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# The morphological basis of increased stiffness of rabbit tibialis anterior muscles during surgical limb-lengthening

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ABSTRACT

When the tibialis anterior muscle of the rabbit is progressively stretched during surgical limb distraction, the muscle fibres lengthen by addition of new serial sarcomeres, provided that stretch is carried out at an appropriate rate. However, in spite of the apparent adaptation to the new functional length, range of joint movement is greatly decreased. In this study we have first, made measurements of the passive tension developed by distracted muscles over the range of joint movement and secondly made quantitative measurements of endomysial and perimysial connective tissue content. It was found that at all ankle joint angles greater than 90°, the passive tension developed by the distracted muscles was greater than both contralateral and sham-operated controls. Image analysis showed that the ratio of collagen to contractile material was increased in distracted muscles compared with muscles from sham-operated controls, due to increased deposition of collagen type III. Scanning electron microscopy showed the presence of a dense perimysial weave surrounding the distracted muscle fibres. These quantitative and qualitative changes in the connective tissue component could account for the increased stiffness demonstrated by the physiological measurements. It would seem that in distracted muscle the connective tissue element adapts less readily than the contractile component, with prolonged stretch leading to damage to the perimysial and endomysial network, with subsequent fibrosis and loss of muscle compliance. Such changes could help explain the loss of range of movement noted in the distracted limbs of patients undergoing surgical limb-lengthening and in other conditions that result in muscle contractures.

*Key words*: Connective tissue; collagen; fibrosis; muscle contracture.

## INTRODUCTION

Surgical lengthening of limbs offers the possibility of studying the adaptation of muscle to an increase in functional length at a more rapid rate than that resulting from the extension of the tissue by the elongation of the skeleton during normal growth. The Ilizarov method (Ilizarov, 1989) has become an important surgical means of correcting discrepancies in limb length. However, the procedure often results in soft tissue complications such as muscle contracture (Paley, 1990; Fitch et al. 1993). Thus clearly there is a need to optimise this method of distraction.

Previous work has shown that distraction of the tibialis anterior muscle of the rabbit, during surgical limb lengthening, leads to the formation of new contractile material by the addition of serial sarcomeres (Williams et al. 1994). If distraction is carried out at an appropriate rate, sufficient sarcomeres are added to enable the muscle to function over the new length, i.e. the active length/tension curve undergoes a shift which is equal to the amount of distraction (Williams et al. 1993; Simpson et al. 1995). However, even at low rates of distraction, it was found that range of joint movement was reduced (Williams et al. 1994). Section of the anterior and posterior tendons at the level of the ankle led to restoration of full ankle movement, demonstrating that loss of movement is due to factors within the muscle-tendon complex. One of the aims of this study was to measure the passive

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tension in the distracted muscles over the range of joint movement and to compare the results with those from animals which had the fixation system applied but no lengthening effected. In this way we could establish that the increase in passive tension occurring in distracted muscle is due to the lengthening process rather than the presence of the distracting device.

Muscle elasticity is provided, at least in part, by the intramuscular connective tissue framework (Cavana, 1997). It seems possible, therefore, that remodelling of the connective tissue may be a rate-limiting factor in the adaptation of the muscles to a new functional length. As collagen is the major connective tissue protein in muscle, the tensile properties of passive muscles are primarily dependant on the amount and structure of intramuscular collagen (Kovanan et al. 1984). For this reason it was decided to investigate the endomysial and perimysial collagen content of the distracted muscles, using image analysis of sections stained for collagen with Picrosirius Red. Collagen volume fraction determined in this way has been shown to be closely related to the concentration of the collagen-specific amino acid, hydroxyproline (Pickering & Broughner, 1990). The main intramuscular collagen is collagen type III and image analysis was therefore also carried out on sections which had immunostained using monoclonal antibody to this collagen type. The second aim of this study was therefore to determine whether changes in passive length/tension parameters are correlated with increases in collagen content in the distracted muscles.

