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ORIGINAL PAPER

Structural changes in the lengthened rabbit muscle

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Abstract This study evaluated the histological changes in muscle tissue after limb lengthening in skeletally mature and immature rabbits and assessed the most vulnerable level of striated muscle. Twenty-three male domestic white rabbits, divided into six groups, were operated on and different lengthening protocols were used in the mature and immature rabbits. The histopathological changes were analysed by a semi-quantitative method according to the scoring system of Lee et al. (Acta Orthop Scand 64(6): 688–692, 1993). After the evaluation of the five main degenerative parameters (muscle atrophy, muscle nuclei internalisation, degeneration of the muscle fibre, perimysial and endomysial fibrosis, haematomas), it is evident that the adults lengthened at a rate of 1.6 mm/day showed more degenerative changes than those lengthened at 0.8 mm/day. The adult 1.6 mm/day lengthened group presented significantly higher damage in the muscle and lower regenerative signs compared with the young 1.6 mm/day lengthened group, according to the summarised degenerative scores.

Résumé Objectif: Cette étude a pour but d'évaluer les changements histologiques des muscles après allongement squelettique chez des lapines matures ou immatures. Elle permet d'évaluer quel est le niveau de lésion des muscles striés. Matériel et méthode: 23 lapins mâles domestiques blancs ont été divisés en six groupes et traités avec différents protocoles d'allongements.. Les changements histologiques ont été analysés avec une méthode semi quantitative utilisant le score de LEE et collaborateurs. Résultats et conclusion: l'évaluation des cinq principaux paramètres de dégénérescence (atrophie musculaire, noyau musculaire, internalisation, dégénérescence des fibres musculaires, fibrose du périmysium et de l'endomysium, hématome). Il apparaît évident que l'allongement chez l'adulte à un rythme de 1,6 mm par jour entraîne plus de lésions dégénératives que les sujets allongés au rythme de 0,8 mm par jour. Le groupe de lapins adultes avec allongement de 1,6 mm par jour présente des lésions hautement significatives du muscle, avec des signes de régénération musculaire moins importants que ceux du groupe de lapins plus immatures allongés au même rythme, ceci en utilisant des scores identiques.

Keywords Callus distraction · Muscle degeneration · Muscle regeneration · Rabbit

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Introduction

Research on distraction osteogenesis and improvements in lengthening devices have led to the increased popularity of limb lengthening [16]. Many previous experimental and clinical studies have reported the astonishing regenerative potential of lengthened bones [10, 15], but the regenerative capacity of the surrounding soft tissues appears to be much more limited [12, 24]. Previously, it was thought that the elongation of muscular tissue was caused by an increase in the length of sarcomeres [14, 21]. It is now supposed that the

lengthening of striated muscles is not only passive stretching, but that the muscle gave an active adaptive response to the lengthening, known as distraction histogenesis [4, 11, 18]. This process contains degenerative and regenerative phases. The fibre necrosis seems to serve as a stimulus for regenerative activity. It may be assumed that the presence of regenerating fibres in the samples, even in the absence of necrotic fibres, is a likely sign of previous necrosis in adjacent muscle that was not sampled. Even before the complete removal of the necrotic sarcoplasmic debris by phagocyte cells, the process of regeneration may have begun, so that myogenesis and phagocytosis can be visualised in the same muscle fibre concurrently [13].

The necrosis may be segmental, disrupting only a portion of the sarcoplasma or along the entire length of the fibre. It is not clear whether damage to a discrete area, say, in the middle, leads to the degeneration of the whole fibre [8].

This study evaluated the histological changes in the muscle tissue after limb lengthening in skeletally mature and immature rabbits and assessed the most vulnerable level of the striated muscle.

Materials and methods

Twenty-three male domestic white rabbits, divided into six groups, were operated on and different lengthening protocols were used (Orthofix MiniRail standard lengthener, M-101, Italy). The surgery was followed by 7 days compression in every lengthened group. In group 1 (four mature rabbits), 0.8-mm distraction once a day was applied until 20% lengthening was achieved. In group 2 (five mature rabbits), the lengthening rate was 1.6 mm (0.8 mm twice per day) until 20% elongation of the leg was achieved. In group 3 (five immature rabbits), 0.8-mm distraction once a day was applied until 20% lengthening was achieved. In group 4 (four mature rabbits), the lengthening rate was 1.6 mm (0.8 mm twice per day) and the increase in length was 20%. Group 5 (two mature rabbits) and group 6 (three immature rabbits) contained the sham-operated animals (the fixator was placed and osteotomy was performed but lengthening was not performed). Young animals were 9 weeks old and mature animals were 28 weeks old [19]. All of the animals were sacrificed immediately after the completion of the lengthening procedure. All animal procedures conformed to national regulations and approval by the Ethical Committee was obtained.

