# The effect of femoral lengthening on skeletal muscle: an experimental study in rats

## Femur uzatma işleminin iskelet kası üzerine etkisi: sıçanlarda yapılan deneysel bir çalışma

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*AMAÇ*

Kırk iki sıçanda femur uzatma işleminin müsküler etkilerini araştırmak ve çok düşük oranda uzatmanın bile kasta geri dönüşsüz patolojik değişikliklere, farklı evrelerde dejenerasyon odaklarının gelişimine neden olduğunu göstermek

*YÖNTEMLER*

Sıçanlar rastgele yöntemle altı gruba ayrılmış ve farklı oranda femur uzatma işlemleri uygulandıktan sonra distraksiyon sürecinin bitiminde feda edilmişlerdir. Tüm örnekler için histopatolojik ve histomorfometrik ölçümler yapılmıştır. Sonuçların istatistiksel analizi için Mann Whitney U testi kullanılmıştır

*BULGULAR*

Uzatma oranı yükseltilerek kas liflerinin dejenerasyonu, vakuollerin sayısı ve hücre infiltrasyonu gibi patolojik değişikliklerin şiddet derecesi artırılmıştır. On dört gün sonra yapılan incelemelerde dejenere olmuş kas liflerinde fagositoz gözlenmiş ve hücrede daha önce oluşmuş infiltrasyonun hızla azaldığı saptanmıştır. Yirmi bir gün süren uzatma işleminden sonra vakuollerde ve yine kas liflerinin etrafında fibroz oluşmuştur. Hücre infiltrasyonu giderek kaybolmuş, kas normal görünümünü kazanmış ve otuz bir gün izlenen grupta endomizyal, perimizyal ve epimizyal fibroz gibi patolojik değişiklikler oluşmuştur. Kas liflerinin % 21'i atrofiye uğramış olmasına rağmen tip I/ tip II kas lifi oranı değişikliğe uğramamıştır.

*SONUÇLAR*

Bu çalışmanın bulguları aynı kasta farklı dejenerasyon dönemlerini gösteren farklı bölgelerin bulunabileceğini ve % 10 oranında bir uzatma işlemi sonrasında bile kasta geri dönüşsüz değişiklikler gelişebileceğini göstermiştir.

***Anahtar sözcükler:*** Uzatma, distraksiyon, fibroz, müsküler değişiklikler, sıçanlar

*BACKGROUND*

To investigate the muscular changes occurring during femoral lengthening performed in 42 rats.

*METHODS*

The rats were randomly divided into six groups and different rates of lengthening was utilized and sacrificed at the end of the distraction. Histopathologic and histomorphometric measurements were done for all specimens. The results were analyzed statistically by Mann-Whitney U test.

*RESULTS*

By increasing the rate of lengthening, the severity of pathologic changes such as degeneration of muscle fibers, number of vacuoles and cellular infiltration were increased. Phagocytosis of degenerated muscle fibers was observed after 14 days and cellular infiltration decreased sharply and fibrosis formed in the vacuoles and around the muscle fibers after 21 days of lengthening. Cellular infiltration was lost, the muscle had gained its normal appearance and endomysial, perimysial and epimysial fibrosis formed in the group, which was observed for 31 days. Twenty one percent of muscle fibers were atrophic but the ratio of type I to type II muscle fibers did not change.

*CONCLUSIONS*

The findings of the current study showed that, different regions with different phases of degeneration occur in the same muscle and irreversible changes in the muscle may develop even in 10 percent lengthening.

***Key words:*** Lengthening, distraction, fibrosis, muscle changes, rat.

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Several experimental studies were performed in the past, to detect muscular changes after limb lengthening procedures) [1-10] and to test the generally accepted hypothesis of Calandriello that suggests that the muscle fibers are being ruptured due to distraction and then they would heal with fibrosis. [1, 5, 9, 11, 12] Although the rate of soft tissue changes thought to be proportional to the rate of distraction, [1, 5, 9, 12] the percentage of lengthening that causes irreversible changes at muscles is still obscure. [5, 9, 10, 13] While several authors have proposed that these changes are myogenic, [1, 5, 9, 11, 14] some claimed a neurogenic etiology. [3, 6] Current study was conducted to solve these problems.

