Histological alterations to the hamstring tendon caused by cleaning during autograft preparation

Y. Okazaki, T. Furumatsu, A. Maehara, S. Miyazawa, Y. Kamatsuki, T. Hino, T. Ozaki

Department of Orthopaedic Surgery, Okayama University Graduate School, Okayama, Japan.

INTRODUCTION

The anterior cruciate ligament (ACL) is a knee joint ligament that is frequently damaged. ACL rupture causes instability of the knee, which increases the risk of meniscus injury and early-stage osteoarthritis (1). Conservative or surgical treatment is performed to restore knee stability to the preinjury level (2). ACL reconstruction is favored for younger and more active patients, allowing for an earlier return to activities and competitive sport (3). Some studies have reported comparable outcomes for double-bundle and single-bundle ACL reconstruction, with no differences in clinical knee scores and knee extension and flexion muscular strength between the two graft types (4,5). Hamstring autograft is primarily used for double-bundle ACL reconstruction. In highly physically active patients, ≤45 years of age, allografts are not recommended because of the high risk of re-rupture (6). Although the maximum failure strength of hamstring tendons is lower than that of bone-patellar tendon-bone grafts, multiple folding of the tendon increases the tensile strength of the graft sufficiently for ACL reconstruction and, therefore, hamstring tendons are frequently the first choice for ACL graft material (7).

Hamstring tendons are categorized as dense connective tissue. Histologically, they have smaller cell nuclei, less proteoglycan, and more type I collagen fibers than the ACL (8). However, after ACL reconstruction, a graft undergoes transformation, from a stage of initial necrosis to revascularization, cell growth, collagen reconstitution, and matura-
tion, a process known as “ligamentization” (9). Through this graft remodeling process, which lasts for a year or more, a reconstructed graft transforms into tissue that resembles a healthy ACL but is not exactly the same (10). Research into the healing process of human grafts has increased in recent years. Clinical factors known to affect graft healing include graft isometricity, anatomical positioning, patient compliance, the healing response, vascularity, biomechanical strength, and post-operative rehabilitation (11). However, much remains unclear about how the healing process is affected by damage during the preparation of the hamstring autograft itself. In practice, excess muscle fibers must be cleaned from the tendon using a periosteal elevator or curette during preparation (12). This cleaning involves scratching the muscle fibers on the tendon multiple times using one of these tools. Scratching can cause microscopic damage to the tendon, but the degree of histological alteration is unclear. Therefore, the purpose of this study was to assess the histological alteration caused by tendon cleaning during the preparation of hamstring autografts. Our hypothesis was that the more times a hamstring tendon is scratched during cleaning, the greater the histological damage. Furthermore, the specific aim of this study was to determine the number of scratches that cause deleterious damage to the hamstring autograft.

MATERIALS AND METHODS

Graft preparations
Institutional review board approval and informed consent were obtained before all experimental studies.

Histological analysis
The ST specimens were fixed with a 10% formalin solution and embedded in paraffin blocks. Sections were created by making 6-µm slices, parallel to the direction of the scratch-
Sections were then subjected to hematoxylin-eosin staining. The degree of histological alteration was assessed using the modified Bonar scoring system (13). This score describes the extent of degeneration of the tendon based on the following 5 features: cell morphology, collagen arrangement, cellularity, vascularity, and ground substance. Each feature has a maximum score of 4 points, and the scores for each of the 5 features are summed to obtain the total score, with a higher score being indicative of greater damage. The Bonar score for the control and 3 scratch types (5, 10, and 15 repetitions) was then compared.

Immunohistochemical analysis
Rabbit anti-type I collagen antibody (ab34710; Abcam, Cambridge, UK) was used to assess deposits of type I collagen in the areas on the tendon that were scratched, as previously described (14). Six-μm sections were deparaffinized in xylene and incubated with 20 μg/ml proteinase K (Abcam). For staining, the rabbit antibody was diluted in a blocking buffer and incubated overnight at 4°C. Samples were washed 3 × 5 min in PBS and incubated for 1 h with secondary antibodies in blocking buffer. Slides were then washed three times in PBS, counterstained with hematoxylin. Photoshop CC (Adobe, San Jose, CA) and Image J v.1.45 was used to extract the areas stained by collagen deposits (15). Next, the same analysis threshold was set for all groups (0, 5, 10, and 15 repetitions) to calculate type I collagen-stained areas (%), as described (16). The collagen-stained area was quantified as a percentage of the total area of the tendon section (17). In addition, the cell numbers within collagen fibers were counted. The cellular density (cell count/mm²) was quantified and the values were compared among the 4 groups. The mean value derived from the five different images was evaluated by two observers.