## METHODS

### *Animal model*

Adult New Zealand white rabbits were anaesthetised by intravenous injection of Hypnorm (0.4 mg $kg^{-1}$) and midazolam (0.2 mg $kg^{-1}$). The muscles in the lower leg of 5 animals were subjected to distraction by applying an external fixation device (Orthofix M100) to the medial surface of the tibia. A middiaphyseal osteotomy was created, the periosteum repaired and the skin closed (Simpson et al. 1995). The animals were able to bear weight freely on the operated leg within 2 h of operation. Lengthening was carried out at 1.6 mm $d^{-1}$, daily increases being divided into 2 increments. Lengthening was continued until a 20% increase of the initial length of the tibia had been achieved. Contralateral muscles acted as controls; however, in order to distinguish between changes produced by the process of distraction and those resulting from the presence of the distractor itself, a sham operation was carried out in a group of 3 rabbits. In these animals, the external fixation system was applied, the osteotomy created but no lengthening effected.

Animals were again anaesthetised by intravenous injection of Hypnorm (0.4 mg $kg^{-1}$ and midazolam (0.2 mg $kg^{-1}$) and measurements of function were made on the tibialis anterior. Results were compared using Student's *t* test or the Mann Whitney U test.

### *Measurement of range of joint motion and muscle length*

The range of ankle joint movement (ROM) was measured using a goniometer. The tibialis anterior muscles were exposed and ink marks made on the distal end of the patellar tendon attachment and on the furthest point of insertion of muscle fibres into the distal tendon. Using these fixed anatomical landmarks, muscle length was measured with the ankle at the extremes of the movement range as well as with the ankle at 90°.

### *Measurement of passive length/tension curves*

The tendon of tibialis anterior was cut distally and the muscle belly dissected free for most of its length. The tendon was then attached with a suture to a lever arm of a recording device that measured the passive force generated by the muscle (Cambridge Tensiometer). The muscle was kept moist with a saline drip and maintained at 35 °C, using a thermistor-controlled lamp. Using a vernier calliper and screw carriage mechanism, the belly of tibialis anterior was set to various lengths between the extremes of its range (as measured before cutting the tendon). The passive force developed at each muscle length was measured and the length/tension curve plotted.

### *Serial sarcomere number*

Following the physiological analysis, the animals were killed with an overdose of anaesthetic and the tibialis anterior muscles removed. A strip of muscle fibres inserting furthest onto the distal tendon was dissected free, pinned to cork then fixed overnight in 4% formalin made up in 0.9% NaCl before being digested in 30% nitric acid for 48 h and stored in 50% phosphate-buffered glycerol (pH 7.0). Small bundles of 3–5 fibres were dissected out under a dissecting microscope. The distances between the proximal and distal myotendinous junctions were measured. Single fibres were then mounted in glycerol jelly and viewed under a microscope. At approximately 45 points along each fibre, the length covered by 10 sarcomeres

Table 1. *Changes in range of joint movement, sarcomere number and passive tension in distracted and sham-operated muscles*

| | Sham-operated Mean ± S.E. | Distracted Mean ± S.E. |
|---|---|---|
| Loss of joint movement (%) | 2 ± 1 | 64 ± 11* |
| Increase in muscle length (mm) | −1 ± 1 | 13 ± 2* |
| Change in number of serial sarcomeres | −200 ± 600 | +3400 ± 350* |
| Passive tension at the 90° ankle position (N) | 0 | 8 ± 4 |

* Significantly different from sham-operated muscles ($P < 0.01$).

was measured using an image analyser (Seescan Solitaire). The total number of sarcomeres in series along the length of each fibre was then calculated from the mean sarcomere length and muscle bundle length. Previous work (Williams & Goldspink, 1985), using a control group of 3 rabbits, has shown that when sarcomere number measurements are made as described, the between-animal variation is low (coefficient of variation = 4.9%).

