EXPERIMENTAL STUDY

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Histomorphological comparison of immobilization and denervation atrophies

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Objective: The aim of this study was to compare the histomorphological changes in the muscle tissue after immobilization and denervation atrophies in an animal model.

Methods: The study included 30 Ross-800 hybrid chickens (60 legs) divided into two study (immobilization and denervation) and two control groups. The knee and ankle joints were fixed with a Kirschner wire in the immobilization atrophy group and sciatic nerve resection was performed in the denervation group. The unaffected side of each group was used as controls. The weight, volume, height, diameter and the rate of elongation of the Achilles tendons, and the amount of fat deposition, degeneration and fibrosis were compared between the two groups at the end of 3 weeks. Hematoxylin-eosin staining was performed for a histopathological assessment of the muscles. The Mann-Whitney U-test was used for comparisons.

Results: Loss of the volume, weight and muscle length was significantly lower in the denervation group than the immobilization group ($p<0.05$). Differences between the diameter of the Achilles tendon and length and diameter of the short heads were not statistically significant. There were statistically significant differences in fat deposition, degeneration and fibrosis between the degeneration group and the immobilization group ($p<0.05$). Pixel counting revealed a significant difference in the number of pixels in the fatty tissue area (white area) between the denervation group and the immobilization group ($p<0.05$).

Conclusion: Our results showed that histomorphological changes were more in the denervation group than the immobilization group in an experimental chicken model.

Key words: Atrophy; denervation; histomorphological; immobilization.

Muscle atrophy, or the shortening of muscle fiber without a change in their number, is a severe complication of conditions such as hunger,[1] prolonged bed rest,[2,3] the removal of mechanical load, denervation, immobilization or decreased gravity[4] and age,[4] as well as various pathological conditions (sepsis, chronic renal failure, diabetes, chronic heart failure, chronic obstructive pulmonary disease, cancer, and more).[5,6] Muscle atrophy leads to a decrease in muscle strength and endurance and causes physical disability. Therefore, it decreases the

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quality of life, has a negative impact on disease progression, extends the duration of hospital stay and increases the mortality rate.

This study aimed to compare the histomorphological changes in the muscle tissue after immobilization and denervation atrophy.

Materials and methods

The study included 30 Ross-800 hybrid broiler chickens (60 legs), weighing between 800 and 1200 grams.

Laboratory animals underwent anesthesia with 50 mg/kg ketamine hydrochloride administered intramuscularly without suppressing respiration. A 200 mg ampicillin/sulbactam group of antibiotics was added to the water of the chickens for surgical prophylaxis before and after surgical intervention.

Chickens were divided into 2 groups. The opposite leg of the 14 chickens in the immobilization group and 16 chickens in the denervation group were used as controls.

For the immobilization group, the hind legs, ankles and knee joints were fixed in the neutral position using a 1.2-mm Kirschner wire after anesthesia and medical dressing was performed.

In the denervation group, the skin was passed subcutaneously using a transverse incision posteromedial to the thigh and the sciatic nerve was reached through the gluteus maximus muscle following anesthesia. An approximately 1.5 cm portion of the nerve was extracted.

The experimental animals were sacrificed by cervical dislocation at the end of the 3rd week and the hip joints underwent disarticulation. Both hind legs were protected in containers containing saline fluid. First, the Achilles tendons were detected and freed. The fascia was grazed from the subjacent flexor muscles up to the origin of the gastrocnemius muscle. The length and diameter of the Achilles tendon of the freed gastrocnemius muscle were measured first.

The distal portion of the femur of the same leg was fixed with two bolt screws. The leg was then fixed in a lengthening device and a clamp was attached to the Achilles tendon for hanging weights.

The amount of elongation was measured with 50, 100, 150 and 200 grams, respectively. The muscle measured for elongation rate was freed from the attachment point of the bone. Of the muscle that was freed, the length of both the head and the diameter of the belly were measured. Finally, the wet weights of the muscles were measured with a precision balance (g/100). The muscles were placed in a beaker of 30 cc distilled water and their volumes measured by the amount of overflow.

All measurements were performed by the same investigator in all groups.

Transverse and longitudinal sections of the tissues were taken for histopathological examination. The transverse cross-sections were also taken from the flap type tendon of the musculotendinous junction of the muscles. Sections were stained with hematoxylin-eosin (H&E).