## MATERIAL AND METHODS

Forty-two Wistar-Albino male rats with an average of 225 ± 25 gr. were used in the study. Under general anesthesia, a specifically designed external fixators (Hipokrat Medical Devices Manufacturing Co., Istanbul, Turkey) were applied to the left femora, and subperiosteal corticotomy was performed. Postoperatively, a single dose of ceftriaxone (50mg/kg) was injected by intraperitoneal route. Throughout the experimental period, the rats were free to ambulate in their cases. The rats were randomly divided into six groups (seven rats in each group). The first group was control group and no distraction was performed. In the other groups femoral lengthening (0.35 mm) was performed for 4 times starting from the first postoperative day. In the second group lengthening was continued for three days (10% lengthening) and the rats were sacrificed at the end of the distraction. In groups 3, 4, 5, 6, lengthening was continued for seven days (30% lengthening), and the rats were sacrificed at 7, 14, 21 and 31 days postoperatively and biopsy specimens (about 3 cm x1cm) were taken from the rectus femoris muscle.

The specimens were fixated in sucrose-gum arabic solution (Merck-4228) and freezed to –20°C after one hour and sections of 8-micron width, perpendicular to the longitudinal orientation of muscle fibers were made. The muscular materials were stained with hematoxylin- eosine (H-E), Van Gieson (VG), trichrome (T), modified trichrome (MT), periodic acid Schiff (PAS), succinic dehidrogenase (SDH), adenosine triphospha-

**Table 1:** Soft tissue changes (microscopic findings)

| | CI | V | CV | Ph | IN | | CCT | RC | APh |
|---|---|---|---|---|---|---|---|---|---|
| Group 1 | - | - | - | - | +/- | | - | - | - |
| Group 2 | + | + | Some | - | + | | Cellular infiltration | | - |
| Group 3 | +++ | +++ | All | + | ++ | | Cellular infiltration and granular tissue | | - |
| Group 4 | ++/- | +++ | PAS (+) Cell +/- | | +++ | +++ | Perimysial fibrosis | | - |
| Group 5 | + | + | PAS (++) Cell +/- | | - | + | Endomysial – Perimysial fibrosis | | + |
| Group 6 | - | + | PAS (+++) Cell - | | - | +/- | Endomysial – Perimysial Epimysial fibrosis | +++ | ++ |

CI: Cellular infiltration
CV: Changes into the vacuoles
IN: Internalization of the nuclei
RC: Regeneration cells
V: Vacuoles
Ph: Phagositosis
CCT: Changes of the collagen tissue
Aph: Acid phosphatase

**Table 2:** Diameter (micron) and ratio of type I and type II muscle fibers

| | SIZE | | RATIO |
|---|---|---|---|
| | Type I | Type II | Type I / Type II |
| Group 1 | 14 | 15 | 1.0 |
| Group 2 | 12 | 13 | 1.1 |
| Group 3 | 11 | 11 | 1.0 |
| Group 4 | 12 | 12 | 0.9 |
| Group 5 | 10 | 12 | 1.0 |
| Group 6 | 10 | 11 | 1.0 |

tase (ATPase) and acid phosphatase (AP). Cellular infiltration, vacuoles, internalization of nucleus, phagocytosis, muscle fiber and collagen tissue degeneration, muscle fiber regeneration, fibrosis, vascular changes, striation and the changes in enzymatic activities were studied histopathologically. Histomorphometrical measurements on of rectus femoris muscles of rats stained with SDH accomplished in ten randomized microscopic fields. The numbers of muscle fibers of type I and type II were determined (400x). The mean diameters of fifty pieces of type I and II muscle fibers were measured in microns. Mean diameters of type II and I muscles and ratio of type II and I were estimated

The results were analyzed statistically by Mann-Whitney U test. The level of significance was considered to be $p<0.05$.