### *Connective tissue analysis*

*Collagen content*. Muscle samples, taken from the mid belly region of each muscle, were frozen in isopentane precooled in liquid nitrogen and sectioned at 10 μm using a cryostat. Total amount and distribution of collagen was determined by image analysis on sections stained with Picrosirius Red (Sweat et al. 1964). Recent work has shown that modification of the Picrosirius Red stain by the addition of Fast Blue prevents any uptake of the Picrosirius Red by the cytoplasm, thus facilitating image analysis by providing good contrast between the connective tissue and intracellular elements (unpublished observations). Slides were fixed in a solution of 10% formalin in 0.9% NaCl for 15–20 min before being rinsed with distilled water. They were then placed in Fast Blue RR solution (0.1% Fast Blue RR, 7 mM magnesium sulphate and 60 mM magnesium borate) at room temperature for 30 min. Slides were then washed thoroughly in distilled water and placed in a solution of Picrosirius Red (0.1% in saturated aqueous picric acid) for 10 min, before being dehydrated, cleared and mounted. Sections were viewed using light microscopy (Leitz). The microscope image was detected by a video camera and transduced to a Seescan Solitaire image analysis system by which the image was digitised. An image outside the tissue was recorded as a reference for shade correction of each section. The threshold was adjusted so as to give the best correspondence between the video and the thresholded image. The sections were scanned using a sampling area of 0.2 $mm^2$ (excluding dense bands of perimysium) and the area occupied by connective tissue, i.e. the number of highlighted pixels, determined. The percentage area within each section that was occupied by connective tissue was then calculated. Results were compared using the Mann Whitney U test.

*Analysis of collagen type III*. Using frozen sections, endogenous peroxidase was quenched with 1.2% hydrogen peroxide at room temperature for 30 min. Following blocking with normal rabbit serum, sections were incubated with primary antibody. The sections were then incubated with biotinylated rabbit antimouse immunoglobulin antibody (1:300)(DAKO) at room temperature for 30 min, followed by streptavidin complexed with biotinylated peroxidases (DAKO) at room temperature for 30 min. The peroxidase complexes were visualised using DAB; washing and dilution buffers were either TBS or Tris. This method of visualisation has been shown to give stronger positive staining without increased background when compared with the indirect peroxidase method, thus providing greater contrast for image analysis (Lowry et al. 1997). Sections were scanned using the Seescan image analyser and the relative area occupied by collagen III determined.

*Electron microscopy*. Small bundles of fibres from the midregion of each muscle were tied to cocktail sticks then fixed in 5% phosphate-buffered glutaraldehyde, postfixed in phosphate-buffered osmium tetroxide, dehydrated in a series of acetones and subjected to critical point drying using carbon dioxide. Samples were mounted on stubs, coated with gold and examined in the scanning electron microscope.

## RESULTS

### *Range of movement and muscle length* (Table 1)

All the distracted limbs showed a considerable loss of ankle joint movement (mean loss 64% ). In the sham operated animals the mean loss of joint movement was only 2%. Measurement of muscle length at the 90° ankle position showed that all distracted muscles