Fat deposition, degeneration, fibrosis and the inflammatory response of the muscles were assessed. The sectional area where the fat deposition and degeneration were predominant was evaluated with the smallest magnification (4 X 0.10). In these sections, the automatic area counting method of the intended cells was performed at the Geodesy and Photogrammetry Engineering Department of Yıldız Technical University. Colored spots of the adipose tissue (white area) and muscle tissue (red area) on the photo were counted.

The threshold method was used for the automatic calculation of the wanted cell areas. This procedure was performed according to the following rules and orders. For each pixel, R (Red) and G (Green) bands were checked among B (blue), R (red) and G (green) bands in the image. If the gray value of R or G were higher than 230, the color of the pixels in the image was white. If the gray value of R was lower than 190 and the gray value of G lower than 200, the color of the pixels in the image was red. To calculate the pixels except for the red and white pixels, the number of pixels of two classes was subtracted from the total number of pixels.

All measurements were evaluated statistically using the Mann-Whitney U-test. P values of less than 0.05 were considered significant.

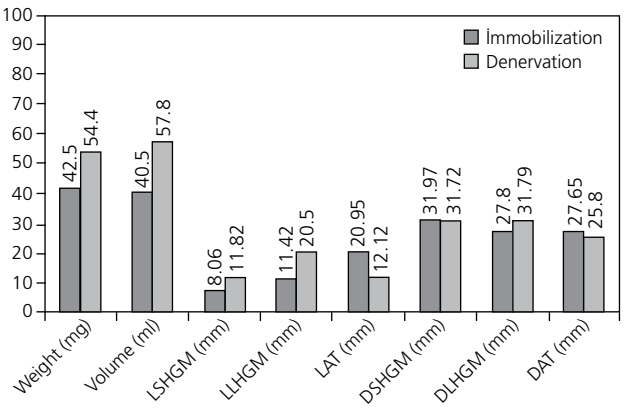

Fig. 1. Percentage distribution of immobilization and denervation atrophy groups. LSHGM: Length of the short head of the gastrocnemius muscle; LLHGM: Length of the long head of the gastrocnemius muscle; LAT: Length of the Achilles tendon; DSHGM: Diameter of the short head of the gastrocnemius muscle; DLHGM: Diameter of the long head of the gastrocnemius muscle; DAT: Diameter of the Achilles tendon.

Results

The loss of weight and volume, difference in length of the short-legs, difference in length of the long-legs, difference in length of the Achilles tendons, and difference in diameter of the long-legs of the immobilization and denervation groups were calculated. All results, with the exception of the difference in the diameter of short-legs and the diameter of the Achilles tendon, were statistically significant ($p<0.05$). However, the loss in the denervation group was greater than in the immobilization and control groups. Figure 1 shows the percentage distribution of the losses in the immobilization and denervation atrophy groups.

When the difference in elongation was compared, there were statistically significant differences of 50, 100 and 150 g in the immobilization group ($p<0.05$). Tables 1 and 2 show the mean values obtained from the measurements.

Histopathological examination evaluated fat deposition, degeneration of fibers, fibrosis and inflammatory response in the muscles. Results are given in Table 3.

Fat deposition, degeneration, fibrosis and inflammatory response were statistically significantly lower in the denervation group than the immobilization group ($p<0.05$).

When the areas of adipose tissue (white area) and muscle tissue (red area) were measured, the white field comprised 18% of the pixels in the control group, 22% in the immobilization group and 42% in the denervation group. Additionally, the red area was measured as 67% in the control group, 42% in the immobilization and 34% in the denervation group.

Table 1. Mean values of the control and immobilization groups.

	Control group (n=14)		Immobilization group (n=14)	
	Mean±SD	**Min.-Max.**	**Mean±SD**	**Min.-Max.**
Weight (mg)	16.32±4.04	6.18-23.08	9.37±2.05	6.0-12.60
Volume (ml)	167±30	117-227	96±21	52-124
LSHGM (mm)	60.50±5.91	53.28-72.75	57.19±4.08	48.50-64.32
LLHGM (mm)	76.41±6.75	70.87-87.62	67.71±5.19	59.18-64.32
LAT (mm)	9.83±2.19	6.76-13.53	7.77±1.89	4.72-11.84
DSHGM (mm)	21.08±2.70	15.54-26.27	14.34±2.03	10.25-17.16
DLHGM (mm)	29.96±4.00	23.35-38.24	21.63±3.27	16.70-27.67
DAT (mm)	6.19±0.87	4.87-7.92	5.16±1.59	4.13-10.36