***Table 3:*** Ratio of atrophy in type I and type II muscle fibers

| | Type I % | Type II % |
|---|---|---|
| Group 2 | 10.3 | 10.5 |
| Group 3 | 22.7 | 22.4 |
| Group 4 | 15.3 | 17.8 |
| Group 5 | 26.7 | 18.4 |
| Group 6 | 23.8 | 20.4 |

## RESULTS

**Group 1:** In H-E dyed specimens; there were muscle fibers with fusiform nuclei separated from each other by interfascicular spaces. One internal nucleus was seen per 2-3 high power fields (Table 1). In specimens that stained with SDH and ATPase , type 1 and 2 muscle fibers showed mosaic architecture and the ratio of type 1 to type 2 fibers was 1:1. The average diameter of Type 1 muscle fibers were 14 microns while those of type 2 was 15 microns (Table 2).

**Group 2:** The major pathological findings were vacuoles and cellular infiltration among muscle fibers (Figure 1). There were too many tiny vacuoles. Cellular infiltration was observed in some of the vacuoles was observed. Cellular infiltration was more diffuse in perifascicular areas. In this study with SDH and ATPase dyed specimens, we noticed three different areas on the cross section of the muscles. Firstly, there were normal mosaic distribution of muscle fibers. Secondly, there we-

