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# 토끼 회전근 개 만성 파열 모델에서, 고콜레스테롤혈증이 회전근 개의 지방 변성과 유합에 미치는 영향

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**2016년 5월**


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# Effect of Hypercholesterolemia on Fatty Infiltration and Quality of Tendon-to-Bone Healing in a Rabbit Model of a Chronic Rotator Cuff Tear

by

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# Abstract

**Introduction:** The prevalence of hypercholesterolemia is rapidly increasing as the population ages. Hypercholesterolemia can result in fatty deposits in the muscle and tendon tissues, vascular endothelial cells, and liver cells. These fatty deposits may aggravate fatty infiltration of the rotator cuff muscles in rotator cuff tears; the deposit of fat or cholesterol byproducts in tendon tissues can weaken the mechanical strength of the repaired tendon and further impair healing. At present, the failure of rotator cuff healing is one of the most common complications after surgical repair, and the fatty infiltration of rotator cuff muscle is a well-known prognostic factor associated with poor anatomical and functional outcomes after rotator cuff repair. The purpose of this study was to verify the effect of hypercholesterolemia (high-cholesterol diet) on fatty infiltration of the rotator cuff muscle and the quality of tendon-to-bone healing and its reversibility by lowering the cholesterol level in a chronic tear model using the rabbit supraspinatus.

**Materials and Methods:** Forty-eight rabbits were randomly allocated into 4 groups (n = 12 each). After 4 weeks of a high-cholesterol diet (groups A and B) and a regular diet (groups C and D), the supraspinatus tendon was detached and left alone for 6 weeks and then was repaired in a transosseous manner (groups A, B, and C). Group D served as a control. Group A continued to receive the high-cholesterol diet until the final evaluation (6 weeks after repair); however, at the time of repair, group B was changed to a general diet with administration of a cholesterol-lowering agent (simvastatin, 3.0 mg/kg/day). The serum lipid levels of all rabbits were checked by extracting blood from an ear vein at

every experimental time. and, to rule out statin-induced muscle damage, the serum creatinine kinase level was checked at 10 and 16 weeks (before and after treatment with simvastatin) for Group B. Histological evaluation of the fat-to-muscle proportion at muscle portion (1 cm proximal to the musculotendinous junction) was performed twice, at the time of repair and the final evaluation, and an electromyographic (EMG) test (area under the negative compound muscle action potential), mechanical test (mode of tear, load-to-failure, and stiffness), and histological test of tendon-to-bone healing at tendon-to-bone interface (collagen fiber continuity, orientation, density, and maturation of the tendon-to-bone interface structure as well as vascularity and cellularity) were performed at the final evaluation.

**Results:** At the final evaluation, a wide dehiscence of the supraspinatus tendon repair was noted in three shoulders (two in Group A and one in Group B); these rabbits were excluded from the final analysis. After 4 weeks of a high-cholesterol diet, the serum blood cholesterol level was significantly increased in Groups A and B (total cholesterol, 1664.50 ± 770.12 mg/dL in Group A, 2186.25 ± 832.37 mg/dL in Group B, 32.41 ± 20.07 mg/dL in Group C, and 28.28 ± 9.70 mg/dL in Group D), and the serum total cholesterol level was decreased significantly to 81.65 ± 51.21 mg/dL ($p < 0.001$) after changing to a general diet with simvastatin administration in Group B. There was no significant difference in the serum creatinine kinase level before and after the use of simvastatin in Group B (serum creatinine kinase levels were 1358.75 ± 474.41IU/L and 1693.50 ±5 86.09IU/L, respectively, $p = 0.676$). For the EMG test, Group A showed a significantly smaller area of compound muscle action potential compared with Groups C and D (all $p < 0.01$), and Group B showed a larger area than Group A, almost up to the level of Group

C ($p = 0.312$). Similarly, Group A showed significantly lower mechanical properties both in load-to-failure and stiffness compared with Groups C and D (all $p < 0.05$). In addition, although not significantly different, the mechanical properties of Group B were higher than those of Group A (load-to-failure: group A=42.01N and group B=58.23, p=0.103; stiffness: group A=36.32N/mm and group B=47.22N/mm, p=0.153). For the histological test, Groups A and B showed a significantly higher fat-to-muscle proportion than Groups C and D at 6 weeks after detachment (all $p < 0.05$), but at the final evaluation, Group B showed a decreased fat-to-muscle proportion (from 64.02 ± 11.87% to 54.68 ± 10.47%; $p = 0.146$), different from Group A, which showed increased fat-to-muscle proportion (from 59.26 ± 17.80% to 78.23 ± 10.87%; $p = 0.015$). Groups B and C showed better tendon-to-bone interface structures than Group A, which showed coarse and poorly organized collagen fibers with fat interposition.

**Conclusion:** Hypercholesterolemia had a deleterious effect on fatty infiltration and the quality of tendon-to-bone repair site, and lowering hypercholesterolemia seemed to halt or reverse these harmful effects in this experimental model. Systemic diseases such as hypercholesterolemia should be tightly controlled during the perioperative period of rotator cuff repair.

**Key Words:** Hypercholesterolemia; simvastatin; fatty infiltration; tendon-to-bone healing; chronic rotator cuff tear; rabbit model

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# I. Introduction

Rotator cuff tear is a common condition that causes pain and functional disability, and more than 50% of patients over age 60 have either a partial or a full thickness rotator cuff tear, yet are asymptomatic.[59] Surgical repair of rotator cuff tears is widely practiced and has been a commonly accepted treatment for full-thickness rotator cuff tears;[20] however, failure of rotator cuff healing after surgical repair is common and is a well-known complication. Recent studies have reported an unacceptably high failure rate of 20% to 94% after surgical repair in spite of improved surgical techniques and instrumentations.[8,9,19,22,24,27,46,56] Fatty infiltration is one of the most important prognostic factors for anatomical and functional outcomes after rotator cuff repair; however, surgical repair of the torn rotator cuff tendon alone may not be able to halt or reverse the progression of fatty infiltration.[23,44]

Meanwhile, the prevalence of hypercholesterolemia (defined as a serum cholesterol concentration greater than 240 mg/dL[25]), a possible metabolic risk factor, is currently rapidly increasing due to an aging population, poor dietary habits, and lack of exercise. Hypercholesterolemia shows a similar age distribution to rotator cuff tears, with the peak ages being the 50s to the70s.[50] Hypercholesterolemia is known to result in fatty deposits in muscle and tendon tissues, as well as vascular endothelial cells or liver cells.[3,16,45] There is a possibility that this fatty deposit process may aggravate the fatty infiltration of rotator cuff muscles in rotator cuff tears, and the deposit of fat or cholesterol byproducts in tendon tissues can weaken the mechanical strength of the repaired tendon and further impair healing.[53,55] However, in spite of a possible relationship between

hypercholesterolemia and the results of rotator cuff repair, no study has evaluated the effect of hypercholesterolemia on fatty infiltration or rotator cuff healing after rotator cuff repair. In addition, even though cholesterol-lowering agents, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), are commonly prescribed to patients with hypercholesterolemia, it is unknown whether lowering the cholesterol level can affect the outcomes of rotator cuff repair.

Thus, the purpose of this study was to verify the effect of hypercholesterolemia on fatty infiltration and the quality of rotator cuff tendon-to-bone healing in a chronic rotator cuff tear model using a rabbit supraspinatus. Furthermore, we aimed to investigate whether controlling hypercholesterolemia by administrating an HMG-CoA reductase inhibitor (simvastatin) could lead to improvement of fatty infiltration and the quality of tendon-to-bone interface after rotator cuff repair. We hypothesized that hypercholesterolemia would be associated with aggravated fatty infiltration and poor rotator cuff properties of repair, and its control would have beneficial effects.

# II. Materials and Methods

All experimental procedures were approved by the Experimental Animal Committee of the Clinical Research Institute of the senior author's institute (IACUC No. BA1307-133/064-02).

