Collagen accumulation in muscles of children with cerebral palsy and correlation with severity of spasticity

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Muscle function often becomes progressively more compromised in children with spastic cerebral palsy, leading to reduced mobility. This study aimed to examine the role that muscle connective tissue plays in this process. Severity of spasticity as determined by a range of clinical measures was assessed in 26 children (14 males 12 females; age range 4 to 17 years) with either diplegic or quadriplegic cerebral palsy (CP). Muscle biopsies from the vastus laeralis muscle were obtained for biomedical and histological analysis during orthopaedic surgery as part of the child's ongoing care. Total collagen was quantified by hydroxyproline determination. Two clinical measures of severity, Modified Ashworth Scale and Balance, were shown to have a highly significant correlation with collagen content, and Ambulatory Status, Clonus, and Selective Muscle Control all showed positive trends. Collagen I accumulated in spastic muscle's endomysium which appeared to be thickened, and fibrotic regions with sparse muscle fibres were evident in more severe cases. This suggests that collagen may be involved in increases in muscle stiffness observed in spasticity. Once developed, these changes are essentially irreversible and we suggest that future treatments should consider including prevention of muscle fibrosis.

CP can affect an individual in one of a number of ways, but the most common is spastic CP where muscle spasticity is characterized by 'a velocity dependent increase in tonic stretch reflexes (muscle tone) with exaggerated tendon jerks' (Lance 1980, p 485).

Our understanding of CP is largely based on the many previous studies which have concentrated on the primary neurological aspects of CP as reviewed by Botte et al.(1988) and Wright and Rang (1990). However, disability also results from changes in children's muscles as spasticity develops (Tardieu et al. 1979, 1982). We believe that there is much to be gained from understanding what is happening within spastic skeletal muscles themselves at the cellular and biochemical level, particularly with respect to their connective tissue. This consists of a hydrated gel of proteoglycans, that contains structural proteins, the collagens, and other non-collagenous proteins such as fibronectin. The amount and type of connective tissue within an organ is important for its development, repair following injury, and in the maintenance of normal physiology. Accumulation of excessive amounts of connective tissue can have serious deleterious effects as is seen in, for example, fibrotic diseases of the liver and lung.

Very little is known of the pathology of spastic muscle in CP, the main emphasis of previous studies (Edstrom 1970, Castle et al. 1979, Rose et al. 1994) being on changes in muscle fibre type and size. The purpose of this study was to ask if connective tissue, and more specifically the structural protein collagen, accumulates within the spastic muscles of children with CP and if so, if there is a correlation between the amount of connective tissue and the severity of spasticity in those individuals.

In normal muscle, collagen is highly organized around fascicles or groups of myofibres in a structure known as the perimysium and around individual myofibres known as the endomysium. Both the perimysium and the endomysium play important roles in force transduction and muscle stiffness. It is possible that if connective tissue accumulates within spastic muscle, the muscle's mechanical properties may be affected such that this accumulation contributes either directly or indirectly to the development of contractures and secondary bony abnormalities thus playing a major role in mobility problems observed in CP.

Method

PARTICIPANTS

Ethical approval was obtained from the Central Oxford Research Ethics Committee and informed parental consent given to take open muscle biopsies from children undergoing orthopaedic surgery as part of their ongoing care.

Participants were 26 children (14 males, 12 females, age range 4 to 17 years, mean 10.6, SD 0.6 years) with either diplegic or quadriplegic spastic CP. Children were recruited before surgery undertaken as part of ongoing management of their condition. Exclusion criteria were any neuromuscular disorder other than CP, previous splinting or immobilization of lower limbs, drug therapy within the previous year targeted at or with known effects on skeletal muscle, previous soft tissue surgery, or injection of the quadriceps muscle or related nerves at any stage. All children were receiving the same program of physical therapy, regardless of severity.

Thirteen patients had spastic diplegia (five males and eight females) and thirteen patients had spastic quadriplegia (nine males and four females).