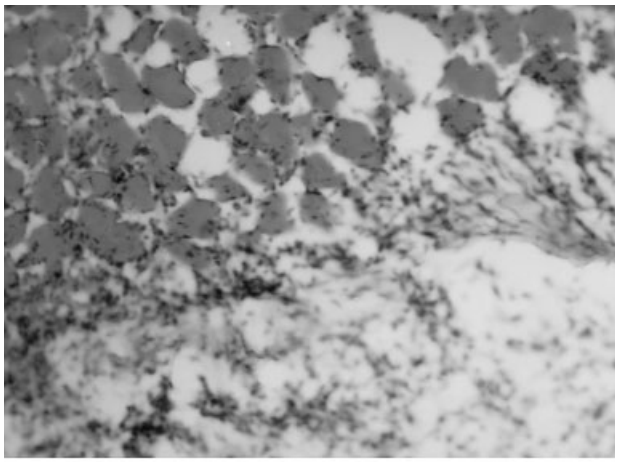

**Figure 1:** Dense cellular infiltration and vacuoles in Group 2 (H-E, x 160)

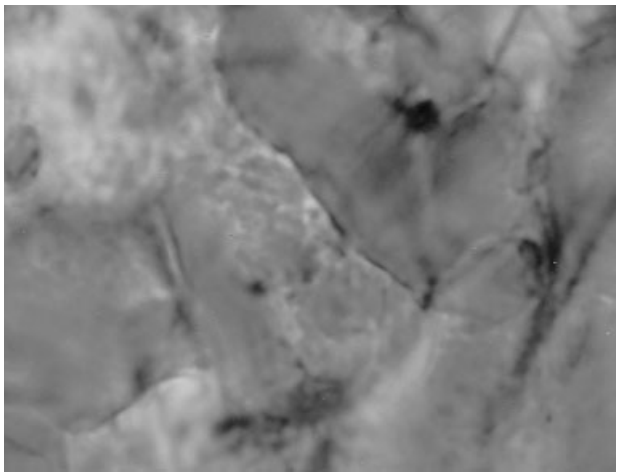

**Figure 2:** Internalization of nuclei in Group 3 (H-E, x 1600)

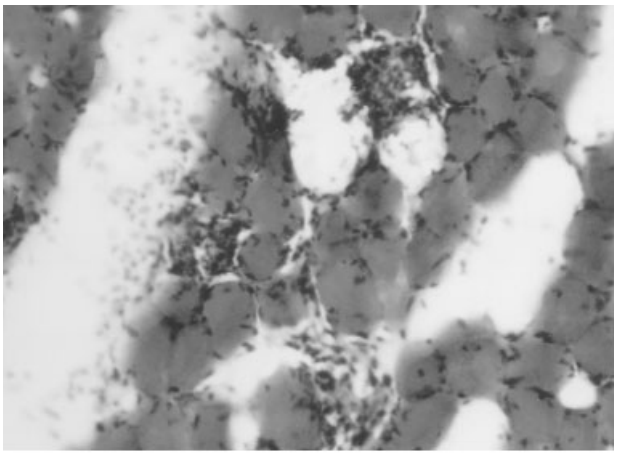

**Figure 3:** Cellular infiltration and phagocytosis in Group 3 (Periodic Acid Schiff, x 160)

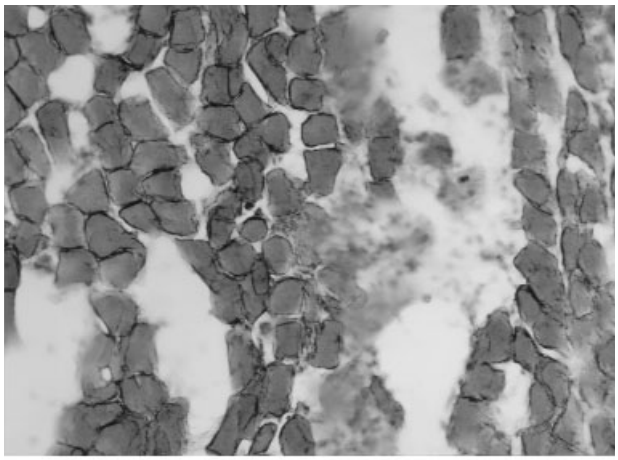

**Figure 4:** Vacuoles and degeneration of collagenous tissue in Group 3 (Van Gieson, x 160)

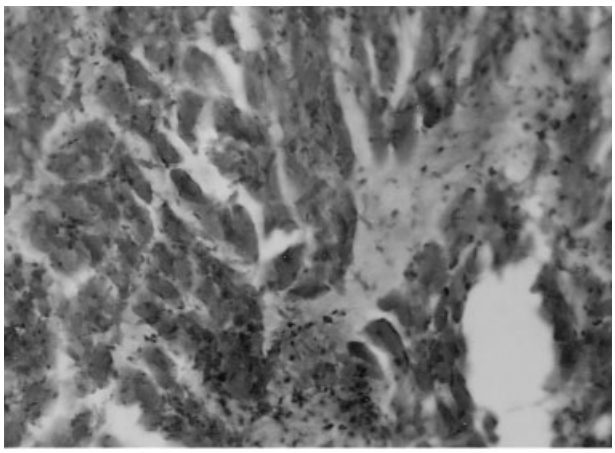

**Figure 5:** Fibrosis and collagenous tissue and PAS (+) vacuoles in Group 4 (periodic acid Sciff, 160)

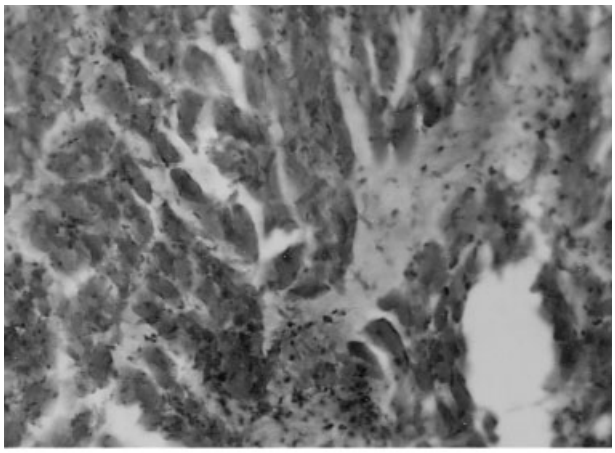

**Figure 6:** Endomysial fibrosis in Group 5 (Trichrome, x 160)

re degenerated muscle fibers. In the third area there were muscle fiber degeneration and diffuse cellular infiltration. Comparing with the control group, the percentage of atrophy was 10.3 % in type I and 10.5 % in type II muscle fibers ($p<0.05$, Table 3). The ratio of type I to type II didn't change ($p>0.05$, Table 2).