## A. Allocation of rabbits (Figure 1)

Power analysis determined that eight specimens were needed per group to detect a 10% difference in fat-to-muscle proportion (α-error = 0.05, β-error = 0.2, drop-out rate = 20%) in histology and to detect a significant difference in ultimate failure load (mean difference = 90 N, standard deviation = 40 N, α-error = 0.05, β-error = 0.2, dropout rate = 20%), based on previous studies.40,54 We added another four rabbits per group for the evaluation of the quality of tendon-to-bone healing (bilaterally, eight shoulders per group). Thus, 48 rabbits (New Zealand white male rabbits weighing about 3.5 kg) were randomly allocated into four groups (12 rabbits per group): Group A (high-cholesterol diet + repair), Group B (high-cholesterol diet + repair + statin), Group C (regular diet + repair), and Group D (regular diet + sham operation, control). The left shoulders of eight rabbits per group underwent evaluation of the fat-to-muscle proportion 6 weeks after detachment (at the time of repair), and histological, mechanical, and physiological evaluation of the right shoulder was performed 6 weeks after repair of the right supraspinatus tendon (final evaluation). In addition, another four rabbits per group underwent evaluation of the

quality of tendon-to-bone healing for bilateral shoulders 6 weeks after repair of the bilateral supraspinatus tendon (final evaluation).

## B. Hypercholesterolemia rabbit model

For the hypercholesterolemia model, rabbits were fed high-cholesterol meals (Purina meals, rabbit chow mixed with 1% cholesterol and 2% corn oil)[48] (Youngbio, Korea). Prior to creating a tear model, the rabbits in Groups A and B received a high-cholesterol diet for 4 weeks,[31] and the high-cholesterol diet continued until the time of repair of the torn supraspinatus (6 weeks after creating the tear). After the repair, the rabbits in Group A continued the high-cholesterol diet until the final evaluation (6 weeks after surgical repair); however, those in Group B changed to a general diet with administration of a cholesterol-lowering agent (simvastatin, CJ, Korea, 3.0 mg/kg/day) by oral gavage to reduce the serum cholesterol level.[10] Rabbits in Groups C and D received a general diet for comparison. The rabbit hypercholesterolemia model and the effect of simvastatin to reduce the serum cholesterol level in rabbits have been well established in previous studies.[10,36,37,47] The serum lipid levels (total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides) of all rabbits were checked by extracting blood from an ear vein at the beginning of the study, at 4 weeks (when creating the tear), at 10 weeks (when conducting the repair), and at 16 weeks (at the final evaluation). In addition, to rule out statin-induced muscle damage, we checked the serum creatinine kinase level at 10 and 16 weeks (before and after treatment with simvastatin) for Group B.

## C. Surgical procedure

A lateral skin incision of the bilateral shoulders followed by omovertebral and deltoid muscle retraction exposed the supraspinatus tendon at its insertion into the greater tuberosity. The chronic tear model[43] was created by completely severing the supraspinatus tendon with a sharp scalpel from the greater tuberosity and wrapping the torn tendon with a silicone Penrose drain 10 mm in length (8 mm in outer diameter, Yushin Corp., Korea) to inhibit adhesion to the surrounding soft tissue. It was then left alone for 6 weeks. Six weeks after creation of the supraspinatus tear, the torn supraspinatus tendon was repaired with 2-0 Ticron (Tyco, Waltham, Massachusetts) in a transosseous manner after creating a bleeding bed at the footprint of the greater tuberosity. Repair procedures were as follows: we removed the Penrose drain wrapped around the supraspinatus tendon and roughened the exposed greater tuberosity with a scalpel blade. Two bone tunnels were created at the articular margin of the footprint to the lateral humeral cortex. The suture (2-0 Ticron: Tyco, Waltham, Massachusetts) was passed through the bone tunnels and tied, reattaching the supraspinatus tendon to the footprint (Figure 2). The wound was closed in layers. For the control group, a sham surgical procedure was performed through the same approach to visualize the rotator cuff without inducing any injury to the tendon. We injected meloxicam (Metacam, Boehringer Ingelheim Vetmedia, St Joseph, MO), 0.3 mg/kg IM to control pain, immediately after operation and every 24 h for 3 days postoperatively. The shoulders were not immobilized postoperatively. Rabbits were housed individually and had free access to water and food.

## D. Electromyographic (EMG) evaluation

Six weeks after repair (at the final evaluation), the compound muscle action potential (CMAP) was measured five times for the right supraspinatus muscle of each rabbit using a portable electro-stimulator (Complex Medical Systems, Ecublens, Switzerland) under anesthesia. To elicit the CMAP of the supraspinatus muscle, the suprascapular nerve area was exposed and then stimulated with a needle electrode at supramaximal intensity (1 Hz frequency, 0.1 ms stimulation duration, 30 mA or greater current). CMAPs were recorded from both an active electrode (a needle electrode placed in the supraspinatus muscle belly) and a reference electrode (a surface electrode at the supraspinatus tendon) with a filtering frequency of 10 Hz to 10 kHz, a sweep speed of 1 ms/division, and a sensitivity of 1 mV/division, using a portable 2-channel EMG/nerve conduction velocity system (Medelec Synergy; Oxford Instrument Medical Systems, Oxford, UK) as previously described (see also Figure 3).[42] From the CMAP data, the average of the areas under the negative CMAP phase was calculated as a parameter; this indicated the level of motor unit recruitment.[52] This EMG evaluation of the supraspinatus muscle of the rabbit was not fully validated. However, the successful EMG evaluation of the rabbit subscapularis muscle was previously well reported.[42]

## E. Biomechanical evaluation

After EMG evaluation, the rabbits were fully anesthetized and euthanized with carbon dioxide, and the entire supraspinatus muscle and tendon of the right shoulder along with

the humeral head of each rabbit were harvested. The muscle portion from 1 cm proximal to the musculotendinous junction was used for histological analysis, and the tendon along with the humeral head was used for mechanical evaluation. The mechanical evaluation parameters were the mode of tear, the load-to-failure, and stiffness, measured at a rate of 1 mm/s with a preload of 5 N after five consecutive preconditioning trials (from 5 N to 50 N at a loading rate of 15 N/s), using a custom fixture clamping system and an Instron materials testing machine (Instron 5565A, Norwood, MA). The supraspinatus tendon was fixed to this system along its anatomical direction to allow tensile loading and tendon-to-bone interface, forming a right angle (Figure 4). The data of tensile load to failure were automatically collected with a PC based data acquisition system.

## F. Histological evaluation

At the time of repair for the eight right shoulders and four bilateral shoulders (6 weeks after creating the tear model), the supraspinatus muscles of the eight left shoulders were harvested and sliced transversely 1 cm proximal to the musculotendinous junction. Specimens were fixed in neutral buffered 10% formalin (pH 7.4), and paraffin blocks were made. Sections 5 μm thick were cut in the transverse plane and stained with hematoxylin and eosin. Using these samples, the fat-to-muscle proportions were assessed using image analyzing software (Image-Pro Plus, Media Cybernetics Inc., Maryland) under ×100 magnification. The amount of fatty infiltration was calculated by subtracting the area of the stained muscle and connective tissue from the total area, and the proportion of fatty infiltration (fat-to-muscle proportion) was calculated by dividing the

amount of fat by the total area. Each slide was examined for a total of five scanned sections per slide, and the average was used for the analysis. At the final evaluation (6 weeks after repair), the same procedures were performed in the eight right supraspinatus muscles.

At the final evaluation, histological evaluation of the quality of tendon-to-bone healing in both shoulders of another four rabbits per group was performed. Using the same method, specimens from the repair site of the supraspinatus tendon and greater tuberosity were fixed in neutral buffered 10% formalin and decalcified, and paraffin blocks were made. Sections 5 μm thick were cut in the coronal plane and stained with hematoxylin and eosin and Masson trichrome. The collagen fiber continuity, orientation, density, and maturation of the tendon-to-bone interface structure as well as vascularity and cellularity were assessed. Each of items was graded semi-quantitatively into 4 stages (grade (G) 0, 1, 2, and 3). For the items of collagen fiber continuity and collagen fibers oriented parallel, we divided their stages by percentage: present with <25% of proportion, 25-50% proportion, 50-75% proportion, and >75% proportion, which corresponded to G0, 1, 2, and 3, respectively. The item of collagen fiber density was graded as very loose, loose, dense, and very dense, which corresponded to G0, 1, 2, and 3, respectively, and that of maturation of the tendon-to-bone interface structure as poorly organized, mildly organized, moderately organized, and marked organized, which also corresponded to G0, 1, 2, and 3, respectively. In addition, for the items of vascularity and cellularity, we graded them as absent or minimally present, mildly present, moderately present, and severe or markedly present, which also corresponded to G0, 1, 2, and 3, respectively. A score of 1 to 4 was given to each grade for the later statistical analysis (1 for G0, 2 for G1,

3 for G2, and 4 for G3). All examinations were performed in a randomized and blinded fashion to eliminate observer bias by a 7-year trained pathologist (J.Y.K.), who was blinded to group assignment. An Eclipse Ni-U microscope (Nikon, Tokyo, Japan) was used, and images were captured and acquired using a Nikon DS-Ri1, and NIS Elements F4.00.00 (version 4.0) acquisition software.