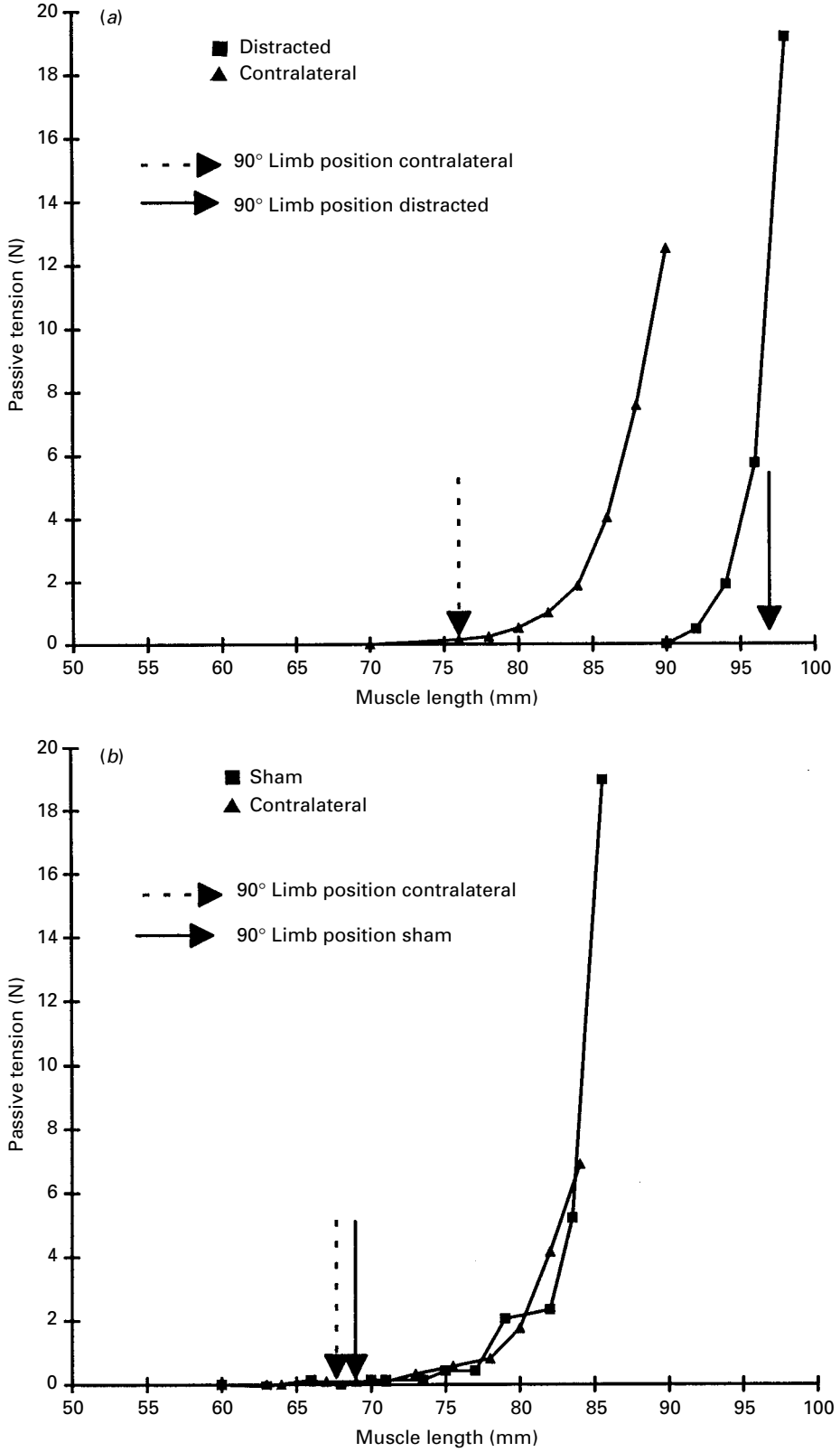


Fig. 1. Passive length/tension curves of distracted (*a*) and sham-operated (*b*) tibialis anterior muscles and their contralateral controls. Due to the addition of new contractile material, the curve for the distracted muscle is to the right of that of the contralateral muscle. The 90° limb position is marked with a vertical line. It can be seen that at the longer muscle lengths, the passive force exerted by the distracted muscle is greater than that exerted by the sham-operated or contralateral control muscles.