The children were clinically assessed before their operation by the same clinician for the presence or absence of clonus, balance (scored excellent, good, moderate, or poor) selective muscle control, (scored good, moderate, or poor) ambulatory status, (scored independent, limited therapeutic, or non-ambulant) and for severity of their spasticity using the Modified Ashworth Scale (MAS) applied to the quadriceps (Gage 1991, Bohannon and Smith 1987). This is summarized in Table I.

Control biopsies were obtained from two normally developing children (two males aged 10 years 4 months and 16 years 3 months) without CP or any other disease or disorder with neurological or skeletal muscle involvement, who were undergoing orthopaedic surgery for different indications. The same exclusion criteria used for the CP group were applied. Each individual was assigned a random patient code number and subsequent analyses were performed blind.

MUSCLE BIOPSIES

Skeletal muscle biopsies were collected from the proximal third of the quadriceps (vastus lateralis) muscle and, with the exception of the control children, were spastic in all cases. Tissue was divided into two parts: the first was snap frozen on dry ice for collagen determination and the second processed for histochemistry by freezing in isopentane cooled on dry ice.

HYDROXYPROLINE DETERMINATION

Total collagen within the muscle biopsy was measured by assaying for the exclusive collagen-specific modified amino acid, hydroxyproline.

All muscle biopsy samples were freeze dried and the dry weight was noted. Each sample was then hydrolysed in a sealed tube in a known volume of 6 M hydrochloric acid at 110°C for 24 hours.

Hydroxyproline was assayed on a microtitre plate using a modification of the method of Bergman and Loxley (1963). Absorbance was measured spectrophotometrically at 570 nm using a Wallac 1420 Victor^2 Multilabel Counter (EG and G Wallac, Turku, Finland). Hydroxyproline concentration was determined by comparison with a standard curve prepared from pure hydroxyproline of known concentrations, which was included on every plate. Results were reported as μg hydroxyproline per mg dry weight of tissue and represent the means of three to five independent assays.

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Collagen I was localized in 5 μm cryostat sections using specific antibodies to these proteins as follows: sections were preincubated in hyaluronidase (4800 U/mL in 0.025 M sodium chloride, 0.05 M sodium acetate buffer, pH 5) for 2 hours at room temperature, washed extensively in tris buffered saline (TBS; 0.05 M tris base, 0.15 M sodium chloride, pH 7.2), and incubated in goat anticollagen I diluted 1:100 in TBS, for 1 hour at room temperature. The sections were washed again extensively before incubating in the fluorescently-labelled secondary