**Group 3:** In this term, the number and diameters of vacuoles were increased; cellular infiltration was more diffuse and dense. Meanwhile, the number of internalization of nuclei was increased (four / five nuclei on each microscopic area, (Figure 2). Phagocytosis were seen on some areas (Figure 3). In group 2 the vacuoles were empty, but in group 3 cellular infiltration into the vacuoles were seen. Three different areas in SDH and ATP' ase ATPase studies were seen in this term. In some degenerated areas (Figure 4), mitochondrial diffusion was seen. Comparing with the control group, the rate of atrophy was 22.7 % in type I and 22.4 % in type II muscle fibers ($p<0.05$, Table 3). The ratio of type I to type II didn't change ($p>0.05$, Table 2).

**Group 4:** The vacuoles were the same in diameters and numbers but the cellular infiltration decreased and a new granular tissue stained with PAS (+) formed (Figure 5). Internalization of nucleus was the same as in group 3 (four / five on each microscopic area). The amount of collagenous tissue increased and perimysial fibrosis was observed in some areas. Vascular structures in muscles were prominent. Three different areas observed in SDH and ATPase studies were also seen in this term. In cross- sections stained with AP, hyperactive areas in collagenous tissue were observed. Comparing with the control group, the atrophy was 15.3 % in type I and 17.8 % in type II muscle fibers ($p<0.05$, Table 3). The ratio of type

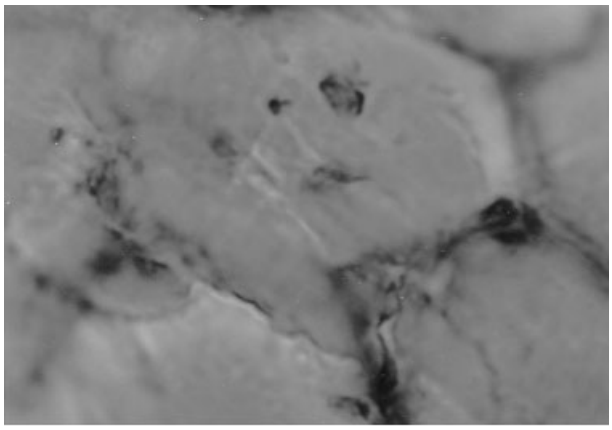

**Figure 7**: Regeneration cell in Group 5 (H-E, x 640)

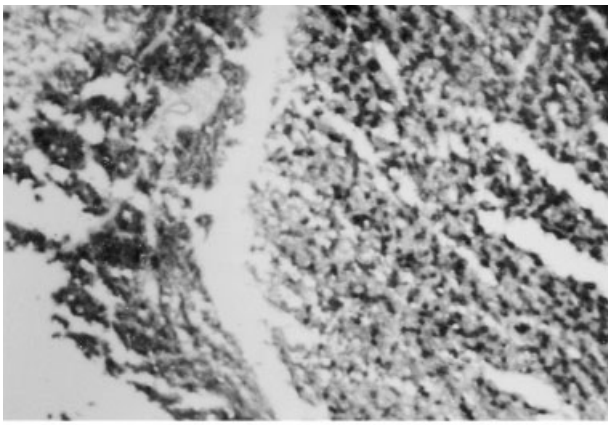

**Figure 8**: Three different areas in Group 6 succinic dehidrogenase, x 32)

I to type II didn't change (p>0.05, Table 2).