## G. Statistics

Data were evaluated using an SPSS software package (version 15.0, SPSS Inc., Chicago, IL), and $p < 0.05$ was considered to be statistically significant. Kruskal-Wallis testing, followed by post-hoc Mann-Whitney U testing with a Bonferroni correction for multiple comparisons, was performed to evaluate if differences existed between groups for the data from the EMG, biomechanical, and histological testing of the fat-to-muscle proportion and the tendon-to-bone interface histology.

# III. Results

Eight rabbits (four in Group A, three in Group B, and 1 in Group C) showed a deep infection with pus discharge at the time of harvest, and another five rabbits died within a few days after the operation of unknown causes (two in Group A, two in Group B, and one in Group C). This number was unacceptable for further assessment; thus, we repeated the experiments from the beginning with 13 additional rabbits with more sterile and careful processes. No further loss due to infection occurred. At the final evaluation, a wide dehiscence of the supraspinatus tendon repair was noted in three shoulders (two in Group A and one in Group B); these rabbits were excluded from the final analysis. Specifically, one in Group A and one in Group B were excluded from the EMG and biomechanical tests, and another one in Group A was excluded from the histology of tendon-to-bone interface.

## A. Blood test (Table 1)

The hypercholesterolemic rabbit model is well established. After 4 weeks of a high-cholesterol diet, the serum blood cholesterol level was significantly increased in Groups A and B (total cholesterol, 1664.50 ± 770.12 mg/dL in Group A, 2186.25 ± 832.37 mg/dL in Group B, 32.41 ± 20.07 mg/dL in Group C, and 28.28 ± 9.70 mg/dL in Group D); however, there was no significant difference between groups A and B ($p = 0.372$). The serum total cholesterol level was decreased significantly to 81.65 ± 51.21 mg/dL ($p <$

0.001) after changing to a general diet with simvastatin administration in Group B. There was no significant difference in the serum creatinine kinase level before and after the use of simvastatin in Group B (serum creatinine kinase levels were 1358.75 ± 474.41IU/L and 1693.50 ±5 86.09IU/L, respectively, p = 0.676). The normal range of rabbit serum blood cholesterol is known to be below 110 mg/dL,[29] and the normal range of rabbit serum creatinine kinase is between 65 and 5583 IU/L.[57]

## B. EMG evaluation

The area under the negative phase of CMAP of Group A (6.69 ± 2.23 ms·mV) was significantly smaller compared with Group C (10.50 ± 2.96 ms·mV, p = 0.008). Even though the difference was not statistically significant (p = 0.112), Group B (9.05 ± 3.23 ms·mV) showed a larger area than Group A, almost to the level of Group C (p = 0.312). The areas of all experimental groups (Groups A, B, and C) were significantly smaller than the control group (Group D, 14.40 ± 2.79 ms·mV; all $p < 0.05$; see also Figure 5 and Table 2).

## C. Biomechanical evaluation

There was no distinct difference in the composition of the tear mode between the groups, except a relatively high incidence of mid-substance tears in Groups A and D (insertional tear: mid-substance tear was 3:4 in Group A, 4:3 in Group B, 5:3 in Group C, and 3:5 in Group D). In the tensile test, load-to-failure and stiffness showed a similar trend. Group A

showed significantly lower load-to-failure and less stiffness (42.0 ± 13.8 N and 36.3 ± 14.7 N/mm) compared with Group C (65.1 ± 22.8 N and 65.3 ± 23.2 N/mm, $p = 0.02$ and $p = 0.006$, respectively). Even though there was no statistically significant difference, the load-to-failure and stiffness of Group B (58.2 ± 22.4 N and 47.2 ± 14.1 N/mm) was higher than those of Group A ($p = 0.103$ and $p = 0.153$, respectively). Group D (normal control) showed a much higher load-to-failure (148.0 ± 26.1 N) than any other group (all $p < 0.001$; Figure 6 and Table 2).

## D. Histological evaluation

At the time of repair (6 weeks after detachment), Groups A and B showed a significantly higher fat-to-muscle proportion (Group A, 59.3 ± 17.8%; Group B, 64.0 ± 11.9%) compared with Groups C (44.3 ± 7.9%, $p = 0.044$ and $p = 0.004$, respectively) and D (8.0 ± 5.3%, all $p < 0.001$). However, at the final evaluation (6 weeks after repair), Group B showed a decreased fat-to-muscle proportion (from 64.0 ± 11.9% to 54.7 ± 10.5%; $p = 0.146$), different from Group A which showed an increased fat-to-muscle proportion (from 59.3 ± 17.8% to 78.2 ± 10.9%; $p = 0.015$) (Figure 7).

The result of this semi-quantitative grading for the tendon-to-bone interface histology was shown in Table 3. The Kruskal-Wallis testing for each of items revealed that there were significant differences in the collagen fiber continuity, orientation, density, and the maturation of the tendon-to-bone interface structure (all $p<0.001$), but no differences in the vascularity and cellularity ($p=0.161$ and 0.538, respectively). The results of the post-hoc Mann-Whitney U testing for each of items were as follows (Table 4): The collagen

fiber continuity, orientation, density, and the maturation of the tendon-to-bone interface structure were poorer in group A compared with group C ($p=0.009$, 0.029, 0.002, and 0.001, respectively). In addition, group B showed superior collagen fiber density and the maturation of the tendon-to-bone interface structure compared with group A ($p=0.029$ and 0.040, respectively). There were no differences in the vascularity and cellularity between each group (all $p>0.05$). Group A showed coarse, poorly organized collagen fibers with fat interposition in the tendon-to-bone interface area (Figure 8). Fat interposition was also found in the mid-portion of the tendons of Group A (Figure 9). Even though the collagen fiber arrangements were still rough and irregular, Groups B and C showed a more organized structure with a higher collagen fiber density than Group A, and more collagen fibers bridged the interface. These differences were somewhat more distinct in Group C, which showed a little better collagen organization with a healthier parallel arrangement of reparative collagen bundles than Group B. However, it was still clearly different from Group D (normal control), which showed dense regular collagen fibers with complete continuity to the bone, and typical mature 4-zone formation was not acquired in any of the experimental groups (Groups A, B, or C). Cellular and vascular fibrous tissues at the tendon-to-bone interface were not evident in all groups (Figure 8).

**Table 1.** The serum total cholesterol and creatinine kinase level

| | | Group A | Group B | Group C | Group D |
|---|---|---|---|---|---|
| Serum total cholesterol (mg/dL) | *Week 0 | 46.37 ± 23.70 | 40.71 ± 21.32 | 46.58 ± 20.55 | 43.28 ± 8.55 |
| | Week 4 | 1664.50 ± 770.12 | 2186.25 ± 832.37 | 32.41 ± 20.07 | 28.28 ± 9.70 |
| | †Week 10 | 2125.00 ± 465.61 | 2777.50 ± 680.37 | 23.16 ± 8.34 | 20.85 ± 5.66 |
| | Week 16 | 2523.28 ± 1153.34 | 81.65 ± 51.21 | 23.83 ± 9.97 | 20.00 ± 8.60 |
| Serum creatinine kinase (IU/L) | †Week 10 | | 1358.75 ± 474.41 | | |
| | Week 16 | | 1693.50 ± 586.09 | | |

*The high-cholesterol diet was started for the group A and B on week 0.
†At week 10, group B was changed to a general diet with administration of a cholesterol-lowering agent (simvastatin), however, group A continued to receive the high-cholesterol diet until the final evaluation.