P value lower than 0.05 ($p<0.05$) (statistically significant) with Mann-Whitney U-test. LSHGM: Length of the short head of the gastrocnemius muscle; LLHGM: Length of the long head of the gastrocnemius muscle; LAT: Length of the Achilles tendon; DSHGM: Diameter of the short head of the gastrocnemius muscle; DLHGM: Diameter of the long head of the gastrocnemius muscle; DAT: Diameter of the Achilles tendon.

Table 2. Mean values of the control and denervation groups.

	Control group (n=16)		Denervation group (n=16)	
	Mean±SD	**Min.-Max.**	**Mean±SD**	**Min.-Max.**
Weight (mg)	22.41±6.32	9.77-30.96	10.44±3.11	5.12-14.39
Volume (ml)	216±47	136-30	101±26	49-147
LSHGM (mm)	68.05±7.50	58.67-84.95	60.01±8.29	49.68-73.62
LLHGM (mm)	84.30±10.41	67.27-107.4	67.31±7.84	54.67-82.90
LAT (mm)	10.32±8.67	15.76-7.14	9.80±3.09	4.95-14.23
DSHGM (mm)	23.24±2.99	18.35-29.71	15.87±4.83	11.23-29.22
DLHGM (mm)	33.51±3.58	25.62-40.46	22.86±4.21	16.86-33.39
DAT (mm)	7.83±1.97	5.72-12.60	4.76±0.54	4.63-7.27

P value lower than 0.05 ($p<0.05$) (statistically significant) with Mann-Whitney U-test. LSHGM: Length of the short head of the gastrocnemius muscle; LLHGM: Length of the long head of the gastrocnemius muscle; LAT: Length of the Achilles tendon; DSHGM: Diameter of the short head of the gastrocnemius muscle; DLHGM: Diameter of the long head of the gastrocnemius muscle; DAT: Diameter of the Achilles tendon.

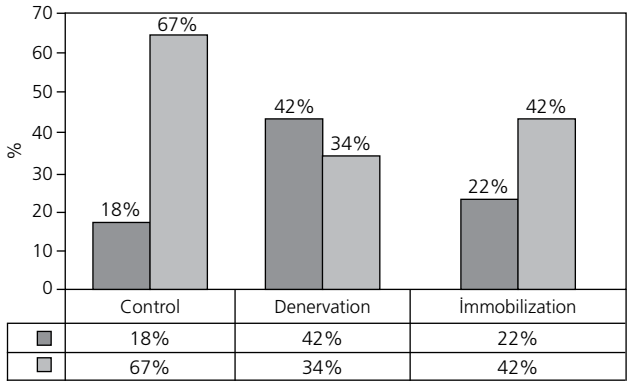

Fig. 2. The percentage distribution of control, denervation and immobilization groups.

According to these results, the adipose tissue area (white area) in the denervation group was significantly larger than the immobilization group ($p<0.05$). The percentage distribution of the red and white areas of the control, immobilization, and denervation groups are given in Figure 2.

With the empirical measurement of the pure muscle tissue of the control, immobilization and denervation groups, the percentage of the red field in the cross-sectional area of all three groups (muscle tissue) multiplied by the mean volume values of that group will give, empirically, the volume of pure muscle tissue in that muscle (Tables 4 and 5).

The percentage of the remaining volume of the pure muscle tissue at the end of 3 weeks is shown in Table 6. While there was a reduction in pure atrophic muscle tissue (motor strength) of 2/3 when compared to the healthy muscle tissue in the immobilization group, there was an empiric reduction in pure atrophic muscle tissue (motor strength) of 3/4 when compared to the healthy muscle tissue in the denervation group.

Discussion

The principal functions of the skeletal muscles are movement, postural behavior and respiration. Atrophy of the muscles is a severe complication that can lead to a decrease in muscle strength and endurance and causes physical disability and is caused by various physiological (aging) and pathological (disuse, denervation and zero-gravity environment) stimulations.