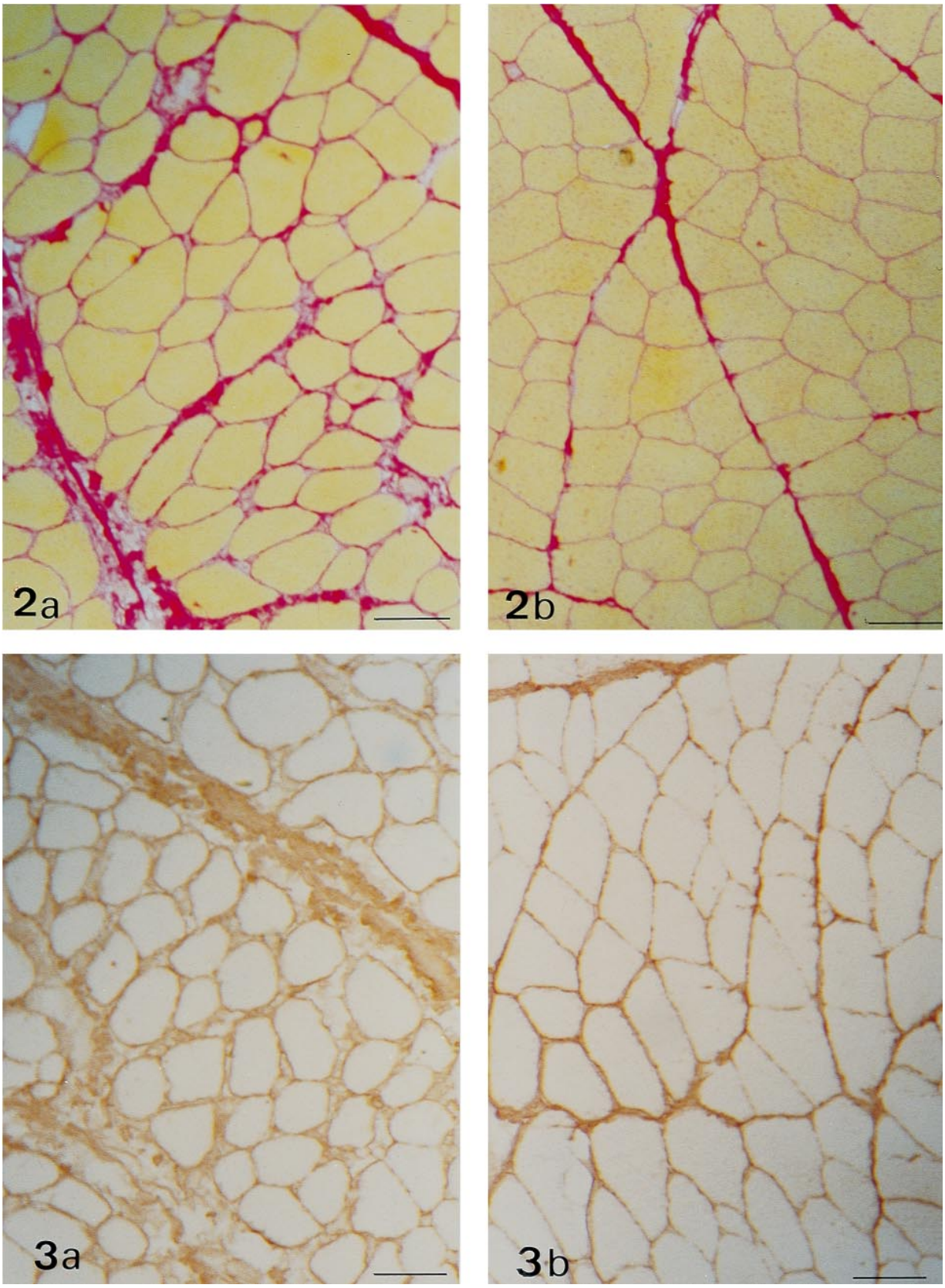


Fig. 2. Connective tissue in distracted (*a*) and contralateral (*b*) muscle. Transverse frozen sections stained with Picrosirius Red. Bar, 100 μm.

Fig. 3. Sections stained with antibody to collagen type III in distracted (*a*) and contralateral (*b*) muscles. Bar, 20 μm.

Table 2. *Semiquantitative measurements of connective tissue content of distracted, contralateral control and sham-operated muscles*

| | Sham-operated Mean ± S.E. | | Distracted Mean ± S.E. | |
|---|---|---|---|---|
| | Experimental | Contralateral | Experimental | Contralateral |
| Area stained by Picrosirius Red (%) | 12.1 ± 2.5 | 10.5 ± 2.6 | 19.3 ± 1.5* | 12.2 ± 0.8 |
| Area stained by antibody to by collagen III (%) | 11.8 ± 2.0 | 11.3 ± 1.5 | 16.5 ± 1.3* | 10.2 ± 0.4 |

Endomysial and perimysial collagen content was measured as a percentage of muscle unit cross-sectional area; n = 5 for distracted and contralateral muscles; n = 3 for sham-operated and contralateral muscles.
* Significantly greater than contralateral and sham-operated control values ($P < 0.01$).