Table I: Participant diagnoses and hydroxyproline content

Patient	*Age y:mo*	*Diagnosis*	*Modified Ashworth Scale*	*Balance*	*Clonus*	*Ambulation*	*Selective control*	*Mean hypro μg/mg dry weight*	*SEM*
1	10:4	Normal	Normal	Excellent	No	Limited distance	Excellent	2.81	0.19
2	16:3	Normal	Normal	Excellent	No	Independent	Excellent	2.55	0.35
3	8:5	Quadriplegic	Mild	Good	No	Limited distance	Good	2.65	0.32
4	10:4	Diplegic	Mild	Good	No	Limited distance	Good	8.29	0.71
5	10:4	Diplegic	Mild	Good	No	Independent	Good	6.42	0.83
6	15:3	Diplegic	Mild	–	No	Independent	–	4.07	0.37
7	15:5	Diplegic	Mild	Good	Yes	Independent	Good	3.84	0.43
8	4:0	Diplegic	Moderate	Good	Yes	Limited distance	Good	3.08	0.30
9	9:0	Diplegic	Moderate	Good	No	Limited distance	Good	5.62	0.29
10	10:1	Quadriplegic	Moderate	Good	Yes	Therapeutic	Moderate	2.67	0.15
11	10:5	Diplegic	Moderate	Moderate	Yes	Therapeutic	Good	13.97	1.04
12	10:6	Diplegic	Moderate	Poor	No	Limited distance	Moderate	3.99	0.63
13	13:10	Diplegic	Moderate	Good	No	Limited distance	Good	3.54	0.29
15	14:1	Diplegic	Moderate	Good	Yes	Limited distance	Good	7.14	1.36
14	15:10	Diplegic	Moderate	Moderate	No	Limited distance	Moderate	5.39	0.59
15	17:6	Diplegic	Moderate	Moderate	Yes	Limited distance	Good	3.81	0.54
16	6:8	Quadriplegic	Mod–severe	Poor	No	Non-ambulant	Moderate	8.19	0.56
17	7:0	Quadriplegic	Mod–severe	Poor	Yes	Therapeutic	Moderate	11.29	0.92
18	7:11	Quadriplegic	Mod–severe	Poor	Yes	Therapeutic	Moderate	5.33	0.64
19	8:1	Quadriplegic	Mod–severe	Poor	Yes	Therapeutic	Moderate	7.60	0.81
20	8:3	Quadriplegic	Mod–severe	Moderate	Yes	Limited distance	Moderate	8.38	0.16
21	9:5	Quadriplegic	Mod–severe	Poor	Yes	Therapeutic	Poor	7.58	0.92
22	11:2	Quadriplegic	Mod–severe	Poor	Yes	Non-ambulant	Poor	5.20	0.71
23	11:9	Quadriplegic	Mod–severe	Poor	Yes	Non-ambulant	Poor	9.62	0.85
24	13:8	Quadriplegic	Mod–severe	Poor	Yes	Non-ambulant	Poor	4.88	0.42
25	7:0	Quadriplegic	Severe	Poor	No	Non-ambulant	Poor	8.94	0.62
26	8:8	Diplegic	Severe	Moderate	Yes	Therapeutic	Poor	13.85	1.06
27	9:6	Quadriplegic	Severe	Poor	Yes	Non-ambulant	Poor	10.44	1.20

Hypro, hydroxyproline; SEM, standard error of mean hydroxyproline concentration.

antibody, FITC-anti goat immunoglobulins (Zymed, San Fransico, CA, USA) diluted 1:100 in TBS, and 10% normal rabbit serum, again for 1 hour and in the dark. After washing once more in TBS, the sections were mounted in Vectashield mountant (Vector Laboratories, Burlingame, CA, USA) and stored in the dark for up to 48 hours before viewing. Samples from control participants were included where the primary antibody was omitted to check for any non-specific binding of the secondary antibody.

Sections, all of which were processed at the same time under identical conditions, were viewed on a Zeiss fluorescence microscope and images collected through the programme Scion Image 9 (version 1.62) using a Cohu 4910 CCD camera (Cohu, San Diego, CA, USA) with a Scion LG3 image grabber card. Six images were integrated on-chip for all of the samples, a level which did not result in the saturation of pixels in the most intensely stained section. No modification of the image was performed so that the relative intensity of the immunofluorescence was preserved.

STATISTICAL ANALYSIS

Statistical analysis was carried out using the S-Plus programme (version 4.5; MathSoft Inc, Seattle, USA 1996). Means were determined using the Welch Modified Two-Sample *t*-test, which takes into account unequal variance. An analysis of variance (ANOVA) model was fitted to hydroxyproline content as a function of the different clinical parameters and their interactions. Mean individual hydroxyproline concentration was used as the response variable and the inverses of the standard errors of the individual measures as weights for the estimation procedure. Data were adjusted for age and sex but as they were found not to be significant effectors, they were excluded from the model. In cases where a significant overall difference was observed at the 99.99% confidence level ($p=0.0001$), we performed pairwise comparisons using Tukey's method (Hsu 1996) to account for multiple test' significance levels.