The rabbits were anaesthetised with a mixture of ketamine (25 mg/kg) and either xylazine (5 mg/kg) or medetomidine (0.5 mg/kg), which was given by intramuscular injection.

A venous catheter was inserted, and the same drugs were used for the maintenance of general anaesthesia. Operations were performed according to the description of the surgical method published by Simpson et al. [20]. Great care was taken to avoid damaging the muscles or any other parts of the soft tissues. All rabbits received one IV bolus of cephalosporins (20 mg/kg). The postoperative radiographs were made in two projections, dorsoplantar and lateromedial (50 kV, 8.0 mAs).

After 7 days of compression, the apparatus was distracted by 1 mm once a day. The goal was a 20% lengthening of the tibia. The length of the required distraction was calculated on plain radiographs. The animals were randomly allocated to the groups.

The flexor digitorum longus and peroneus quartus muscles from all of the control and experimental rabbit legs (and from the two sham groups) were fixed in 10% buffered formalin solution for 48 h. Transverse sections were cut from the border between the proximal third and the middle third of the muscle belly, and from the border between the middle and distal third of the muscle belly of the flexor digitorum longus and peroneus quartus. Thereafter, a routine paraffin-embedding method was used and series of 5-μm-thick sections were cut from the blocks. The slides were stained with haematoxyline and eosin (H and E) Weigert-van Gieson trichrome using the Masson trichrome method.

The histopathological changes were analysed by a semi-quantitative method according to the scoring system of Lee et al. [9]. This system consists of rating muscle specimens on a scale of 0 to 3, where 0 is normal. Lee et al.'s system was slightly modified and enlarged from five parameters to nine, consisting of: (1) size variation of muscle fibres; (2) internalisation of the nuclei of muscle fibres; (3) degeneration of muscle fibres; (4) regeneration of muscle fibre; (5) endomysial and perimysial fibrosis of muscle; (6) internalisation of the muscle fibre nuclei at the myotendinous junction (MTJ); (7) cell number at the MTJ line; (8) the number of blood vessels at the MTJ; (9) haematomas at the MTJ [9] (Table 1). The slides were evaluated by Zeiss ICM 405 inverted microscope with an MC63 exposure unit and an M35 camera unit (Zeiss, Germany). The histopathological signs were counted in 20 fields to determine the average occurrence.

The statistical tests of the histopathological scores, based on the ordinal scale, among the lengthened groups were done by the Kruskal-Wallis test, followed by the Wilcoxon rank-sum test or individual comparisons among the lengthened groups and within each lengthening group between the lengthened side and the control side.

Results

The adult 1.6 mm/day lengthening rate group (G2) presented significantly higher mean muscle fibre-size

Table 1 The rating of muscle specimens according to the system of Lee et al. [9]

Features of fibres	Mature		Immature		Mature	Immature
	0.8 mm once/day (G1)	0.8 mm twice/day (G2)	0.8 mm once/day (G3)	0.8 mm twice/day (G4)	Sham (G5)	Sham (G6)
Muscle atrophy	1.5	2.35	1.2	1.375	0	0.0833
Internalisation of muscle nuclei	0.75	1.35	0.8	0.9375	0.125	0.0833
Muscle degeneration	1.1875	1.95	0.85	1.25	0.125	0.0833
Muscle regeneration	0.375	0.95	0.8	1.6875	0	0
Endomysial and perimysial fibrosis of muscle	1.0625	2.85	0.85	1.4375	0	0
Internalisation of muscle fibre nuclei at the MTJ	0.4375	1.45	1.2	2	0	0.0833
Cell number at the MTJ	0.5625	1.95	0.95	1.625	0	0
Number of the blood vessels at the MTJ	0.25	1.1	0	1.375	0.125	0
Haematomas at the MTJ	0.1875	1.85	0	0.125	0	0

