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Steroids in Duchenne muscular dystrophy: from clinical trials to genomic research

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Abstract

Steroids represent the only pharmacological palliative treatment for Duchenne muscular dystrophy. However, they do have side effects and despite a large number of published studies showing their efficacy, they are still not universally used. This is largely due to the lack of functional outcome and quality of life measures in most of the published studies and suggests that further trials might be required to answer some of the still unclear aspects of their role. Another important aspect of steroid therapy in Duchenne dystrophy is that we do not know how they work in dystrophic muscle. We have initiated a collaborative study on gene profiling using microarray in steroid-treated *mdx* mice. cDNA microarray studies were performed to examine the levels of skeletal muscle gene expression in a pool of *mdx* mice treated with prednisolone for 1 and 6 weeks. Interesting preliminary data on untreated *mdx* mice suggest that the gene profiling of young (7 weeks) versus older (12 weeks) mice is very significantly different. Furthermore, a large number of genes showed significant changes in expression at the mRNA level on treatment with prednisolone. These included structural protein genes; signalling genes and genes involved in immune response. Hopefully, analysis of this pattern of steroid-induced gene expression will provide some insight into understanding how glucocorticoids improve strength in Duchenne dystrophy, and may help in developing more effective and less toxic therapeutic approaches.

Keywords: Duchenne muscular dystrophy; Steroids; Gene profiling; Clinical trial; *mdx* mouse

1. Duchenne muscular dystrophy and steroids: clinical trials

Duchenne muscular dystrophy is a common and progressive form of muscular dystrophy for which no curative treatment is available. Corticosteroid therapy has been shown by a number of studies to have some positive effect on Duchenne dystrophy: at least 16 studies on nearly 1000 affected children have been published in the last three decades (reviewed in Ref. [1]). Most studies have demonstrated transitory improvement and/or stabilization of muscle strength, but the trials were relatively short-term and the side effects significant. The long term benefit of steroid therapy on walking ability and later morbidity have not been clearly documented in randomized studies and no consensus guidelines on the type or dosage of steroids to use are available to the clinician. It is clear from the published studies that high doses of corticosteroids (continuous administration of more than 1 mg/kg per day of prednisolone), although beneficial on muscle strength, are not sustainable over a long period of time because of the severe systemic side effects. The group of Moxley recently presented the long-term result of a prospective follow-up study of the patients initially reported in 1989 [2], originally treated with daily prednisolone at a dose of 0.75 mg/kg or higher. After continuing treatment for some 13–15 years, the dose of prednisolone had to be reduced because of the systemic steroids side effects to a mean of 0.35 mg/kg per day, with a range from 0.15 to 0.75 mg/kg per day [3].

This introduction highlights some of the controversial aspects related to the use of steroids in muscular dystrophy. Firstly, how much steroids to use and which regime to use? At what age to start and what to expect in terms of functional benefit (i.e. prolongation of ambulation)? Secondly, how do steroids work in Duchenne muscular dystrophy? And how is it that a treatment that may induce muscle weakness in normal muscle, is able to reduce loss of muscle power in muscular dystrophy? If one had a clear understanding of the mechanism of action of steroids, it might be possible to devise more effective treatments with less severe side effects.

Regarding the issue on how much steroids to use and for

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how long, it is difficult to provide an evidence-based answer. A systematic review of steroids/prednisolone has not been reported yet, although the project 'Steroids in Duchenne Muscular Dystrophy' has recently been registered with the Cochrane Neuromuscular Diseases Review Group. The 47th European Neuromuscular Centre International Workshop on treatment of Duchenne muscular dystrophy held in 1997 concluded that "there was currently evidence of potential value of steroids in treatment of Duchenne dystrophy and recommended further collaborative studies to answer specific questions" [4]. The need for such a long-term study was discussed again at the most recent European Neuromuscular Centre International Workshop on treatment of Duchenne dystrophy held in December 1999 [5].

Why so many uncertainties after several decades of clinical use of steroids? One important point relates to the practicalities of organizing a large randomized clinical trial in which significant functional endpoints such as loss of independent ability to rise from the floor or loss of independent ambulation are taken into account. In order to overcome these difficulties, surrogate endpoints are used instead of functional endpoints. For most of the studies on steroids published so far, these are functional endpoints represented by Medical Research Council (MRC) strength measurements. A number of studies have shown unequivocally an effect of corticosteroids on preservation of 'average muscle scores' as measured with MRC [1,2,6]. However, in absence of demonstration of a functional advantage, the question of whether the improved average muscle score has had a significant and long-lasting impact on patient disease course cannot be easily answered. This is probably the main reason why corticosteroids are currently not universally used in Duchenne dystrophy. When designing therapeutic trials it is therefore important to consider both functional outcomes and quality of life issues in order to be able to prove that a treatment might be effective and useful in clinical practice. However, this has quite significant implications for the duration and hence the funding necessary to run such a trial.