Results

HYDROXYPROLINE CONCENTRATIONS IN CP VERSUS CONTROL MUSCLE

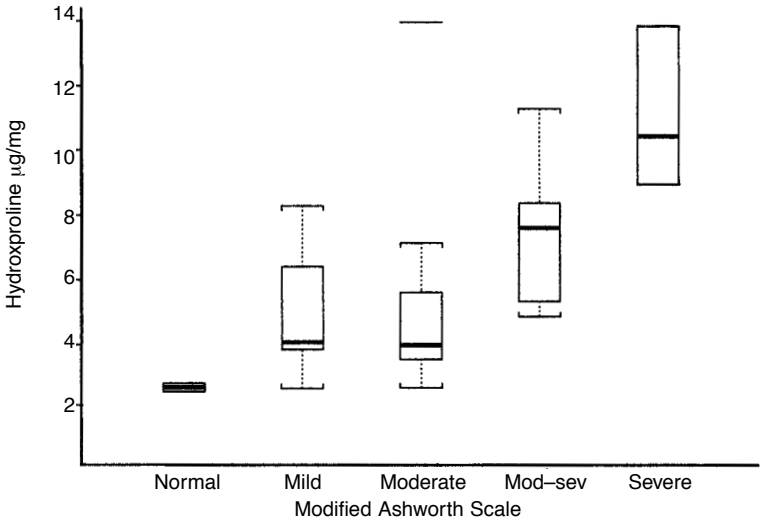
The total muscle hydroxyproline content was found to be significantly higher at the 99% confidence level ($p=0.0004$) in the spastic muscles of children with CP compared to control children without CP with a mean hydroxyproline value of 6.5 (SD 0.63 μg/mg dry weight in spastic muscle compared to 2.8 and 2.6 μg/mg dry weight in control muscles respectively; average 2.7) using the Welch Modified Two-Sample *t*-test.

We then went on to ask which of the clinical parameters correlates with elevated collagen in the spastic muscle of children with CP.

CORRELATION OF HYDROXYPROLINE CONTENT WITH MODIFIED ASHWORTH SCALE

Fitting an ANOVA model, we found a highly significant relation at the 95% confidence level between MAS and hydroxyproline content ($p=0.0025$). Using Tukey's method we found that there was a significant difference at the 95% confidence level between children in the mild group compared with the severe group, between those in the moderate group and the moderately severe group and also between those in the moderate group compared with the severe group on the MAS. (see Table I for classes of severity). When the two control individuals were included, significant effects were also seen between the control children and children with moderately severe and severe scores.

A box and whisker plot of hydroxyproline concentration against the MAS shows a trend in the location of collagen in the different categories increasing in parallel to severity (Fig. 1). Taken together with the statistical analyses, these results



Median	2.68	4.07	3.99	7.60	10.44
SD	0.19	2.27	3.49	2.15	2.51
Range	2.55–2.81	2.65–8.29	2.67–13.97	4.88–11.29	8.94–13.85
Nr	2	5	9	9	3

Figure 1: *Collagen content (μg hydroxyproline/mg dry weight) for all children at each level of Modified Ashworth Scale showing median (box=25th and 75th centiles).*

suggest that children with CP fell into two broad categories: mild and moderate, and moderately severe and severe. Grouping the data into these two categories and refitting the ANOVA model, we were able to show that the collagen content in the spastic muscles in these two groups were highly significantly different to each other at the 99% confidence level ($p=0.0005$).

CORRELATION OF HYDROXYPROLINE CONTENT WITH BALANCE

An ANOVA model showed a highly significant relation between hydroxyproline concentration and balance ($p=0.0032$). Using Tukey's method we found that there was a significant difference at the 95% confidence level between children with good and moderate balance and also between those with good and poor balance. A significant effect was also seen between the control children and children with moderate balance and also between the control children and those with poor balance.

A box and whisker plot of the results (Fig. 2) illustrates the suggestion that the data can once more be separated into two broad categories: those children with CP who have excellent and good balance and those with moderate and poor balance. Pooling the data and fitting an ANOVA model, the collagen content of the two groups was shown to be highly significantly different at the 99% confidence level ($p=0.0008$).

CORRELATION OF COLLAGEN CONTENT WITH OTHER CLINICAL MEASURES

Fitting an ANOVA model to hydroxyproline content as a function of neurological classification (spastic diplegia versus spastic quadriplegia), the presence or absence of clonus, selective control, or ambulatory status showed no significant effects. (Fig 3). However, in all cases the box and whisker plots suggest a trend towards increasing collagen content with increased severity of involvement and it is possible that some of these clinical measures could become significant if more data were available.