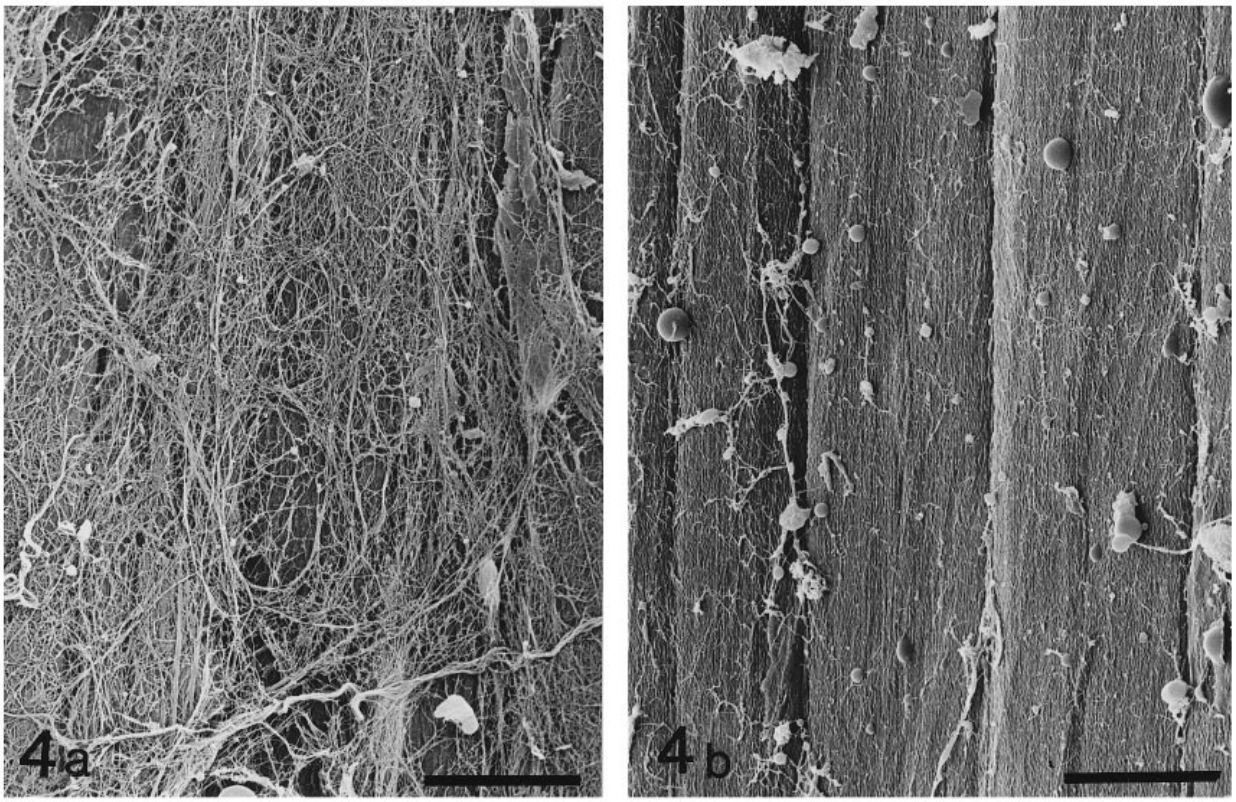


Fig. 4. Scanning electron micrographs showing thick collagen fibrils surrounding muscle fibres taken from distracted muscle (*a*) contrasting with the fine network of fibrils surrounding control muscle fibres (*b*). Bar, 50 μm.

were longer (mean increase 13 mm) than the contralateral controls.

### *Length/tension properties*

Distraction resulted in the serial addition of approximately 3000 sarcomeres. There was no significant difference between the sarcomere length (measured at the 90° limb position) between the sham-operated (2.4 ± 0.03 μm) and the distracted (2.5 ± 0.04 μm) muscles (Fig. 1). Thus new contractile material had been added which represented an adjustment to the longer functional length. However, the passive tension exerted by the distracted muscles was found to be increased compared with both the contralateral and sham-operated control muscles. In the 90° limb position contralateral and sham-operated control muscles exerted no passive tension whereas the distracted muscles exerted a mean passive tension of 8 Newtons.

*Connective tissue content*

Image analysis of collagen content using sections stained with Picrosirius Red (Fig. 2) showed that there is a significant increase in the collagen volume fraction in the perimysium and endomysium of the distracted muscles compared with both the contralateral controls and the sham-operated animals (Table 2). Immunocytochemistry showed that there was a highly significant increase of collagen III in the distracted muscles (Fig. 3).