**Group 5:** The muscle started to return to its normal appearance. Cellular infiltration was seen in some areas. Internalization of nucleus decreased (two / three on each microscopic area). Phagocytosis was not observed. Cellular infiltration in vacuoles decreased and some of them filled with granular tissue stained with PAS (+). Vascular structures gained their normal texture Perimysial and endomysial collagenous tissue increased with fibrosis (Figure 6). Regenerated muscle cells with small size, large nuclei and basophilic cytoplasm cytoplasms were observed (Figure 7, Table 1). Although normal mosaic appearance was detected on some areas, type 1 muscle fibers were observed adjacent to collagenous tissue on the areas with the diffuse inflammation. In the specimens stained with AP, the number of hyperactive areas were fewer. Comparing with the control group, the rate of atrophy was 26.7 % in type I and 18.4 % in type II muscle fibers (p<0.05, Table 3). The proportion ratio of type I to type II didn't change (p>0.05, Table 2).

**Group 6:** The muscle returned to its normal appearance generally. Vacuoles were completely filled with PAS (+) collagenous tissue and cellular infiltration was almost nonexistent. Rarely internal nucleus was observed (one / two on each microscopic area field). Too many regeneration cells were observed. Epymisial, perimysial and endomysial fibrosis were conspicuous (Table 1). Three different areas were observed in SDH and ATPase stained areas (Figure 8). In some microscopic fields, normal mosaic appearance was noted. Type I muscle fibers were sharply seen on the second area and collagenous tissue almost completely replaced the muscle fibers on the third area. Collagenated tissue regenerated in the specimens stained with AP. Comparing with the control group, the percentage atrophy was 23.8 % in type I and 20.4 % in type II muscle fibers (p<0.05, Table 3). The ratio of type I to type II didn't change (p>0.05, Table 2).

## DISCUSSION

Although, there are several reports in the literature regarding the soft tissue changes after bone lengthening procedures, no consensus is shared among the authors. Most of the authors have found a strong correlation between the rate of distraction and the histological appearance of the muscle that, when the rate of distraction increases, proportional rate of changes also occur. [1, 5, 9, 10, 12] According to these authors, 10 to 15% lengthening is a borderline in that the musculature loses its adaptive compliance to the bone lengthening beyond this limit. [1, 5, 9, 10, 12] In the present study, the results are similar to previous studies,in that irreversible muscle changes became significant, proportional to the rate of distraction (Table 1). However, contrary to the previous reports, we observed irreversible changes in the muscle tissue, even after 10% lengthening, characterized by the presence of vacuoles, cellular infiltration, and fibrosis. Similarly, we observed internalization of the nuclei in all distraction groups. This is also contrary to the common belief that internalization occurs only after more than 10% of lengthening. [1, 5]

According to Calandriello, rupture of the muscle fibers occurs beyond a certain point of distraction and muscle cells proliferate associated with con-

nective tissue fibrosis within these areas. [11] Although Lindboe found no evidence for such a pathogenetic mechanism, [6] we observed vacuoles between muscle fibers whose numbers and the diameters of the vacuoles increased in accordance with the rate of distraction. Interestingly, these vacuoles were present even after 10% lengthening (Table 1).

The most important factor of the present study, in our opinion, was the presence of three different regions in the muscle when stained with SDH and ATPase. These regions showed, different stages of muscle degeneration and this might be the explanation of controversies among histological changes, reported. Possibly, the region where the biopsy specimens were retrieved, gave different results.

In addition to myogenic changes, some authors [3,6] had noticed the signs of neurogenic muscular atrophy with denervation processes during the distraction phase and reinnervation processes during the consolidation period. In the present study, we failed to observe target cells that represent neurogenic atrophy.

As a result, our findings showed that, different regions with different phases of degeneration can occur in the same muscle. Certain evaluation of all these regions revealed that, irreversible changes in the muscle occur, and even in 10 percent lengthening these procedures should be handled with caution.

## ACKNOWLEDGMENTS

The authors thank Türe Tunçbay, M.D., from Ege University Hospital and İzge Günal, M.D., from Dokuz Eylül University Hospital, İzmir, Turkey for their contribution to the study.

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