Statistical Analysis
All data were expressed as means ± standard deviation (SD). Histological scoring was performed by 2 independent investigators, who were blinded to the results. Interclass correlation coefficients (ICCs) for intra-observer and inter-observer agreement were calculated. Differences among groups were compared using the Mann-Whitney U-test. Statistical significance was established at P<0.05.

RESULTS
Histological analysis
Figure 2 shows the hematoxylin-eosin stained specimens for each number of scratches. As the number of scratches increased, cell counts decreased and fiber bundles sepa-
rated. As for vascularity, a small number of vascular structures were observed between fibers in the no scratching and 5-repetition groups, with no vascular structures observed in the 10- and 15-repetition groups, the latter being scored as ‘0’ on the Bonar scale. The modified Bonar score was 0.8±0.4 for the control (no scratching group), increasing for the three scratching groups as follows: 5-repetitions, 3.2±1.5; 10-repetitions, 5.7±1.5; and 15-repetitions, 7.2±0.4 (Table I). The Bonar score increased as a function of the number of scratches (Figure 3A), with a significant difference in score between the control (no scratch) group and the 5-repetition group (P=0.003), as well as between the 5- and 10-repetition groups (P=0.023). The Bonar score for collagen arrangement was significantly different between the three groups (Figure 3B), with cellularity being significantly increased between the 5- and 10-repetition groups (P=0.009). The intra-observer and inter-observer ICCs for the Bonar scoring system in this study were 0.82 and 0.77, respectively.

Immunohistochemical analysis
The type I collagen-stained specimens are shown in Figure 4. The mean type I collagen-stained area was 88.4±5.8% in the control (no scratch) group, decreasing to 71.4±7.0% in the 5-repetition group, 50.0±8.2% in the 10-repetition group, and 35.6±6.8% in the 15-repetition group (Table I). The type I collagen-stained area decreased as the number of scratches increased, with a significant difference between all groups (P<0.05, Figure 5A). The mean cellular density was 44.9±11.3/mm² in the control (no scratch) group, decreasing to 38.7±11.6/mm² in the 5-repetition group, 17.6±5.7/mm² in the 10-repetition group, and 13.6±5.6/mm² in the 15-repetition group (Table I). The density in the 10-repetition group was less than half the density in the 5-repetition group (p=0.004, Figure 5B). Intra- and inter-observer reliabilities were excellent (ICC>0.91).

DISCUSSION
The most important finding in this study is that cleaning hamstring tendons by scratching causes histological alterations to the tendons. To our knowledge, this is the first histological analysis of artificial damage to the hamstring tendon during cleaning in the process of graft preparation for ACL reconstruction. In our tendon samples, the modified Bonar score increased as a function of the number of scratches performed, indicative of greater histological degeneration. Moreover, as the number of scratches increased, both the area of staining for type I collagen and the cellular density decreased.

Human biopsy studies have defined the stages of graft remodeling, recellularization, revascularization, and changes in collagen crimp and collagen fibril density during the proliferation phases, suggesting that the human ACL graft might have its lowest mechanical strength at 6–8 weeks after reconstruction (10). Three factors are known to exert adverse effects on the mechanical strength of the graft: increased revascularization and extracellular infiltration (18), loss of the regular collagen crimp pattern, and...
Figure 4. A tendon sample for type I collagen-staining. The triangles show spindle-shaped fibroblasts for the control (no scratching) group (A), and the 5- (B), 10- (C), and 15-repetition groups (D). Inlets, negative controls. Bars, 50 μm.