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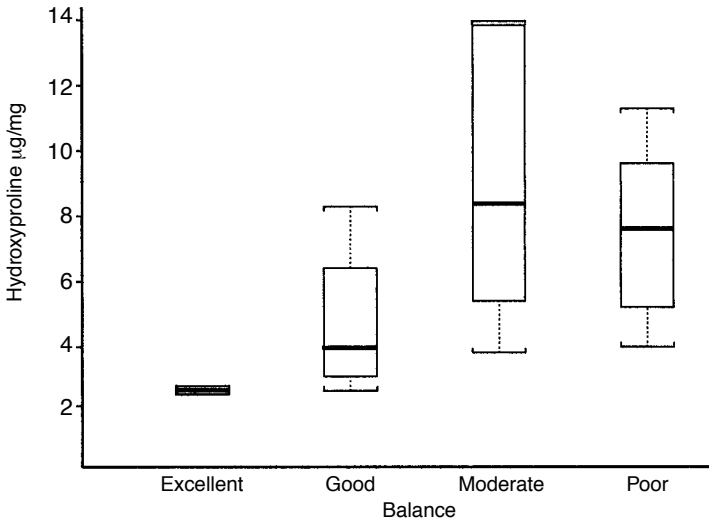
Skeletal muscle morphology was found altered in the spastic muscles of children with CP and our findings suggest that these changes are most pronounced in the more severely affected group of patients. These changes range from rounding of muscle fibres and increased interfibre space to hypertrophic and atrophic fibres associated with the presence of excessive connective tissue, which parallel those seen with the biochemical assays for total collagen.

In spastic muscles classified as mild on the MAS (Fig. 4a), myofibres were found to be tessellated similar to normal muscle. However, with progressing severity, the fibres became rounded and the interfibre space increased (Fig. 4b, c, and d: moderate, moderately severe, and severe respectively).

Individuals in the moderately severe and severe groups (see Fig. 4c and d) showed far more heterogeneity in muscle fibre size and distribution with abnormal collagen I-containing connective tissue-rich areas (black and white asterisk) often containing sparse, hypertrophic fibres (white asterisk) and there also appears to be some evidence of muscle fibre atrophy in these regions as well. It is speculated that these very large fibres are not a result of growth hypertrophy but are undergoing apoptosis or other degenerative changes. The more abnormal, fibrotic morphology was generally associated with children with poor balance (see Fig. 4c) compared to those with moderate balance (see Fig. 4d) and these fibrotic regions were interspersed within less affected muscle giving a very heterogeneous picture.

There was also an increase in collagen I in the endomysium of spastic muscle around the myofibres in association with the basal lamina (arrows), which also appears to became thicker with severity. As the images were prepared and collected

Figure 2: *Collagen content (μg hydroxyproline/mg dry weight) for all children at each level of Balance showing median (box=25th and 75th centiles).*