Our group is presently involved in setting up a large collaborative randomized placebo controlled therapeutic trial aimed at assessing the long-term functional outcome of an intermittent regime of prednisolone in Duchenne dystrophy. A pilot study performed at the Hammersmith in the past has clearly indicated that an intermittent regime of prednisolone, designed to minimize side effects (0.75 mg/kg for 10 days each month), had a significant effect on slowing down the rate of progression of muscle weakness [7]. More recently we have used a regime of 10 mg/kg per day for 10 days on and 10 days off and showed in an open study that this treatment is effective and well tolerated with negligible side effects even if started at an early age [8].

In order to answer some of the questions related to the role of steroids in Duchenne dystrophy, we are currently aiming to arrange a further trial on the role intermittent steroids focused on quality of life issues and two main functional endpoints: loss of the ability to rise from the floor and loss of independent ambulation. We have set up a network of 15 Paediatric Neuromuscular centres in the United Kingdom and are currently seeking funds to carry out this study. A detailed protocol has been agreed between the collaborating centres following a series of meetings held during the last 2 years by the both the lead clinicians and the physiotherapists; the list of centres participating in this initiative is indicated in Appendix A. We estimate that a minimum of 190 boys will be required to demonstrate clinically significant benefit of a placebo-controlled study aimed at establishing if steroids are capable of prolonging ambulation. The recruitment of the children for such a study will take up to 3 years. Of the two functional endpoints chosen, it is estimated that 5 years will be required in order to show a statistical difference in the loss of the ability to rise from the floor, and 8 years to document an alteration in the loss of independent ambulation. These figures clearly illustrate some of the difficulties encountered when trying to set up a study focused on functional aspects. On the other hand the very fact that no consensus has been reached to date regarding the use of steroids on Duchenne dystrophy strongly suggests that analysis of functional aspects together with assessment of quality of life issues will have to be addressed in trial planning in the future.

2. Mechanism of action of steroids in skeletal muscle

The mechanism of action of steroids in Duchenne muscular dystrophy is not known, but a number of theories have been put forward. These include:

(a) positive effect of steroids on myogenesis [9,10];
(b) anabolic effect on muscle, resulting in increased muscle mass [11];
(c) stabilization of muscle fibre membranes [12];
(d) attenuation of muscle necrosis [13], although this is controversial [14,15];
(e) effect on intracellular calcium concentrations [16,17];
(f) immunosuppressive effect with reduction of mononucleated cells, in particular cytotoxic $CD8^+$ cells [18].

However, similar effects on T-cell subpopulation are also found in Duchenne patients treated with azathioprine, a drug that is not effective in slowing down progression of disease [19,20]. This led to the hypothesis that immunosuppression is not the main mechanism of action of corticosteroids.

2.1. Genetic profiling in mdx mice treated with steroids

In order to obtain unbiased information on the mechanism of action of steroids in dystrophin deficient muscle, our group, has started a study focused on the gene profiling of steroid-treated *mdx* mice. The *mdx* mouse was chosen as a model for dystrophin deficiency. Despite significant limitations of this animal model compared to children with Duch-

enne, steroids have been previously used in *mdx* mice and shown to significantly protect the skeletal muscle from degeneration [21,22]. We have chosen to study both an 'acute' model of steroid administration (as significant functional improvement can be observed in children with Duchenne as early as after 7–10 days [1,7,8]); and a chronic model of continuous steroid administration, as most studies have used this protocol. cDNA microarray studies were therefore used to examine the levels of skeletal muscle gene expression in a pool of *mdx* mice treated with prednisolone for 1 and 6 weeks. We administered continuous prednisolone at a dose of 1 mg/kg per day, as this was the dosage previously used in the *mdx* [22]. The mice were started on treatment at the age of 6 weeks, well after the acute muscle degeneration phase, and analysed at 7 and 12 weeks of age. Fifteen *mdx* mice were studied for each time point along with age-matched controls. In addition, steroids were administered to a group of wild-type control mice to study if the effect on gene profiling of steroids in *mdx* mice is similar to the one obtained in normal muscle.

The mice were killed and the gastrocnemius muscles removed, and immediately snap frozen and stored in liquid nitrogen. Total RNA was then extracted carefully. The skeletal muscle was weighted and carefully analysed histologically and histochemically (number of internal nuclei; fibre size; fibre typing; degeneration/regeneration).

