 and TNFα can be found in I-K. All mRNA values were normalized to the reference genes B2M and PGK1 and are presented as mean±SEM. Patients (untreated and/or treated) were significantly different from normal muscle, *p<0.05, **p<0.01, ***p<0.001. All targets were evaluated by linear test for trend and # indicates a significant trend over the groups (untreated > treated > normal), p<0.05.

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 *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 C-*MET* is important for myoblast motility and efficient myoblast fusion[25], while *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 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 *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 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 NEONATAL MYOSIN in patient biopsies did not show any obvious differences in the presence of positive myofibers. Thus the change in gene expression of *MYH8* is not observed

this early after treatment using histological evaluation.

Dystrophic muscle is characterized by chronic inflammation[32], 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[16]. Glucocorticoid treatment also improves dystrophic muscle pathology by activation of the NFAT pathway by up-regulating the activity of CALCINEURIN[33], while others have found that part of the beneficial effect can be attributed to a reduction in Ca^{2+} influx and of the size of Ca^{2+} pools in dystrophic muscle fibres[34]. Since CD68-positive macrophages are involved in the inflammatory process and development of tissue necrosis[35], and necrotic muscle fibres undergoing phagocytosis display strong expression of TNFα[36], our findings of reduced levels of *CD68* and *TNFa* 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[17]. 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[37]. 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[38]. 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)[39] might reduce the unwanted side effects. With the present data in mind, revealing effects of glucocorticoid treatment on a range of parameters and indicating that multiple pathways are affected after only three months of treatment, it is crucial to search all potential effects of glucocorticoid treatment in order to determine the importance of each. Based on our data, larger studies are warranted to elucidate whether a single parameter of the glucocorticoid treatment or the combined effects are most important for the final treatment result. A recent study in mice using the highly potent glucocorticoid dexamethasone indicates that the metabolic transcription factor KLF15 might play an important role in mediating the performing enhancing effects of glucocorticoids[40], which should be investigated in a human setting in the future.

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.

Acknoledgements

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 analysed the data; 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.

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