After 4 weeks of a high-cholesterol diet, the serum blood cholesterol level was significantly increased in Groups A and B compared with groups C and D (all $p < 0.001$), however, there was no significant difference between group A and B ($p = 0.372$).

In group B, the serum total cholesterol level was significantly decreased ($p < 0.001$), after changing to a general diet with simvastatin administration, however, there was no significant difference in the serum creatinine kinase level before (week 10) and after (week 16) the use of simvastatin ($p = 0.676$).

**Table 2.** The results of electromyographic and biomechanical tests

| | Group A (n = 7) | Group B (n = 7) | Group C (n = 8) | Group D (n = 8) |
|---|---|---|---|---|
| Compound muscle action potential (area under curve, ms·mV) | 6.69 ± 2.23 | 9.05 ± 3.23 | 10.50 ± 2.96 | 14.40 ± 2.79 |
| Insertional tear : mid-substance tear | 3:4 | 4:3 | 5:3 | 3:5 |
| Load to failure (N) | 42.01 | 58.23 | 65.12 | 148.01 |
| Stiffness (N/mm) | 36.32 | 47.22 | 65.31 | 62.51 |

*The electromyographic test was performed using a portable 2-channel electromyography/nerve conduction velocity system, and the biomechanical test was performed using a custom fixture clamping system and an Instron materials testing machine

**Table 3.** The result of this semi-quantitative grading for the tendon-to-bone interface histology

| **Items** | Group A (n=7) | | | | Group B (n=8) | | | | Group C (n=8) | | | | Group D (n=8) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G 0 | G 1 | G 2 | G 3 | G 0 | G 1 | G 2 | G 3 | G 0 | G 1 | G 2 | G 3 | G 0 | G 1 | G 2 | G 3 |
| Collagen fiber continuity | 4 | 3 | 0 | 0 | 1 | 5 | 2 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 8 |
| Collagen fibers oriented parallel | 6 | 1 | 0 | 0 | 3 | 4 | 1 | 0 | 2 | 3 | 3 | 0 | 0 | 0 | 0 | 8 |
| Collagen fiber density | 5 | 2 | 0 | 0 | 1 | 5 | 2 | 0 | 0 | 3 | 4 | 1 | 0 | 0 | 0 | 8 |
| Maturation of the tendon-to-bone interface structure | 6 | 1 | 0 | 0 | 2 | 5 | 1 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 8 |
| Vascularity | 5 | 2 | 0 | 0 | 5 | 2 | 1 | 0 | 4 | 3 | 1 | 0 | 8 | 0 | 0 | 0 |
| Cellularity | 6 | 1 | 0 | 0 | 7 | 1 | 0 | 0 | 6 | 2 | 0 | 0 | 8 | 0 | 0 | 0 |

G, grade

**Table 4.** The result of multiple comparisons using post-hoc Mann-Whitney U testing with Bonferroni correction for each of items of the tendon-to-bone interface histology

| **Collagen fiber continuity** | Group A | Group B | Group C | Group D |
|---|---|---|---|---|
| Group A | | p=0.072 | p=0.009 | p<0.001 |
| Group B | | | p=0.328 | p<0.001 |
| Group C | | | | p<0.001 |
| Group D | | | | |
| **Collagen fibers oriented parallel** | Group A | Group B | Group C | Group D |
| Group A | | p=0.121 | p=0.029 | p<0.001 |
| Group B | | | p=0.382 | p<0.001 |
| Group C | | | | p<0.001 |
| Group D | | | | |
| **Collagen fiber density** | Group A | Group B | Group C | Group D |
| Group A | | p=0.029 | p=0.002 | p<0.001 |
| Group B | | | p=0.130 | p=0.002 |
| Group C | | | | p<0.001 |
| Group D | | | | |
| **Maturation of the tendon-to-bone interface structure** | Group A | Group B | Group C | Group D |
| Group A | | p=0.040 | p=0.001 | p<0.001 |
| Group B | | | p=0.105 | p<0.001 |
| Group C | | | | p<0.001 |
| Group D | | | | |
| **Vascularity** | Group A | Group B | Group C | Group D |
| Group A | | P=0.694 | p=0.463 | p=0.397 |
| Group B | | | p=0.721 | p=0.234 |
| Group C | | | | p=0.105 |
| Group D | | | | |
| **Cellularity** | Group A | Group B | Group C | Group D |
| Group A | | P=0.955 | p=0.779 | p=0.694 |
| Group B | | | p=0.721 | p=0.721 |
| Group C | | | | p=0.442 |
| Group D | | | | |

**Figure 1.** Study flowchart. EMG, electromyographic.

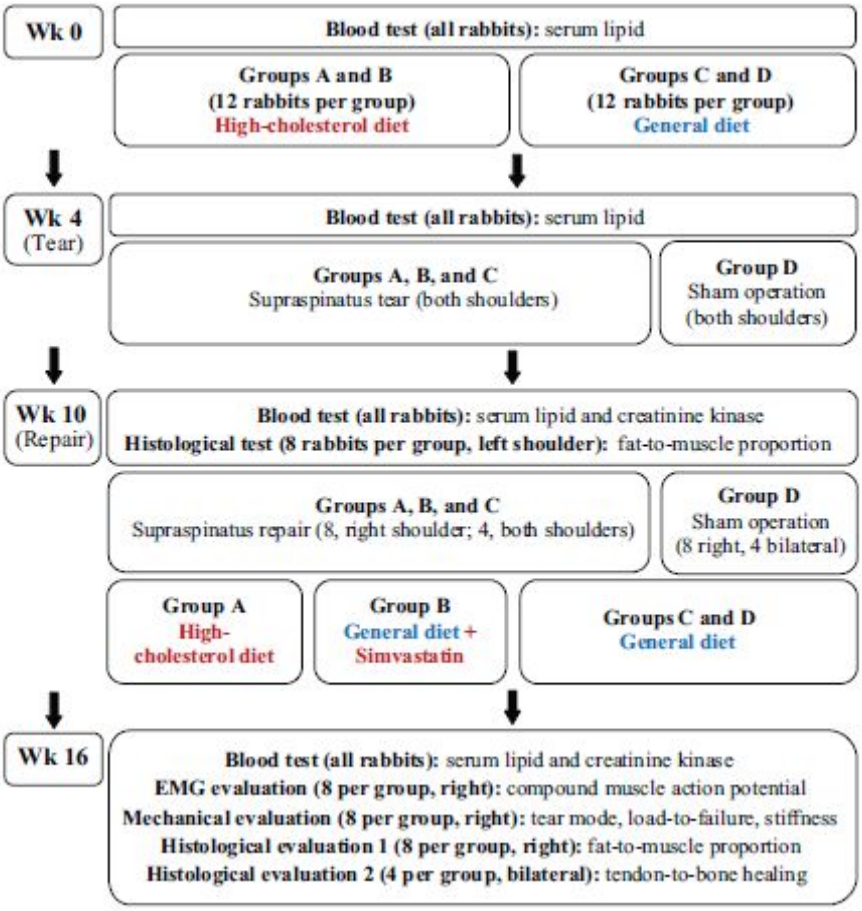


**Figure 2.** The repair procedure of the torn supraspinatus tendon in a transosseous manner is shown. Two bone tunnels were created at the articular margin of the footprint to the lateral humeral cortex. The suture was passed through the bone tunnels and tied, reattaching the supraspinatus tendon to the footprint.