2.2. cDNA microarrays

The arrays used were Clontech Mouse Broad Range 1.2 arrays, a Nylon membrane-based system containing over 1000 polymerase chain reaction products of known genes, including a range transcription factors, structural proteins, proto-oncogenes and signalling genes. After hybridization of a cDNA probe prepared according to the manufacturer's instructions, the arrays were then analysed upon a Phosphorimager after 3 days on a Phosphorimager screen. The entire process was then repeated twice for each RNA sample as means of confirming the arrays. The Phosphorimager images of each file were obtained using the ImageQuant software package and analysed using the Clontech Atlas-Image software. Normalization using the sum of global intensities of the arrays was chosen with the local background for each cDNA spot taken into account. A significance level of a 2-fold difference in gene expression was selected, so as to decrease biological noise. The data were considered only if the differential expression was confirmed in a set of three independent experiments. The gene expression profile was compared with age-matched control *mdx* mice and with the control mice treated with steroids.

2.3. Preliminary results

We have completed the analysis of all the microarrays and are currently involved in validating the results obtained with two other independent techniques: RNA quantitation using Northern blot analysis and protein studies for all molecules that appear to be differentially regulated and for which antibodies are available. As this study is ongoing, we will refer only to the general trend observed, as a detailed discussion of results will only be possible after having completed the validation process.

From a morphological point of view the main difference was the finding that steroid-treated *mdx* mice had less type 1 fibre predominance compared to the untreated *mdx* mice.

Regarding the gene profiling results, a number of genes were differentially regulated in treated *mdx* mice; interestingly there was only a very limited overlap between the differentially regulated genes observed in steroid-treated wild-type mice compared to steroid-treated *mdx* mice. This suggests that the mechanism of action of steroids in dystrophin-deficient muscle is likely to be different from their action in normal muscle.

There were also very striking differences between the gene profiling of untreated young (7 weeks old) *mdx* mice compared to untreated older (13 weeks old) mice. This is very interesting as it suggests that the dystrophic process is very dynamic, with significantly different sets of genes up- and down-regulated at different stages of the disease. The only available previous study on gene profiling on *mdx* mice focused on a single time point (8 weeks) [23]. Furthermore, these preliminary results demonstrate the potential limitation of pooling muscle of Duchenne patients of different ages [24,25], highlighting the difficulties and limitations of gene profiling studies in the patients.

The comparison of the differentially regulated genes in the 7-week-old *mdx* treated for 1 week versus aged-matched controls showed eight differentially regulated genes. The comparison of the older mice treated for 6 weeks identified 26 differentially regulated genes, with only partial overlap with the genes differentially regulated at 7 weeks (after only 1 week of treatment).

Regarding which genes were differentially regulated, these included structural protein genes (up-regulated by steroids); signalling genes (up- or down-regulated by steroids) and genes involved in immune response (down-regulated by steroids).

In conclusion, our study has identified a number of genes differentially regulated in steroid-treated *mdx* mice. It is likely that the changes in these genes in steroid-treated animals may be acting to improve strength and function of dystrophic muscle in the *mdx* mouse [21,22]. Our preliminary data also suggest that not all genes differentially regulated by chronic steroid administration are also differentially regulated following a shorter duration treatment in *mdx* mice. This is interesting, considering that both continuous and intermittent steroid treatment appear to be beneficial in slowing down disease progression in Duchenne dystrophy.

We think that analysis of this pattern of gene expression will help our understanding on how corticosteroids influence disease progression in Duchenne muscular dystrophy, and might help in developing novel therapeutic approaches to this disease. Our preliminary studies also show striking differences in the gene profiling of young versus mature *mdx*

mice, further supporting the concept that muscular dystrophy is a dynamic process in which different mechanisms might play a different role at different stages of the disease.

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Appendix A. UK centres that have agreed to take part to the UK steroid trial study

Dr Helen Roper	Birmingham Heartlands Hospital
Dr A.Y. Manzur	Hammersmith Hospital, London
Dr Paola Nicolaides	Royal Liverpool Children's Hospital
Dr Peter Baxter	Sheffield Children's Hospital
Professor Kate Bushby	Newcastle General Hospital
Dr Rosalind Quinlivan	Royal Shrewsbury Hospitals NHS Trust
Dr Imelda Hughes	Royal Manchester Children's Hospital
Dr Philip Jardine	Frenchay Hospital, Bristol
Dr Neil Thomas	Southampton General Hospital
Dr Colin Ferrie	Leeds General Infirmary
Dr Michael Pike	John Radcliffe Hospital, Oxford
Dr Stephanie Robb	Guy's Hospital, London
Dr Robert McWilliams	Royal Hospital for Sick Children, Glasgow
Dr David Mellor	University Hospital, Nottingham
Dr Katherine Deville	Great Ormond Street Hospital, London

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