Muscle atrophy: 0=normal; 1=atrophied muscle fibres less than 2/3rds the size of the normal contralateral side in less than 20% of the field; 2=atrophied fibres less than 2/3rds the size of the control side in between 20–40% of the field; 3=atrophied fibres less than 2/3rds of the normal size in more than 40% of the field

Internalisation of muscle nuclei: 0=normal; 1=3–5 muscle fibres with central nuclei in 10 fields; 2=muscle fibres with central nuclei from 6 in 10 fields to 5 in one field; 3=more than 5 muscle fibres with central nuclei in one field

Muscle degeneration: 0=normal; 1=1–2 degenerating muscle fibres in 10 fields; 2=degenerating muscle fibres from 3–10 in 10 fields; 3=more than 10 degenerating muscle fibres in 10 fields

Muscle regeneration: 0=normal; 1=1–2 regenerating muscle fibres in 10 fields; 2=number of regenerating muscle fibres from 3 in 10 fields to 10 in 10 fields; 3=more than 10 regenerating muscle fibres in 10 fields

Endomysial and perimysial fibrosis of muscle: 0=normal; 1=mild focal fibrosis; 2=fibrosis between score 1 and 3; 3=severe multifocal perimysial and endomysial fibrosis of the muscle

Internalisation of the muscle fibre nuclei at the myotendinous junction (MTJ): 0=normal, less than six nuclei internalisation in one field (half field if the MTJ line is in the diameter of the field); 1=internalised muscle fibre nuclei between six and 10 in one field; 2=the number of internalised nuclei is more than 10 but less than 21 in one field; 3=more than 20 in one field

Cell number at the MTJ line: 0=normal, less than 10 cells in one field (the MTJ line is placed in the diameter of the field); 1=cell number between 10 and 20 in one field; 2=cell number between score 1 and 3; 3=cell number more than 50 in one field (the normal range was determined by the results of the control side samples)

The number of the blood vessels at the MTJ: 0=0–5 vessels at the MTJ line in 10 fields (the MTJ line was in the diameter of the field); 1=number of vessels more than 5 but less than 10 in 10 fields; 2=blood vessels between score 1 and 3; 3=number of blood at MTJ line more than 15 in 10 fields (the normal range was calculated by the results of control side samples; the blood vessels which contained red blood cells using standard staining methods (H and E, Weigert-van Gieson trichrome) were counted)

Haematomas at the MTJ: 0=normal, no haematoma; 1=a few with small extension; 2=the occurrence of the haematoma at the MTJ line between score 1 and 3; 3=many haematomas with huge extension between the muscle fibres and sometimes signs of infiltration

variation score level than the adult 0.8 mm/day lengthening rate group (G1) when comparing the summarised data of the flexor digitorum longus, peroneus quartus, proximal and distal sections ($p<0.005$). This difference was not significant between the young 0.8 mm/day lengthening rate group (G3) and the young 1.6 mm/day lengthening rate group (G4), but in the latter group, the mean score was slightly elevated.

After the comparison with the young rabbits' experimental side data, the adult experimental sides showed significantly higher mean score values in both the 0.8 mm/day and the 1.6 mm/day lengthening rate groups ($p<0.05$) (mean score: G1: 1.5; G2: 2.35; G3: 1.2; G4: 1.375) (Fig. 1).

The mean scores of fibre-size variation were slightly greater in the distal samples compared to the proximal sections. This did not reach statistical significance (G1 PROX: 1.375; G1 DIST: 1.625; G2 PROX: 2.1; G2 DIST: 2.6).

The mean score of muscle nuclei internalisation was significantly higher ($p<0.05$) in the adult G2 group than in

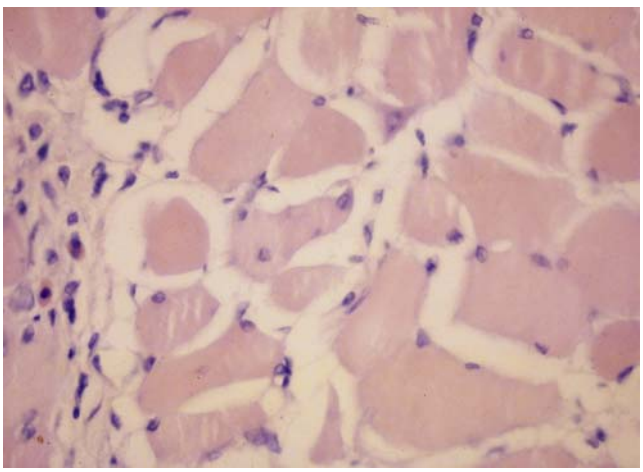

Fig. 1 Muscle fibre atrophy. Muscle fibre-size variation score=3. Adult rabbit lengthened (1.6 mm/day, 20%) peronaeus quartus. HE×400