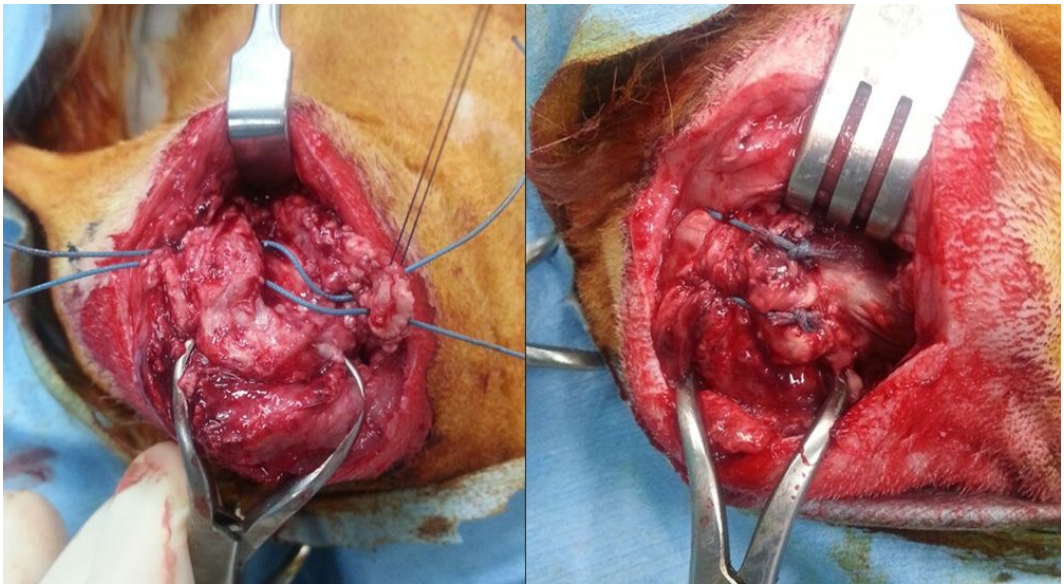


**Figure 3.** The electromyographic evaluation procedure is shown. The supraspinatus nerve area is stimulated with a needle electrode (white arrow), and the compound muscle action potential is recorded from an active needle electrode (black arrow) and a reference surface electrode (curved arrow).

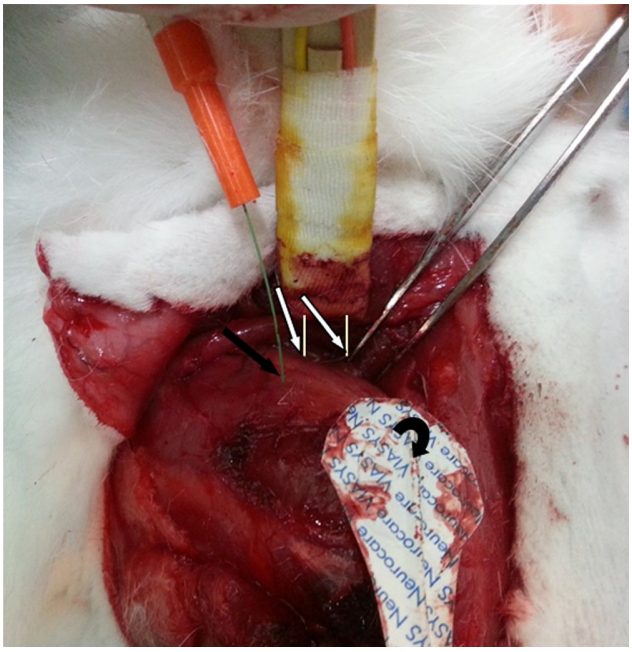


**Figure 4.** The custom fixture clamping system attached to the material testing machine is shown. The supraspinatus tendon was fixed to this system along its anatomical direction to allow tensile loading and tendon-to-bone interface, forming a right angle.

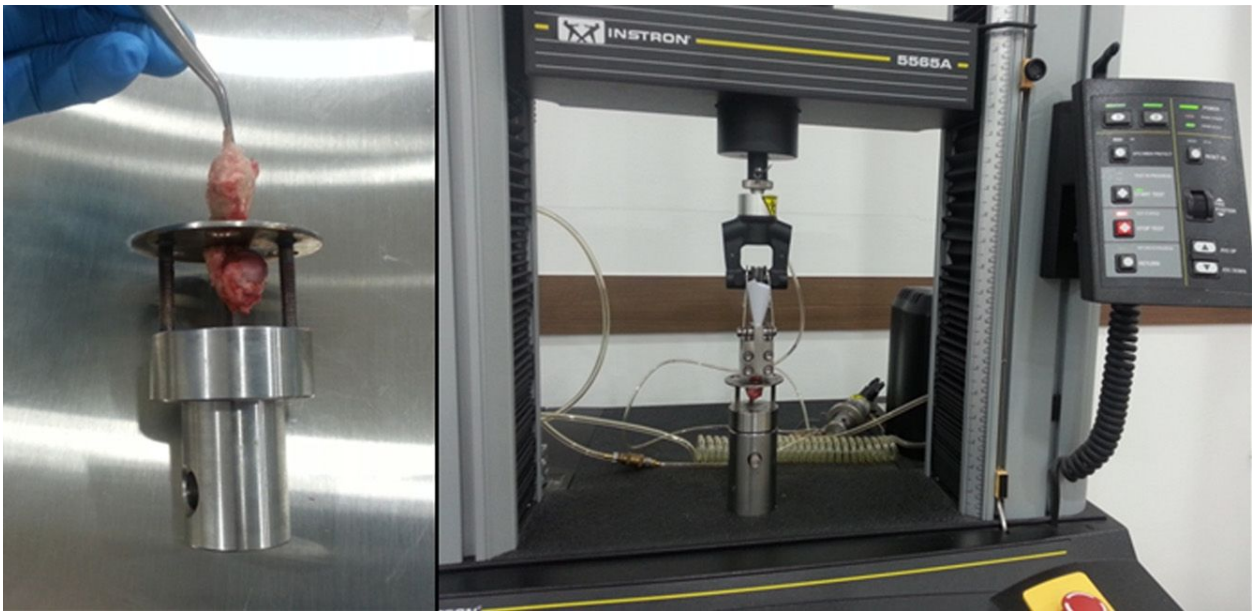


**Figure 5.** Areas under the negative phase of compound muscle action potential (CMAP) of each group during the electromyographic evaluation are shown. The area under the negative phase of CMAP of Group A was significantly smaller than that of Group C ($p = 0.008$). Even though there was no statistically significant difference ($p = 0.112$), Group B showed a larger area than Group A, almost to the level of Group C. The areas of all experimental groups (Groups A, B, and C) were significantly smaller than those of the control group (Group D, $p < 0.05$).

*Statistically significant

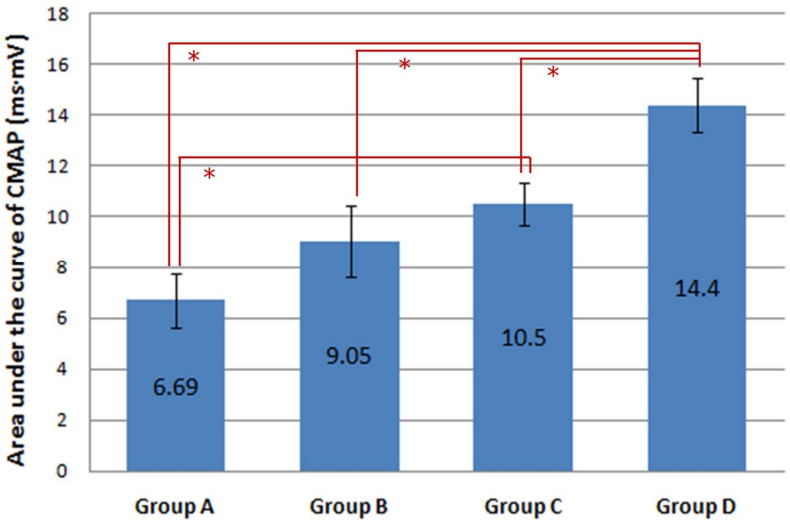


**Figure 6.** (A) Load-to-failure and (B) stiffness on biomechanical evaluation. Group A showed significantly lower load-to-failure and less stiffness than Group C ($p = 0.02$ and $p = 0.006$, respectively). Even though there was no statistical significance ($p = 0.103$ and $p = 0.153$, respectively), the load-to-failure and stiffness of Group B was higher than those of Group A. Group D (normal control) showed a much higher load-to-failure than any other group (all $p < 0.001$).