The scanning electron micrographs showed a proliferation of connective tissue in the distracted muscle. It can be seen that individual fibres are surrounded by a dense weave of both endomysial and perimysial collagen fibres (Fig. 4).

## DISCUSSION

Surgical lengthening of the limbs in patients is often accompanied by muscle contractures (Paley et al. 1991) and loss of range of ankle movement (Sofield et al. 1958). Animal studies have shown, either directly (Williams et al. 1994) or indirectly (Matano et al. 1994), that distraction results in increased muscle length due to an increase in the number of serial sarcomeres. The sarcomere addition appears to be associated with the incorporation of satellite cells (Day et al. 1997). Animal studies have also shown that above a certain rate of distraction, loss of movement results, at least in part, from the failure of the muscle fibres to add on sufficient new contractile material (serial sarcomeres) to adapt to the imposed increase in length. This results in over-stretching of the sarcomeres, muscle weakness and loss of range of movement (Williams et al. 1994). In the present study, the muscles were distracted at a rate which resulted in good adaptation (increase in serial sarcomeres). However, the parallel elastic component adapted to prolonged stretch less well than the contractile component: the muscles were less compliant than the contralateral muscles. Muscles from the sham-operated animals did not show comparable changes, demonstrating that loss of compliance is associated mainly with limb lengthening rather than the operative procedure or the presence of the distracting device.

The marked increase in stiffness in the anterior muscles would mean that the posterior muscles would need to exert more force to produce the same ankle movement. In addition, distracted muscles have been shown to undergo muscle atrophy (Lee et al. 1993; Williams et al. 1994) and loss of maximum force (Williams et al. 1995). This would be expected to reduce further the ability of the posterior muscles to effect extension of the anterior muscles.

The connective tissue framework of muscle consists of fibrillar collagen which provides both myofibre to myofibre connections (Bourg & Caulfield, 1980) and a 3-dimensional support structure with important elastic properties (Trotter & Purslow, 1992). Thus even small changes in the concentration of this element would be expected to have a substantial effect on muscle function. The results presented here show that in the distracted muscles there was a significant increase in the percentage of interstitial collagen. A study of passive properties of the rat medial gastrocnemius as a function of muscle dimensions showed a correlation between the relative amount of connective tissue and the passive tension ($g/mm^2$) which is independent of muscle dimensions (Heerkins et al. 1987). This means that when the percentage connective tissue content of a muscle is increased, the passive tension will also be increased, given equal material properties. Thus the increase in percentage of collagen found in the distracted muscles appears to explain the increased stiffness shown by the physiological measurements. This increase in collagen may also be accompanied by qualitative changes such as alterations in thickness or configuration of the endomysial and perimysial collagen fibres.

This work indicates that during prolonged stretch the connective tissue elements remodel less readily than the contractile component. This may lead to damage to the perimysial and endomysial network with subsequent fibrosis. Alternatively, fibrosis may be subsequent to muscle fibre damage (Foidart et al. 1981). Distraction has previously been shown to result in some muscle fibre damage (Williams et al. 1995) and during the connective tissue analysis, in addition to the generally high levels of collagen, pockets of dense connective tissue were noted where it appeared that myofibres had been replaced by fibrotic tissue (Fig. 3). Similar observations were made by Lee et al. (1993) in a study of changes in the gastrocnemius muscle of the rabbit following distraction of the tibia.

This study demonstrates that the intramuscular connective tissue framework does not readily adapt to passive stretch beyond the normal physiological range. As well as providing fundamental knowledge of muscle adaptation, the data obtained are of considerable practical value, having important implications for the treatment of patients undergoing surgical distraction to correct limb-length discrepancies. It is possible that slower rates of distraction, at a rate which is just above that at which the bone fuses, would enable the connective tissue, as well

the contractile material, to adapt more completely to the increased functional length of the muscle.

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REFERENCES

Borg TK, Caulfield JB (1980) Morphology of connective tissue in skeletal muscle. *Tissue & Cell* **12**, 197–207.

Cavana GA (1977) Storage and utilisation of elastic energy in skeletal muscle. In *Exercise and Sport Sciences* 5 (ed. Hutton RS), pp. 89–129. Santa Barbara: Journal Publishing Affiliates.