the young G4 group according to the summarised data of the flexor digitorum longus and peroneus quartus muscles (Fig. 2). On the other hand, this value was about the same in the adult and young 0.8mm/day lengthening rate groups (G1; G3) (mean score: G1: 0.75; G2: 1.35; G3: 0.8; G4: 0.9375).

When the muscle nuclei internalisation histopathological scores of the proximal and distal sections were compared among the adult groups, in the G1 group, the value was significantly increased in the distal portion ($p<0.05$), but no difference was found in the G2 group (mean scores: G1 PROX: 0.5; G1 DIST: 1; G2 PROX: 1.3; G2 DIST: 1.4) This degenerative histological sign was more dominant in the distal portion of the muscles in the young age group ($p<0.01$) (mean scores: G4 PROX: 0.625; G4 DIST: 1.25).

The muscle fibre degeneration was significantly increased in the adult group compared to the young group lengthened either at 0.8 mm/day or at 1.6 mm/day rate according to the summarised data of the flexor digitorum longus and peroneus quartus muscles ($p<0.05$) (mean scores: G1: 1.1875; G2: 1.95; G3: 0.85; G4: 1.25) (Fig. 3).

There was no significant difference in the muscle fibre degeneration score between the proximal and distal sections.

When the muscle fibre regeneration score of the adults lengthened at the rate of 0.8 mm/day or 1.6 mm/day was compared with young animals lengthened at the same rate, the latter was significantly increased ($p<0.001$). Comparing the mean scores of the young 0.8 mm/day and the young 1.6mm/day lengthened groups, the latter score was double that of the former group ($p<0.001$) (mean scores: G1: 0.375; G2: 0.95; G3: 0.8; G4: 1.75). Both young groups had a significantly stronger regenerative response compared to the adult groups ($p<0.001$) (Fig. 4).

There was no significant difference between the regeneration outcomes in the proximal and distal sections.

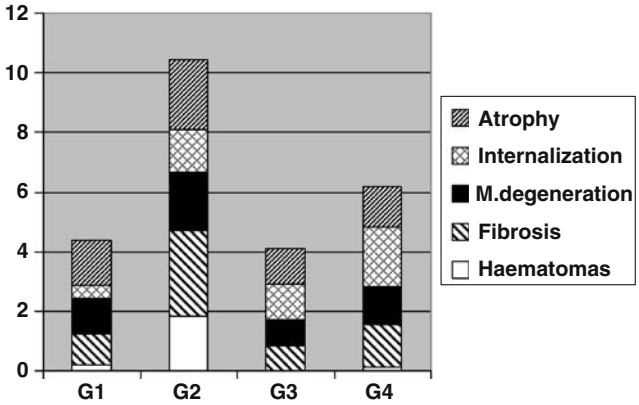

Fig. 3 Summarised scores of the five main degenerative parameters

The adult animals showed significantly greater amounts of peri-endomysial fibrous tissue than the young animals, and the animals lengthened at the rate of 1.6 mm/day had a greater amount of fibrosis compared to those lengthened at the rate of 0.8 mm/day (G1–G2: $p<0.001$; G3–G4: $p<0.01$).

No significant difference was found in the fibrous histopathological response scores between the proximal and distal sections of the muscles.

The animals lengthened at 1.6 mm/day had a significantly greater muscle nuclei internalisation response at the MTJ than those lengthened at 0.8 mm/day (G1–G2: $p<0.001$; G3–G4: $p<0.001$). In addition, the young groups showed increased muscle nuclei internalisation scores compared to the adult groups.

The animals lengthened at a faster rate had a greater cell density at the MTJ (G1–G2: $p<0.001$; G3–G4: $p<0.01$). No significant difference was found when comparing the data of the proximal and distal sections of the flexor digitorum longus and peroneus quartus muscles (Fig. 5).