*Statistically significant

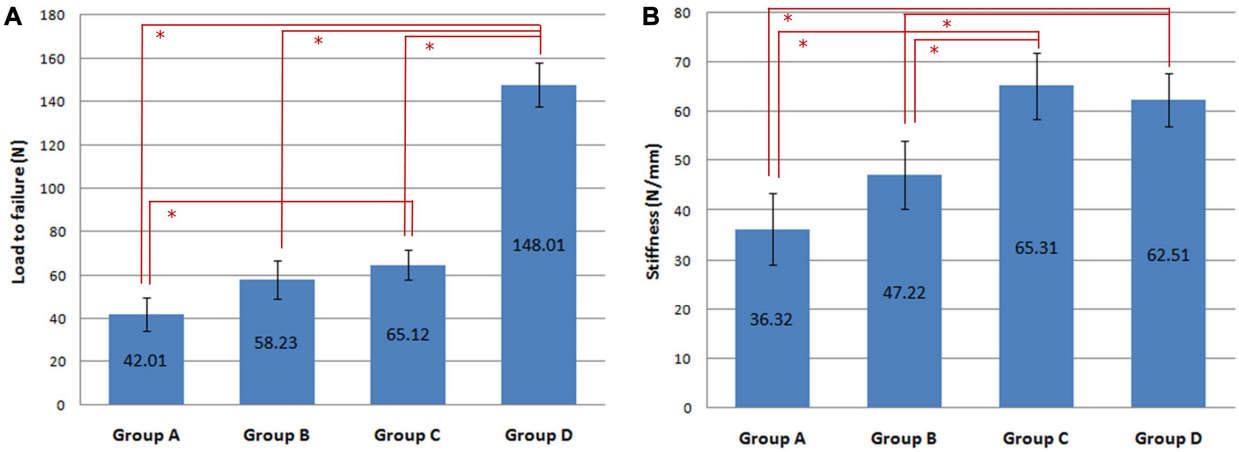


**Figure 7.** Fatty infiltration of the supraspinatus muscle of each group at the time of repair (6 weeks after detachment) and at final evaluation (6 weeks after repair) in hematoxylin and eosin stain (×100). At the time of repair, Groups A and B showed a significantly higher fat-to-muscle proportion (Group A, 59.26 ± 17.80%; Group B, 64.02 ± 11.87%) than Groups C (44.26 ± 7.85%, $p = 0.044$ and $p = 0.004$, respectively) and D (8.02 ± 5.29%, all $p < 0.001$). However, at the final evaluation, Group B showed a decreased fat-to-muscle proportion (from 64.02 ± 11.87% to 54.68 ± 10.47%; $p = 0.146$), different from Group A, which showed an increased fat-to-muscle proportion (from 59.26 ± 17.80% to 78.23 ± 10.87%; $p = 0.015$).

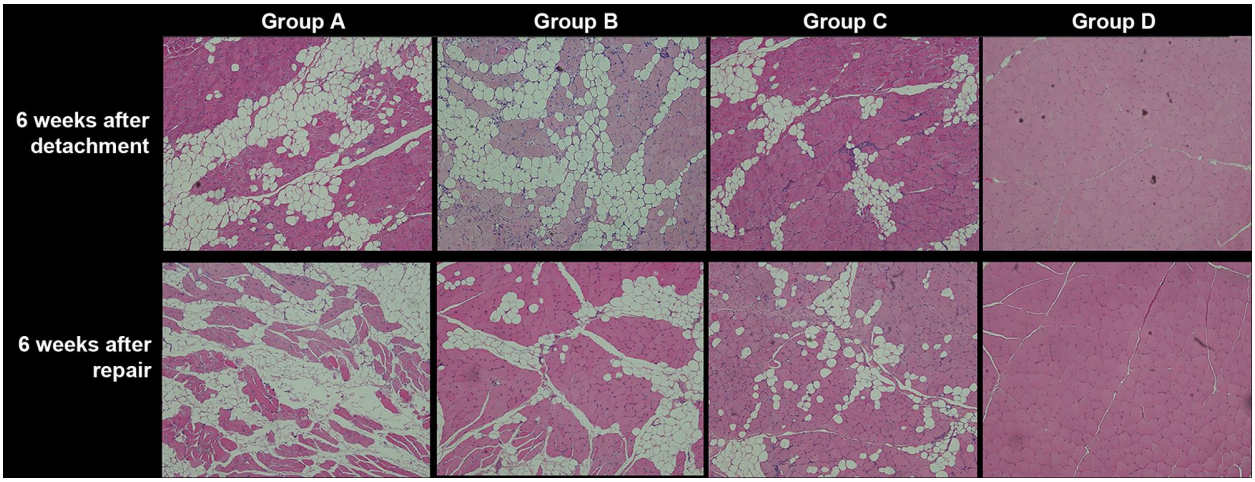


**Figure 8.** Histology of the tendon-to-bone insertion site of each group on Masson trichrome stain (×100). Group A showed coarse, poorly organized collagen fibers with fat interposition in the tendon-to-bone interface area. Even though the collagen fiber arrangements were still rough and irregular, Groups B and C showed a more organized structure with a higher collagen fiber density than Group A, and more collagen fibers bridged the interface. These differences were somewhat more distinct in Group C, which showed a little better collagen organization with a healthier parallel arrangement of reparative collagen bundles than Group B. However, it was still clearly different from Group D (normal control), which showed dense regular collagen fibers with complete continuity to the bone, and typical mature 4-zone formation was not acquired in any of the experimental groups (Groups A, B, or C). Cellular and vascular fibrous tissues at the tendon-to-bone interface were not evident in all groups.

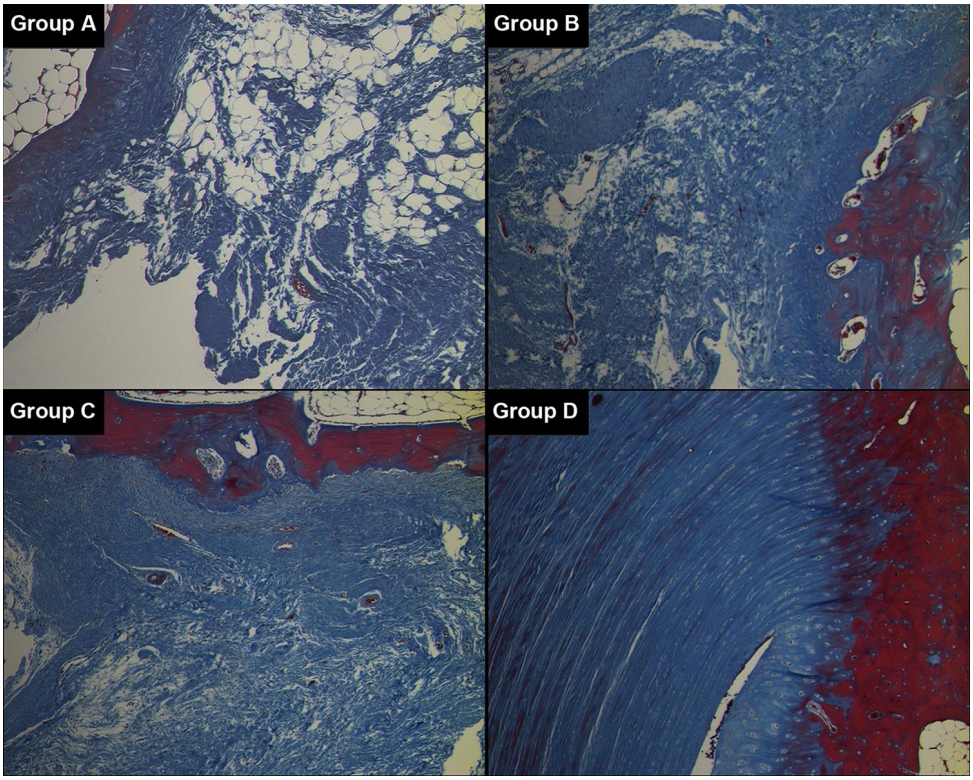
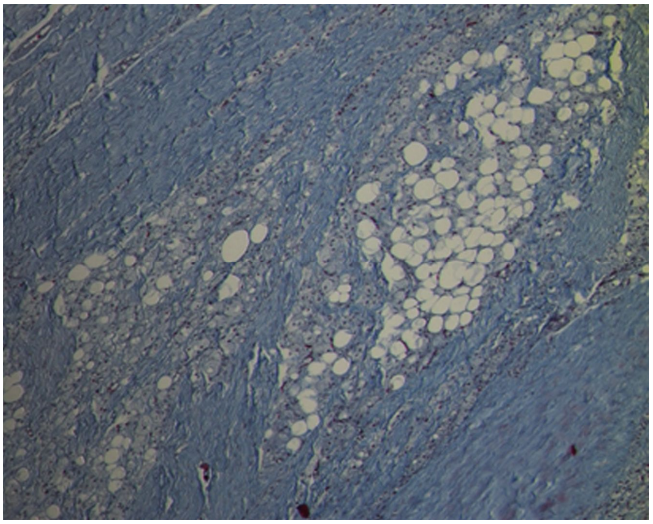


**Figure 9.** Fatty interposition is also evident in the tendon proper of Group A.



# IV. Discussion

In this study, we found notable fatty infiltration in the rotator cuff muscles after rotator cuff tendon detachment in hypercholesterolemia, and the fatty infiltration was aggravated even after repair of the torn tendon if the rabbit was exposed to a persistent hypercholesterolemic condition. Several authors have suggested a possible relationship between hypercholesterolemia and fatty infiltration of the muscle. Esenkaya et al.[15] suggested that hyperlipidemia and insulin resistance were associated with fat deposition in the muscles, and similarly Durheim et al.[14] reported that adipose tissue stored in ectopic locations outside the subcutaneous tissue, such as in the muscle, would be linked with increased total cholesterol level. In addition, Bath et al.[5] showed xanthomatous fatty infiltration within the rotator cuff muscles resulting from hyperlipidemia.