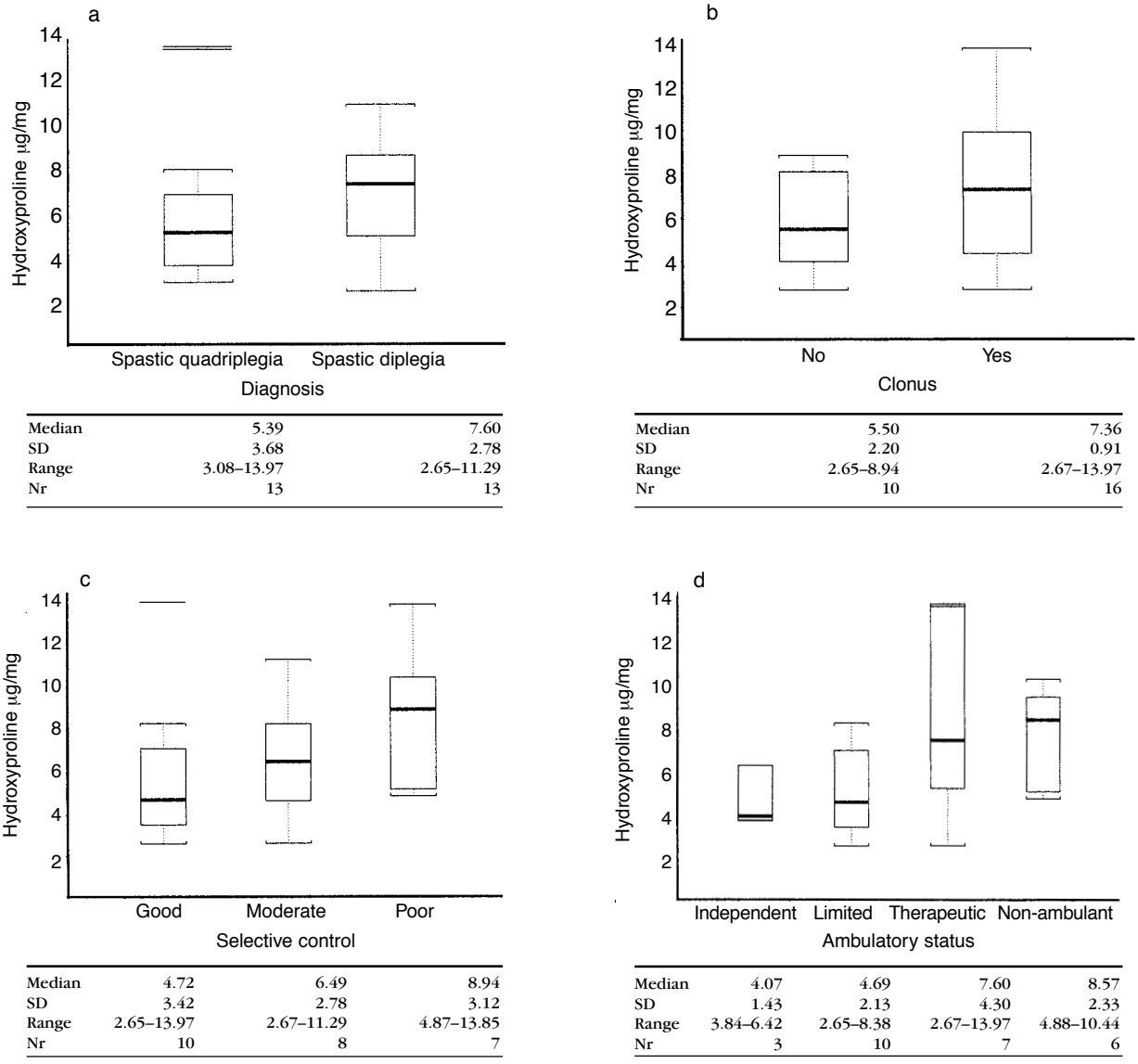
Median	2.68	3.84	8.38	7.60
SD	0.19	2.11	4.70	2.43
Range	2.55–2.81	2.65–8.29	3.81–13.97	3.99–11.29
Nr	2	9	5	11

under identical conditions, the intensity of the immunofluorescence is directly comparable, and was found to be most intense in the more severely affected individuals (see Fig. 4d).

Discussion

In this study we have demonstrated a significant relation between the accumulation of hydroxyproline in spastic muscles of children with CP and the severity of their condition as judged by the MAS, which is an estimate of muscle tone, and by balance. The other clinical parameters show a positive trend, which might become significant if the number of individuals in the study were to be increased. This suggests that the collagen content of spastic muscle integrates a range of different clinical measures. As hydroxyproline is a modified amino acid exclusive to collagen where its proportion to other amino acids is constant, an estimate of its concentration is directly related to the collagen content of the tissue. This relative increase in collagen cannot be accounted for as a simple consequence of loss of muscle content through muscle fibre atrophy as the spaces between the muscle fibres and the intensity of the basal lamina immunofluorescence both appear to increase with severity, thus affecting the total muscle volume to fibre content ratio. Furthermore, muscle atrophy was not seen in all cases: the muscles of some patients had clearly undergone hypertrophy.