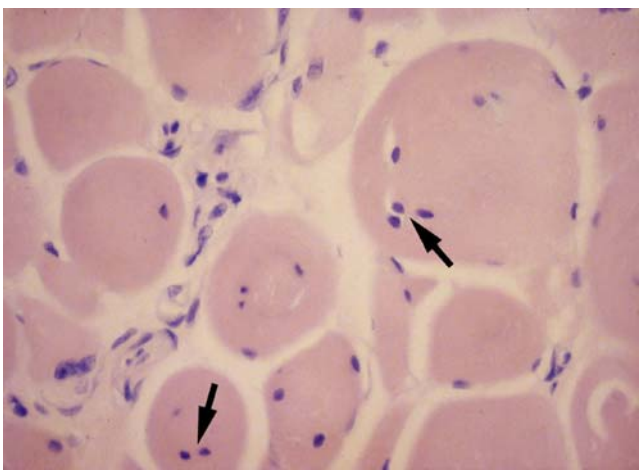

Fig. 2 Muscle nuclei internalisation (*arrows*). Adult rabbit lengthened (1.6mm/day, 20%) peroneus quartus. HE×400

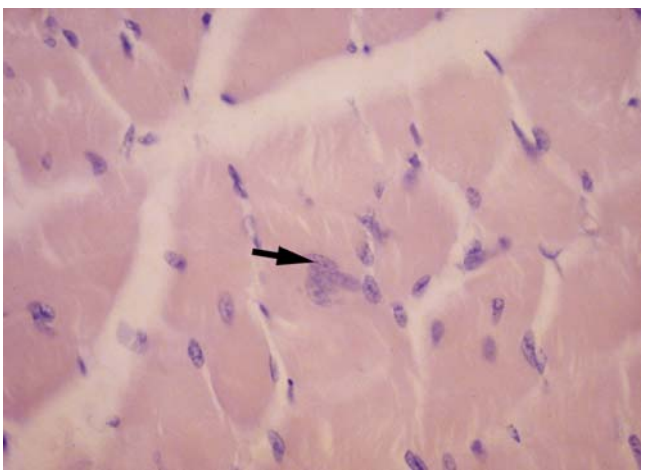

Fig. 4 Regenerating muscle fibre with large nuclei and prominent nucleolus and slightly more basophilic cytoplasm (*arrow*). Young rabbit lengthened (1.6mm/day, 20%) flexor digitorum longus muscle. HE×400

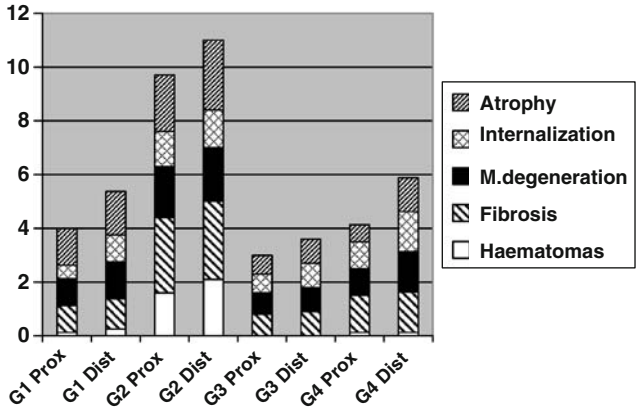

Fig. 5 Summarised scores of the five main degenerative parameters in the proximal and distal sections of different groups

The young animals lengthened at the rate of 1.6 mm/day (G4) had a significantly increased number of capillaries at the MTJ, while the young 0.8 mm/day lengthened rate group (G3) showed normal occurrence in the number of capillaries in the above-mentioned region.

There was a larger number of haematomata in the faster lengthening group compared with the slow group (G1–G2: $p<0.001$) and in adults compared to the young animals. In the young G4 group, the mean score was about the same as in the adult G1 group. As far as the young 0.8 mm/day lengthened group is concerned, this histopathological phenomenon to lengthening did not appear at all (G1: 0.1875; G2: 1.85; G3: 0; G4: 0.1).

In the adult 1.6 mm/day lengthened group, the distal sections presented this histopathological appearance more than the proximal samples. There was no difference in this parameter in the other groups (Fig. 5).