Day CS, Morland MS, Floyd SS, Huard J (1997) Limb lengthening promotes muscle growth. *Journal of Orthopaedic Research* **15**, 227–234.

Fitch RD, Thompson JG, Rizk WS, Seaber AV, Garrett WE (1993) The effects of the Ilizarov distraction technique on bone and muscle in a canine model: a preliminary report. *Iowa Orthopaedic Journal* **16**, 10–19.

Foidart M, Foidart J, Engel WK (1981) Collagen localisation in normal and fibrotic human skeletal muscle. *Archives of Neurology* **38**, 152–157.

Heerkens YF, Wottiez RD, Keila J, Huijing PA Huson A, van Ingen Schenau GI et al. (1987) Mechanical properties of passive rat muscle during sinusoidal stretching. *Pflugers Archiv-European Journal of Physiology* **409**, 438–447.

Ilizarov GA (1989) The tension- stress effect on the genesis and growth of tissues. Part I—the influence of stability of fixation and soft-tissue preservation. *Clinical Orthopaedics & Related Research* **238**, 249–280.

Kovanan V, Suominin H, Heikkinen E (1984) Mechanical properties of fast and slow skeletal muscles with special reference to collagen and endurance training. *Pflugers Archiv* **409**, 438–447.

Lee DY, Choi IH, Chung CY, Chung PH, Chi JG, Suh YL (1993) Effect of tibial lengthening on the gastrocnemius muscle. A histopathologicand morphometric study in rabbits. *Acta Orthopaedica Scandinavica* **64**, 688–682.

Lowry A, Wilcox D, Masson E, Williams PE (1997) Immunocytochemical methods for semiquantitative analysis of collagen content in human peripheral nerve. *Journal of Anatomy* **191**, 367–375.

Matano T, Tamai K, Kurokawa T (1994) Adaptation of skeletal muscle in limb lengthening: a light diffraction study on the sarcomere length in situ. *Journal of Orthopaedic Research* **12**, 193–196.

Paley D (1990) Problems, obstacles and complications of limb lengthening by the Ilizarov technique. *Clinical Orthopaedics* **250**, 81–104.

Paley D, Rumleyto, Kovelman H (1991) The Ilizarov technique: a method to regenerate bone and soft tissue. *Advances in Plastic and Reconstructive Surgery* **7**, 1–43.

Pickering JG, Boughner DR (1990) Fibrosis in the transplanted heart and its relation to donor ischemic time: assessment with polarized light microscopy and digital image analysis. *Circulation* **81**, 949–958.

Simpson AHRW, Williams PE, Kyberd P, Goldspink G, Kenwright J (1995) The response of the rabbit anterior tibialis muscle to stretch during surgical limb lengthening. *Journal of Bone & Joint Surgery* **77B**, 630–636.

Sofield HA, Blair SJ, Millar EA (1958) Leg lengthening: a personal follow-up of forty patients some twenty years after the operation. *Journal of Bone and Joint Surgery* **40A**, 311–322.

Sweat F, Pulcher H, Rosenthal SI (1964) Sirius Red F3BA as a stain for connective tissue. *Archives of Pathology* **78**, 69–72.

Trotter JA, Purslow PP (1992) Functional morphology of the endomysium in series-fibred muscles. *Journal of Morphology* **212**, 109–122.

Williams PE, Goldspink G (1985) Effect of stretch combined with electrical stimulation on the type of sarcomeres produced at the ends of the muscle fibres. *Experimental Neurology* **93**, 500–509.

Williams PE, Simpson Ahrw, Kyberd P, Goldspink G, Kenwright J (1993) Changes in length/tension properties and fatigue resistance in distracted muscles. *Journal of Anatomy* **183**, 184.

Williams PE, Simpson AHRW, Kyberd P, Kenwright J, Goldspink G (1994) Structural changes in the rabbit tibialis anterior muscle following distraction of the tibia. *Basic & Applied Myology* **4**, 431–437.

Williams PE, Simpson AHRW, Kenwright J, Goldspink G (1995) The influence of passive stretch on muscle fibre phenotype and contractile characteristics of the rabbit tibialis anterior. *Journal of Physiology* **479P**, 6–7.