In a study conducted by Tomanek and Lund, chickens were studied due to the higher rate of atrophy in white muscle fibers than in red muscle fibers and the easy application of established denervation and immobilization techniques.[7,8] In the current study, we selected the method of immobilization using external fixation of the ankle and knee joints with Kirschner wire instead of immobilization using a plaster as described by Tomanek and Lund.[8] As in the study conducted by Camillo et al., a 1 cm portion of the sciatic nerve was dissected carefully from the surrounding tissues and resected in the denervation group.[9] Using this method, any re-innervations that might occur after denervation could be prevented.

In both groups, animals were sacrificed at the end of the 3rd weeks. Muscle mass decreased by 54.4% in the animals in the denervation group and 42.5% in the im-

Table 3. Histopathological assessment of the denervation and immobilization groups.

	Mild		Severe		p
	n	%	n	%	
Fat deposition					
Denervation	5	19.2	21	80.8	0.037*
Immobilization	12	46.2	14	53.8	
Degeneration					
Denervation	7	26.9	19	73.1	0.005*
Immobilization	18	69.2	8	30.8	
Fibrozis					
Denervation	19	79.2	5	20.8	0.007*
Immobilization	17	94.4	1	5.6	
	No		**Yes**		
	n	**%**	**n**	**%**	**p**
Inflammation					0.027*
Denervation	11	42.3	15	57.7	
Immobilization	3	11.5	23	88.5	

*P value lower than 0.05 ($p<0.05$) (statistically significant) with Mann-Whitney U-test.

Table 4. The volume of pure muscle tissue in the control and immobilization groups.

	Control group (n=14)	Immobilization group (n=14)
Volume	118 ml	40.32 ml

Table 5. The volume of pure muscle tissue in the control and denervation groups.

	Control group (n=16)	Denervation group (n=16)
Volume	144 ml	34.34 ml

Table 6. Percentage of the volume of pure muscle tissue in the control and the denervation groups.

	Control group (n=16)	Denervation group (n=16)
Percentage	33	23

mobilization group when compared to their own healthy sides. Zarzhevsky et al. determined a decrease in muscle mass of 32 to 42% after immobilization with external fixation for 4 weeks.[5] In a long-term study assessing the effect of denervation atrophy on striated muscles in rats, Sunderland and Ray determined a decrease in muscle mass of approximately 40 to 60% within the first 30 days.[10] Volume loss was 57.5% in the denervation group and 40.5% in the immobilization group. The loss of weight (54.4%) and volume (57.5%) in the denervation group was higher than that of the immobilization group (42.5% and 40.5%, respectively). In a study investigating the effects of denervation on striated muscles, Sunderland and Ray[10] evaluated the linings and changes in the connective tissue and irregularity of the fibers by light microscopy after they stained the transverse and longitudinal sections of muscles with H&E staining. They detected vacuolation in the sarcoplasm, irregularity, collapse and disintegration of muscle fibers and an increase in connective tissue and fibrosis. The rate of fat deposition and degeneration of muscle fibers was 80% and 73%, respectively, in the denervation group and 53% and 30%, respectively, in the immobilization group. The rate of fibrosis that is considered as connective tissue change was 20.8% in the denervation group and 5.6% in the immobilization group. In this study, as emphasized in the study conducted by Tanaka et al.,[11] the most severe atrophy occurred in the denervation group according to both morphological and histopathological findings.

The rate of atrophy was 11.82% in the short head length and 20.50% in the long head length of the gastrocnemius muscle in the denervation group and 8.06% and 11.42%, respectively, in the immobilization group. The atrophy in the denervation group was higher than that of the immobilization group.

In this study, morphological measurements showed that there was a decrease in the length of the Achilles tendon of 20% in the immobilization group and 12% in the denervation group when compared to the control groups. There was a decrease in the diameter of the Achilles tendon of 27.65% in the immobilization group and 25.80% in the denervation group. The changes in the immobilization group were more severe than in the denervation group. According to the histological findings of the musculotendinous and tendinous structures, while the rate of severe changes in the fibers of the tendon was 7.7% in the immobilization group, there was no severe change in the denervation group. The morphological measurements and histological-pathological findings supported the atrophic changes in the immobilization group. While atrophic changes of the muscle fibers were more significant in the denervation group, the atrophic changes of the tendon were more significant in the immobilization group. According to the histopathological findings of the musculotendinous junction, the rates of fat deposition and fibrosis were 36.4% and 30%, respectively in the denervation group and 15.4% and 14.3%, respectively, in the immobilization group.