Figure 5. Immunohistochemical analysis. (A) Type I collagen-stained area (%) decreased with significant differences between all the groups. (B) The cellular density was significantly lower for the 10- than 5-repetition group; *P<0.05.
Histological alterations to the hamstring tendon

Some authors have described that the decrease of collagen fibril density is associated with the maximum loss of graft strength and mechanical restitution of graft healing (20,21). Moreover, a biopsy study has shown an increase in the collagen III content in the connective tissue of the hamstring autograft (22). Bosch et al. also found an increased expression of collagen type III in healing grafts (23). The mechanical strength of collagen III synthesis is lower than that of type I collagen (24). A previous review reported collagen III synthesis within the gaps between collagen fibrils, caused by damage to type I collagen fibrils (25). In this context, the decrease in the area of staining for type I collagen that we observed is indicative of a decrease in collagen fibril density during the proliferation phase. The greater damage to type I collagen by scratching would likely lead to more collagen III synthesis in collagen gaps, which could reduce the mechanical strength of the ACL hamstring autograft.

Fibroblasts have been shown to play an important role in the reconstruction of collagen fibrils (10). These cells are partially involved in collagen crimp formation and have been shown to have a significant impact on ACL graft remodeling (26). The cellular density of fibroblasts increases until the end of the proliferation phase, followed by a gradual decrease toward the density in the intactACL (19,20). It is also considered that the number of fibroblasts and their activity are an indicator of active ACL hamstring tendon autograft remodeling (20,27). The mean cellular density in the present study was 38.9/\text{mm}^2 in the 5-repetition group and 17.6/\text{mm}^2 in the 10-repetition group, causing a marked reduction of fibroblasts. This is also lower than the cellular density (30.2) of a graft measured 0.75 months after ACL reconstruction in a previous study (28). In addition, a systematic review found that the timeline of biological changes in grafts differs greatly between animals and humans. Graft remodeling in humans was found to be much slower compared to that in animals (29). Taken together, a marked decrease in the number of cells with ≥10 scratches is indicative of the adverse effect of this extent of scratching on the final ligamentization phase of graft remodeling in humans.

This study has a number of limitations that need to be acknowledged. First is the small sample size, with a larger number of patients being needed to confirm our findings. Second, we did not measure the minimum amount of scratches necessary to remove all muscle tissues, which would have provided additional clinical implications for graft preparation. Third, we also did not perform histological assessment of the reconstruction implanted graft at the proliferation phase or the final phase of graft remodeling. It remains to be clarified as to whether the reduced tissue integrity in the pre-grafted tissue would always indicate deterioration or failure of tendon regeneration.

Based on our findings, we suggest that surgeons should limit scratching of the tendon with a curette to <10 repetitions because ≥10 repetitions markedly reduced type I collagen-stained area and cellular density. We also conclude that excessive curettage, ≥10 repetitions, could be deleterious to the tissue health of a hamstring autograft.

### Table I. Histological alterations for each scratching repetitions.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number of scratching repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonar score (total)</td>
<td>0.8 ± 0.4 3.2 ± 1.5* 5.7 ± 1.5* 7.2 ± 0.4*</td>
</tr>
<tr>
<td>Bonar subscore</td>
<td></td>
</tr>
<tr>
<td>Cell morphology</td>
<td>0.2 ± 0.4 1.0 ± 0.1 1.3 ± 0.5 1.8 ± 0.4</td>
</tr>
<tr>
<td>Collagen alignment</td>
<td>0.3 ± 0.5 2.3 ± 0.5* 3.0 ± 0*</td>
</tr>
<tr>
<td>Cellularity</td>
<td>0.7 ± 0.5 0.5 ± 0.5 0 0</td>
</tr>
<tr>
<td>Ground substance</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>Type I collagen-stained area (%)</td>
<td>88.4 ± 5.8 71.4 ± 7.0* 50.0 ± 8.2* 35.6 ± 6.8*</td>
</tr>
<tr>
<td>Cellular density (/mm(^2))</td>
<td>44.9 ± 11.3 38.7 ± 11.6 17.6 ± 5.7* 13.6 ± 5.6*</td>
</tr>
</tbody>
</table>

Data are displayed as a mean ± standard deviation. *Significance was determined using Mann-Whitney U test. *P<0.05 vs. 0 times repetitions (control)
Conflict of interest
The authors declare that they have no conflict of interest.

Acknowledgements
We would like to thank Editage (www.editage.jp) for English language editing.

REFERENCES
27. Dustmann M, Schmidt T, Gangey I, Unterhauser FN, Weiler A, Scheffler SU. The extracellular remodeling of
Histological alterations to the hamstring tendon