One explanation could be that this metabolic process of fat deposition due to hypercholesterolemia may exacerbate fatty infiltration of the rotator cuff muscle in patients with rotator cuff tears, and this vicious cycle does not seem to halt or reverse even after repair if the hypercholesterolemia persists. In severely fat-infiltrated rotator cuff muscles, worse EMG or electrophysiological results, as evidenced by a smaller area under the curve in CMAP in this study, may be natural. Kertesz et al.[33] demonstrated that diet-related hypercholesterolemia deteriorated the electrophysiological activity of a rabbit myocardium. Although their detailed evaluation method was different from the current one, it seems to be evident that hypercholesterolemia may impair the electrophysiological activity of the affected muscle. The CMAP reports the sum of a group of almost simultaneous action potentials from several muscle fibers in the same area, evoked by

stimulation of the supplying motor nerve, and this represents the level of recruitment of functioning muscle fiber.[21] From the EMG evaluation of the supraspinatus muscle, we were able to assess the functional effect of hypercholesterolemia-induced fatty infiltration on the repaired supraspinatus muscle.

Furthermore, fat deposition was found even in the tendon substance of hypercholesterolemic rabbits, and poorer maturation of the tendon-to-bone interface and mechanical properties were associated with hypercholesterolemia in this study. This finding is consistent with the previous report of Jozsa et al.[30] that lipid droplets can be deposited along collagen fibers and disrupt the interaction of the fiber network in the pathogenesis of tendolipomatosis, making it more prone to tendon rupture or injury. Even though there is some controversy,[32,39] the deleterious effect of hypercholesterolemia on tendons has been reported. Ozgurtas et al.[45] showed that the concentration of serum lipids is higher in patients with Achilles tendon rupture than in controls, and similarly, Von Bahr et al.[55] showed that the esterified fraction of cholesterol is elevated in biopsies from degenerated Achilles tendons. Moreover, Abboud et al.[1] demonstrated that total cholesterol and triglyceride concentrations are higher in patients with rotator cuff tears. Thus, we can guess that lipid deposits in tendon tissue may weaken the mechanical strength of the repaired tendon, as verified by previous studies.[6,7]

Other authors have reported similar results with worse mechanical properties of the repaired rotator cuff tendon in rabbits with uncontrolled hypercholesterolemia. When considering the biomolecular process, we think that cholesterol deposition inside the tendons could initiate and maintain persistent inflammation, which may cause chronic tendon degeneration and weakness.[6] Several pro-inflammatory cytokines, such as

interleukin-6 or tumor necrosis factor alpha, which could be released from the accumulated macroscopic or microscopic fatty tissue, may negatively affect tendon health and further inhibit tendon-to-bone healing.[18,60] From these studies, we can speculate that hypercholesterolemia may similarly worsen the healing of the repaired rotator cuff tendon. The occurrence of a wide dehiscence and poor maturation of the tendon-to-bone interface with poor mechanical properties in rabbits with hypercholesterolemia predicts a low healing potential in uncontrolled hypercholesterolemia. The local collagen degradation accompanying hypercholesterolemia[49] or reduced cell replication elicited by the wound in hyperlipidemia[17] may be further explanations for the worsened healing observed in hypercholesterolemia. Moreover, the impairment of micro- and macro-circulation by hypercholesterolemia may affect rotator cuff healing by inducing ischemia at the critical zone of the rotator cuff tendon or repair site.[38]

We do not know exactly how hypercholesterolemia exacerbates fatty infiltration of the torn rotator cuff muscle or tendon. However, we think that certain pathways of fat accumulation processes may be accelerated by uncontrolled hypercholesterolemia, which are manifested as lipid accumulation in existing adipocytes, proliferation of adipocytes, or differentiation of pluripotent cells of myoblastic or fibroblastic lineage toward adipocytes.[41] Several molecular pathways of adipogenesis such as peroxisome proliferator-activated receptors or Bmp-receptor 1a signaling may be more upregulated under hypercholesterolemic conditions.[28,51] In addition, trauma to the tendon and muscle during surgery and the inflammatory condition created by surgery may aggravate these adipogenic processes. Moreover, the mechanical unloading condition caused by detachment of the rotator cuff tendon could be a further accelerating factor for the

upregulation of adipogenic genes in the rotator cuff muscle.[34] Further biomolecular studies to establish molecular pathways by detecting accelerated adipogenic gene markers in hypercholesterolemia may be needed as a next step of this study.

The fatty infiltration of the rotator cuff muscle did not reverse after repair but instead worsened if hypercholesterolemia persisted. However, in the current study, we first showed the possibility of fatty infiltration reversal, if the cholesterol level was lowered by diet control or statin medication. In addition, the EMG and biomechanical properties and the condition of the tendon-to-bone interface were shown to be superior in hypercholesterolemia-controlled rabbits (Group B) compared with those with persistent hypercholesterolemia.

The dietary effect of hypercholesterolemia control and the resultant diminishing lipid depositions have been previously suggested.[26] Hadjiisky et al.[26] showed that changing from a hyperlipidemic diet to a normal one caused serum cholesterol levels to revert to normal values and initiated the disappearance of intima lipoidosis from rat aortas. In addition to diet control, concomitant cholesterol lowering medications, such as statins, seem to aid in decreasing serum cholesterol levels and lipid depositions. Yamamoto et al.[58] and Lee et al.[35] similarly showed the effect of cholesterol-lowering medications on xanthoma regression. We think that the effect of this reduction of fatty infiltration by cholesterol lowering could have positive results on the electrophysiological and biomechanical properties of the repaired rotator cuff muscle and tendon. In addition, the inflammatory action of lipid deposition, which is associated with degeneration and weakness of the tendon and muscle,[6] can be decreased by lowering the cholesterol level, which may positively affect the muscle and tendon health.

Previously, Aikawa et al.[2] showed that lipid lowering could reduce the expression of catabolic proinflammatory factors such as matrix metalloproteinases -3 and -9. Moreover, the beneficial pleiotropic effects of statins, other than their cholesterol lowering actions, may improve to some degree the electrophysiological and biomechanical properties and tendon-to-bone healing.[11-13] Di Napoli et al.[12] demonstrated the anti-ischemic and anti-apoptotic effects of simvastatin on rat cardiac myocytes via nitric oxide mechanisms, and Dolkart et al.[13] showed that statins enhanced tendon healing by stimulating tenocyte proliferation, migration, and adhesion via prostaglandin E2 receptor 4 signaling.