In CP, neuronal stimulation of spastic muscles is increased. This suggests that the increase in collagen content we observed could arise because collagen genes are under direct neuronal



	Spastic quadriplegia	Spastic diplegia
Median	5.39	7.60
SD	3.68	2.78
Range	3.08–13.97	2.65–11.29
Nr	13	13

	No	Yes
Median	5.50	7.36
SD	2.20	0.91
Range	2.65–8.94	2.67–13.97
Nr	10	16

	Good	Moderate	Poor
Median	4.72	6.49	8.94
SD	3.42	2.78	3.12
Range	2.65–13.97	2.67–11.29	4.87–13.85
Nr	10	8	7

	Independent	Limited	Therapeutic	Non-ambulant
Median	4.07	4.69	7.60	8.57
SD	1.43	2.13	4.30	2.33
Range	3.84–6.42	2.65–8.38	2.67–13.97	4.88–10.44
Nr	3	10	7	6

Figure 3: *Collagen content (µg hydroxyproline/mg dry weight) for all children at each level of (a) diagnosis, (b) clonus, (c) selective control, and (d) ambulatory status represented as box and whisker plots, showing median (box=25th and 75th centiles).*

control. Paradoxically, there is evidence to suggest that not only chronic overstimulation of muscle (Henriksson et al. 1986, Bailey et al. 1996) but also denervation-induced loss of neuronal activity (Salonen et al. 1985, Savolainen et al. 1988) result in an increase in skeletal muscle collagen content. Virtanen and colleagues (1992) have shown that denervation-induced increases in collagen synthesis return to normal levels after reinnervation. Furthermore, Williams and coworkers (1988) have shown that the accumulation of collagen in muscles immobilized in the shortened position is prevented by direct electrical stimulation, and Koskinen and colleagues (2000) suggest that type IV collagen turnover is significantly increased following functional electrical stimulation of the paralysed muscles of patients with spinal cord injury. This suggests that neuronal activity may regulate collagen synthesis and could be acting alone or in combination with other factors to bring about the changes in connective tissue observed in spastic CP.

Alternatively, collagen synthesis in spastic CP muscle may be stimulated directly through exposure of spastic muscle to abnormal, non-physiological mechanical loads, possibly induced via the abnormal neuronal firing pattern, cocontraction of agonist–antagonist muscle groups and/or by reduced passive stretching through the development of contractures in the more severe cases. experiments have shown that passive stretching induces skeletal muscle hypertrophy (Laurent et al. 1978, Holly et al. 1980, Ashmore and Summers 1981, Goldspink et al. 1995, Kelley 1996) and it has been demonstrated in both chicks (Laurent et al. 1978) and rodents (Williams and Goldspink 1981) that this growth is associated with alterations in both collagen synthesis and degradation rates to accommodate the increases in fibre size observed. However, these experiments model normal growth rather than a pathological process. Effects of spasticity on skeletal muscle collagen accumulation found here appear to be more in keeping with those observed following immobilization in a shortened position (Williams and Goldspink 1984), tenotomy (Jozsa et al. 1990), and denervation (Salonen et al. 1985, Savolainen et al. 1988, Virtanen et al. 1992): all of which result in muscle wasting or atrophy. As already discussed, the picture appears to be more complex in CP with spastic muscles of some children showing obvious hypertrophy while others show muscle atrophy.

However, the amount of collagen within a tissue at any particular time is not necessarily directly related to its synthesis rate as its breakdown is tightly controlled by a number of mechanisms including regulation of activity of matrix-degrading enzymes such as the matrix metalloproteinases. Muscle collagen can also undergo posttranslational modification, protecting itself from degradation by the formation of stable hydroxylysylpyridinoline cross-links (Eyre 1995). These increase with age and have also been shown to increase in the collagen, which accumulates in denervated muscle in rodents (Miller et al. 1999). It would be interesting to determine if such cross-links accumulate in spasticity as it may go some way to explain the increases in stiffness seen in spastic muscle.