Discussion

After the summary of scores of the five main degenerative parameters (muscle fibre-size variation: muscle atrophy, muscle nuclei internalisation, degeneration of the muscle fibre, perimysial and endomysial fibrosis, and haematomata at the MTJ), it is evident that the adults lengthened at 1.6 mm/day (G2) showed greater degenerative changes than those lengthened at 0.8 mm/day (G1). The adult 1.6 mm/day lengthened group (G2) presented significantly greater damage in the muscle compared with the young 1.6 mm/day lengthened group (G4) according to the summarised degenerative scores (Fig. 3), and a tendency was visible—although it was not significant—that the distal part of the muscle could be more sensitive to lengthening.

In adult animals, the 1.6 mm/day lengthening rate produced a high number of haematomata along the lengthened MTJ. In adults, this histological phenomenon occurred, even in animals lengthened at a rate of 0.8 mm/day. The haematomata lie near the MTJ and often expand into the gaps between the muscle fibres. In parts of these haematomata, a mononuclear infiltration had started, which confirms that haematomata are not formed artificially by manipulation during the histological preparation.

The muscle fibre degeneration was more frequent in the adult groups compared with the young groups. In the adult groups, the distal sections were more seriously damaged by this parameter compared to the proximal sections. The "slight" or initial degenerative phenomena (fibres with bright eosinophilic or with a pale shade pink colour, and a coarsely granular sarcoplasmic appearance) gathered mainly near the MTJ. It seems that the tension during callus distraction leads to muscle fibre damage parallelled by the increase in necrotic muscle fibres and disturbed membrane integrity [5, 6]. According to our results, the muscles of older animals were more sensitive to the distraction than the younger rabbits. There was an increase in the number of internalised muscle nuclei near the lengthened MTJ. This increase was significantly higher in the immature animal group. As we mentioned previously, before the complete removal of the necrotic sarcoplasmic debris, the process of regeneration may have begun, so that myogenesis and phagocytosis can be visualised in the same muscle fibre concurrently [13].

We found an increase in the cell number along the lengthened MTJ. This was previously described by Caiozzo et al. [2] that myosatellite cells might have a key role in sarcomerogenesis. This response of the MTJ could be an active, proliferative reaction of this area to lengthening. It has been reported that satellite cells can be activated and proliferated in response to stretching [3, 22] and the regeneration of skeletal muscle may be achieved by inducing the activation and proliferation of satellite cells, which fuse with pre-existing muscle fibres or fuse to form new muscle fibres [7, 17]. It is possible that the activation of satellite cells depends on the amount of lengthening achieved [23]. Caiozzo et al. [2] suggest that the activation of satellite cells begins when the sarcomeres' lengths exceed a set point. The number of satellite cells in a muscle undergoes an ordered progressive decrease over the course of post-natal life, and Shisha et al. [19] also observed significantly fewer satellite cells in the mature than in the young animals. This could be the reason why the greater amount of lengthening in immature rabbits results higher regeneration scores. The most frequent location of satellite cell activation is the MTJ, so it has a remarkable synthetic capacity for producing sarcomeres. It could be that the MTJ would act as a regenerative reserve capacity for the muscle [2].

The appearance of the peri- and endomysial fibrosis correlated with the amount of lengthening and the age of the rabbits. Williams et al. [25] found that the connective tissue and collagen content were increased in the muscles distracted at the medium (1.6 mm/day) rate compared to the low

(0.8mm/day) rate. The increase in connective tissue could cause a loss of movement in the lengthened extremity. However, the connective tissue may have developed to replace necrotic muscle parenchyma, as in chronic muscle disorders [2].

We found hypervascularisation of soft tissues during the distraction. This phenomenon was more intensive in both of the younger groups. The hypervascularisation could be part of the repair mechanisms after tissue damage [1].

The occurrence of histopathological signs of muscle fibre regeneration was seen more frequently in the young animal groups. No significant difference was found between the scores for different section levels, although the regenerative signs were concentrated near the MTJ.

This histopathological score system has been used by other researchers [9] and was found to be useful. This paper shows that the young animals have a greater ability to respond to lengthening than the adults. The distal part of the lengthened muscle is slightly more involved by the degenerative effects during limb lengthening. It seems that the muscle has an active adaptive response to the lengthening. The necrotic tissue is replaced by connective tissue, fat and regenerating fibres. The rate of this depends on the age and the amount of lengthening.

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