In the current study, the rate of elongation was 4.8% in the immobilization group and 2.5% in the denervation group. In a study conducted by Tabary et al,[12] the authors emphasized that the number of sarcomeres affects the rate of elongation. In this study, muscular degeneration was higher in the denervation group. The decrease in the sarcomere number may be greater in the denervation group than in the immobilization group. This may be due to the fact that the legs in the immobilization group were fixed in the neutral position. This did not cause a significant decrease in the number of sarcomeres and their ability to elongate may be better than the denervation group.

The method of automatic determination of the required cell area in histopathological images as used in a study, conducted by Toraman et al.,[13] was used in this study. The number of pixels was obtained for each example. All cross-sectional areas consisted of 270,000 pixels. While the rate of the white area (adipose tissue area) was 18% in the control group, it was 42% in the denervation group and 22% in the immobilization group. The rate of red areas (area of muscle tissue) was 67% in the control group, 34% in the denervation group and 42% in

the immobilization group. The amount of the fat deposition (white area) in the denervation group was higher than that of the immobilization group.

Pure muscle tissue in the gastrocnemius muscle was measured as 33% in the immobilization group and 23% in the denervation group. These values were considered to represent the mass of contractile muscle tissue in both groups and the actual contractile muscle tissue. After a 3-week immobilization and denervation period, the rate of the empirical loss of pure muscle tissue was 2/3 in the immobilization group and 3/4 in the denervation group. The loss of contractile muscle tissue in the denervation group was higher than that of the immobilization group.

In conclusion, after a 3-week period of immobilization and denervation in a chicken model, the amount of atrophy was greater in the denervation group than in the immobilization group.

Conflicts of Interest: No conflicts declared.

References

1. Busquets S, Alvarez B, López-Soriano FJ, Argilés JM. Branched-chain amino acids: a role in skeletal muscle proteolysis in catabolic states? J Cell Physiol 2002;191:283-9.
2. Bloomfield SA. Changes in musculoskeletal structure and function with prolonged bed rest. Med Sci Sports Exerc 1997;29:197-206. CrossRef
3. Powers SK, Wiggs MP, Duarte JA, Zergeroglu AM, Demirel HA. Mitochondrial signaling contributes to disuse muscle atrophy. Am J Physiol Endocrinol Metab 2012;303:E31-9. CrossRef
4. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302-10. CrossRef
5. Zarzhevsky N, Coleman R, Volpin G, Fuchs D, Stein H, Reznick AZ. Muscle recovery after immobilisation by external fixation. J Bone Joint Surg Br 1999;81:896-901.
6. Coutinho EL, Gomes AR, França CN, Oishi J, Salvini TF. Effect of passive stretching on the immobilized soleus muscle fiber morphology. Braz J Med Biol Res 2004;37:1853-61. CrossRef
7. Tate CA, Bick RJ, Myers TD, Pitts BJ, Van Winkle WB, Entman ML. Alteration of sarcoplasmic reticulum after denervation of chicken pectoralis muscle. Biochem J 1983;210:339-44.
8. Tomanek RJ, Lund DD. Degeneration of different types of skeletal muscle fibres. I. Denervation. J Anat 1973;116:395-407.
9. Camillo AC, Rocha Rde C, Chopard RP. Structural and microvascular study of soleous muscle of Wistar rats after section of the sciatic nerve. Arq Neuropsiquiatr 2004;62:835-8. CrossRef
10. Sunderland S, Ray LJ. Denervation changes in mammalian striated muscle. J Neurol Neurosurg Psychiatry 1950;13:159-77. CrossRef
11. Tanaka T, Kariya Y, Hoshino Y. Histochemical study on the changes in muscle fibers in relation to the effects of aging on recovery from muscular atrophy caused by disuse in rats. J Orthop Sci 2004;9:76-85. CrossRef
12. Tabary JC, Tabary C, Tardieu C, Tardieu G, Goldspink G. Physiological and structural changes in the cat's soleus muscle due to immobilization at different lengths by plaster casts. J Physiol 1972;224:231-44.
13. Toraman S, Türkoğlu I. Automatic identification of selected cell areas in histopathological image. 5th International Advanced Technologies Symposium, May 13-15, 2009. Karabük, Türkiye.