We have shown that collagen is increased in the spastic muscles of children with CP and that the amount of total collagen correlates with the severity of their disorder. The distribution of this increased collagen is consistent with it playing a role in increased muscle stiffness. We speculate that it may play a role in the formation of contractures and, together with the

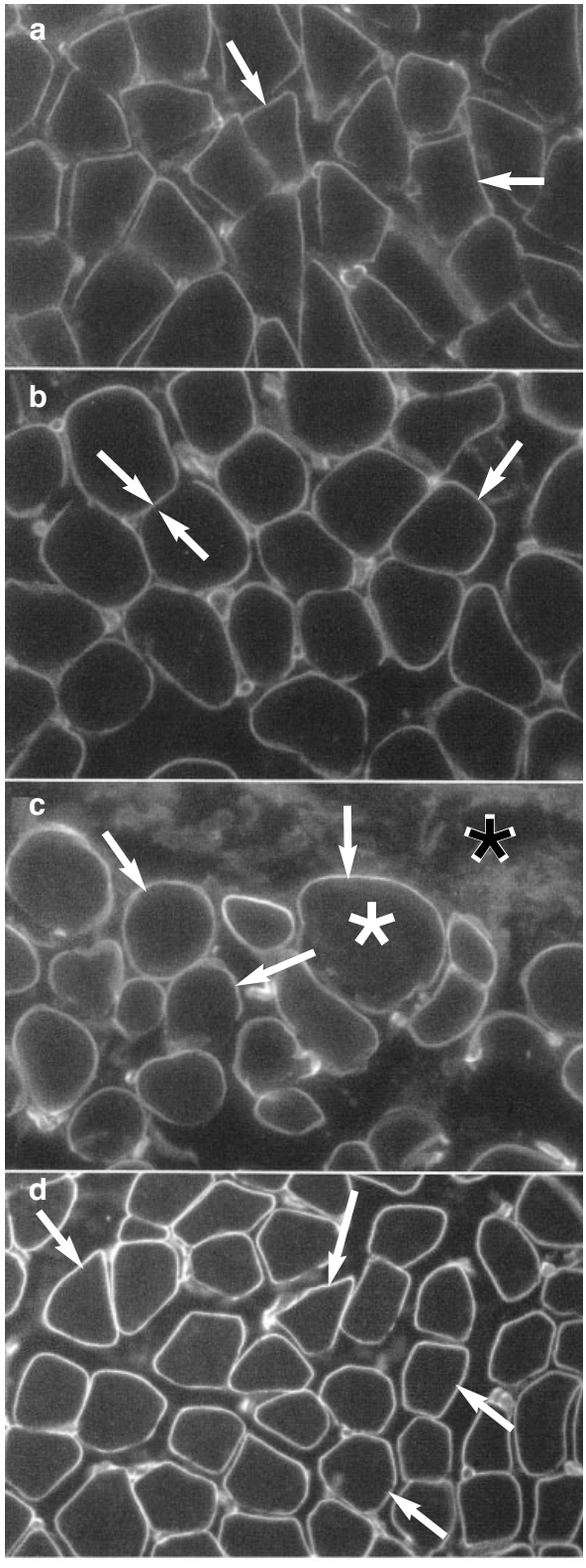


Figure 4: *Collagen I immunohistochemistry in spastic muscle of children with CP at different severities on Modified Ashworth Scale (MAS) and Balance (B). (a) MAS mild, B good; (b) MAS moderate, B good; (c) MAS moderate–severe, B poor; (d) MAS severe, B moderate.*

relative lack of growth in length of spastic muscle, may ultimately play a causative role in the formation of bony abnormalities. Once severe fibrotic changes have occurred in CP, muscle function will be impaired and cannot be reversed. We therefore suggest that it would be beneficial if treatment for children with CP concentrated on preventing these fibrotic alterations.

Accepted for publication 22nd November 2000.

Acknowledgments
We would like to thank Dr Vic Duance and Dr John Wardale for their help and support in providing the anticollagen antibodies and advice on the collagen assays and also the Wishbone Trust who have provided financial support for this project.

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