The present study was the first to demonstrate the effect of hypercholesterolemia on fatty infiltration and the quality of tendon-to-bone healing and the possibility of reversing fatty infiltration and therefore improving the electrophysiological, mechanical, and biological properties by lowering cholesterol via diet change and statin medication. Nevertheless, this study has several limitations that require consideration. First, this study was an animal study. The different anatomy, different injury and healing reactions, and the different lipid metabolism between humans and rabbits limit generalization of the results. In addition, the condition of extremely high total cholesterol levels in the hypercholesterolemic rabbits in this study is somewhat different from real clinical situations; thus, we have to be cautious in the interpretation of the results. Second, the side effects of statins, such as myositis or rhabdomyolysis, may affect the results, even though these are very rare complications.[4] However, as no rabbits showed extraordinarily increased serum creatinine kinase (muscle enzyme) levels after statin administration, we could rule out the occurrence of stain-induced myositis or rhabdomyolysis. Third, we

investigated the change of fatty infiltration and the status of rotator cuff tendon-to-bone repair, only at the early to mid-term period up until 6 weeks after repair, and we had no information on further changes in the late postoperative period. Although we successfully improved results in Group B compared with Group A by lowering the cholesterol level, there could be more mechanical and biological changes after 6 weeks. Fourth, the level of pain and stress of rabbits which may be associated with the hypercholesterolemia and surgery would affect the results, but this factor was not considered in the present study. Fifth, rabbits were not isogenic. The individual differences of each rabbit may affect the results. The isogenic animals would be better to improve comparability between the groups. Finally, the molecular pathway involved in the exacerbation of fatty infiltration in uncontrolled hypercholesterolemia was not identified in this study and that should be the next step in future research.

# V. Conclusion

In conclusion, hypercholesterolemia resulted in a deleterious effect on fatty infiltration and the quality of tendon-to-bone healing assessed by EMG, biomechanical, and histological evaluation, and controlling hypercholesterolemia seemed to be able to halt or reverse these harmful effects to some degree even after rotator cuff repair surgery. Considering that hypercholesterolemia is a very prevalent condition in adults but controllable by dietary treatment, exercise, or medication, we believe that the findings of the current study may have a far-reaching impact on the treatment of rotator cuff tears.

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# 국문초록

**서론:** 고콜레스테롤혈증은 높은 빈도를 보이며 빠르게 증가하는 대사 질환으로, 혈관내피세포나 간세포 이외에 근육이나 힘줄 조직에도 지방의 침착을 유발할 수 있음이 알려져 있다. 이러한 고콜레스테롤혈증에서의 지방 침작은 회전근 개 파열에서 발생하는 회전근 개 근육 조직의 지방 침착을 더욱 악화시킬 수 있는 개연성이 있으며, 봉합된 회전근 개 힘줄의 기계적 강도를 약화시키고 나아가 회전근 개 봉합의 유합 실패를 초래할 가능성이 있다. 회전근 개 유합 실패는 회전근 개 봉합 수술 후 발생하는 가장 대표적인 합병증이고, 이러한 회전근 개 유합 실패에 회전근 개 근육의 지방 침착이 매우 중요한 영향을 미침이 잘 알려져 있다. 본 연구에서는 만성 토끼 회전근 개 파열 모델에서 고콜레스테롤혈증이 회전근 개 근육의 지방 변성과 회전근 개 봉합술 후 힘줄과 뼈의 유합에 미치는 영향에 대해 알아보고자 하였다.

**재료 및 방법:** 48마리의 토끼를 A, B, C, D 각 그룹당 12마리씩 무작위 배정하여, 그룹 A, B는 고콜레스테롤 식이를, 그룹 C, D는 일반 식이를 시행하였다. 4주 뒤 그룹 A, B, C 토끼의 극상건을 절단하고 6주간 방치하여 만성 회전근 개 파열 모델을 만들었다. 그룹 D는 파열을 만들지 않는 대조군으로 하였다. 극상건 절단 후 6주 뒤 다시 건 봉합술을 시행하는데, 그룹 A는 마지막 평가 때까지 고콜레스테롤 식이를 유지하지만, 그룹 B는 봉합 시행 시점부터 일반 식이로 전환함과 동시에 simvastatin(3.0 mg/kg/day)를 써서 고콜레스테롤혈증의 조절을 시행하였다. 매 검사 시기마다 혈중 지질 농도를 측정하고,

simvastatin 유도성 근육 손상에 의한 영향을 배제하기 위해 그룹 B에서는 simvastatin 투여 전후 시기에 혈중 creatinine kinase 농도를 측정하였다. 결과 평가를 위해 조직학적 평가, 생역학적 평가, 전기생리학적 평가를 모두 시행하였다. 지방 침착 정도의 조직학적 평가는 봉합 시행시와 최종 평가시 (봉합 후 6주) 두 차례에 걸쳐 시행하며, 근-건 결합부 1cm 근위부의 근육 부분에서 평가하였다. 기타 회전근 개 건-골 유합의 조직학적 평가(건-골 이행부의 콜라겐 섬유의 연결성, 방향 및 밀집도, 건-골 이행부의 성숙도, 혈관 밀집도 및 세포 밀집도), 생역학적 평가(파열 부위, 최대 파열 강도 및 강성도), 전기생리학적 평가(복합근육활동전위)는 봉합 후 6주 최종 평가시 시행하였다.

**결과:** 최종 평가시기에 3마리의 토끼에서(그룹 A에서 2마리와 그룹 B에서 1마리) 회전근 개 건의 봉합 부위에서 건이 완전히 떨어져 있었고, 이들은 평가를 진행할 수 없어 최종 평가에서 제외하였다. 고콜레스테롤 식이를 진행한 그룹 A와 B에서 총콜레스테롤 농도가 일반 식이를 진행한 그룹 C와 D에 비해 뚜렷하게 증가되어 있음을 4주째 지질 검사를 통해 확인할 수 있었고(총콜레스테롤 농도: 그룹 A에서 1664.50 ± 770.12 mg/dL, 그룹 B에서 2186.25 ± 832.37 mg/dL, 그룹 C에서 32.41 ± 20.07 mg/dL, 그리고 그룹 D에서 28.28 ± 9.70 mg/dL이었다.), 그룹 B에서 일반 식이로 전환하고 simvastatin을 투여한 6주째에 총콜레스테롤 농도가 유의하게 감소하였다(총콜레스테롤 농도 최종 검사시 그룹 B에서 81.65 ± 51.21 mg/dL ($p < 0.001$)). 그룹 B에서 simvastatin 복용 전후 혈중 creatinine kinase 농도는 유의한 차이가 없었다 (혈중 creatinine kinase 농도

가 simvastatin 복용 전후 각각 1358.75 ± 474.41IU/L와 1693.50 ±5 86.09IU/L이었다) ( p = 0.676). 회전근 개 건의 전기 생리학적 평가 결과, 그룹 A는 그룹 C와 D에 비해 복합근육활동전위 면적이 유의하게 작았다(모든 $p < 0.01$). 또한, 그룹 B는 그룹 A에 비해 복합근육활동전위 면적이 컸는데, 이러한 그룹 B의 복합근육활동전위 면적은 그룹 C의 면적에 근접하는 양상을 보였다($p = 0.312$). 생역학적 검사에서도 유사한 양상을 보였는데, 그룹 A는 그룹 C와 D에 비해 파열 강도 및 강성도가 유의하게 작았고(모든 $p < 0.05$), 통계적으로 유의하진 않았으나 그룹 B는 그룹 A에 비해 파열 강도 및 강성도가 더 큰 양상을 보였다. 조직학적 검사 결과, 극상건 절단 후 6주 때(봉합 시행 시점) 그룹 A와 B는 그룹 C와 D에 비해 지방 대 근육 비율이 유의하게 높았는데 (모든 $P < 0.05$), 그룹 B에서는 마지막 평가 때(봉합 시행 후 6주) 봉합 시행 시에 비해 지방 대 근육 비가 감소한 양상을 보였다($p = 0.146$). 이러한 결과는 봉합 시행 시점보다 마지막 평가 때 오히려 지방 대 근육 비가 더 증가했던 그룹 A의 결과($p = 0.015$)와 극명하게 대비되는 모습이었다. 극상건-골 결합의 조직학적 검사 결과, 그룹 B와 C에서 그룹 A에 비해 건-골 결합부의 콜라겐이 더 잘 조직화되고 밀도가 높은 양상을 확인할 수 있었다.

**결론:** 고콜레스테롤혈증은 회전근 개 근육의 지방 변성 및 회전근 개 봉합술 후 건-골 결합에 악영향을 미치며, 고콜레스테롤혈증의 조절을 통해 이러한 악영향을 멈추거나 반전시킬 수 있을 것으로 보인다.

**색인 단어:** 고콜레스테롤혈증, simvastatin, 지방 변성, 건-골 유합, 만성 회전근 개 파열, 토끼 모델

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