

Accelerated Achilles tendon healing with interleukin-1 receptor antagonist protein in rabbits

Marko PECIN^{1*}, Mario KRESZINGER¹, Snjezana VUKOVIC², Marija LIPAR¹, Ozren SMOLEC¹, Berislav RADISIC¹, Josip KOS¹

¹Surgery, Orthopedics, and Ophthalmology Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia
²Department of Anatomy, Histology, and Embryology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

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Abstract: The current study describes the use of interleukin-1 receptor antagonist protein (IL-1Ra) as a possible strategy for optimizing tendon healing and repair by presenting histologically visible changes in rabbit Achilles tendon tissue after longitudinal tenotomy. The study was carried out on 26 Californian rabbits divided into two equal groups. One was the experimental IRAP (interleukin-1 receptor antagonist) group and the second one was the PBS (purified buffered saline) group. The PBS group was the control group. After longitudinal tenotomy in both groups of rabbits, in the IRAP group IL-1Ra was applied locally, whereas PBS was applied in the control group. IL-1 concentration in Achilles tendon tissue was measured with an ELISA IL-1 rabbit kit. Local application of IL-1Ra resulted in 2.5-fold lower IL-1 tissue concentration and prevented cytokine cascade activation and its proinflammatory effects. Consequently, it prevented chronic tendon inflammation and improved Achilles tendon healing in rabbits. Histological changes in tendon tissue samples were evaluated by Bonar scale score with statistically significant differences between groups ($P \leq 0.01$).

Key words: Tendon healing, Bonar scale, interleukin-1, inflammation, rabbit

1. Introduction

Tendons connect muscle to bone and allow the transmission of forces generated by muscle to bone, resulting in joint movement. Therefore, tendon disorders are frequent in animals and are responsible for substantial morbidity both in domestic and companion animals, especially in racing horses and dogs. The Achilles tendon is the strongest tendon in the body and its function is of great importance for animal mobility. Nevertheless, when injury of the tendon occurs, it lacks healing properties and morbidity lasts several months or even a year despite what is considered appropriate management. The basic cell biology of tendons is still not fully understood, and the management of tendon injury poses a considerable challenge for veterinary clinicians. Newer methods of accelerating muscle and tendon healing are used recently with local application of autologous conditioned serum and interleukin-1 receptor antagonist protein (IL-1Ra). The goal of such therapies is to prevent acute and chronic inflammation of tendons by blocking proinflammatory activities of interleukin-1. Therefore, guided by recent publishes studies, we decided to explore the possibilities of accelerating tendon healing using autologous serum rich with IL-1Ra and describe it with histologically visible findings.

2. Materials and methods

The current study included 26 Californian rabbits, 14 males and 12 females, weighing approximately 3 kg (from 2.8 to 3.1), aged 2 years, randomly divided into two equal groups. Each group had 13 rabbits, 7 males and 6 females. One was the IRAP group with IL-1Ra and the second was a control group with purified buffered saline (PBS) applied locally. In a sterile IRAP® 10-mL syringe, using a closed venipuncture system, blood was taken from each rabbit. Syringes were stored in an incubator at 37 °C for approximately 6 h in order to separate the serum from the cellular blood elements. Thereafter, the samples were subjected to the process of centrifugation at 3000 rpm for 10 min. Centrifuging resulted in conditioned serum containing large quantities of antiinflammatory autologous IL-1Ra.

2.1. Animals, anesthesia, and surgical procedure

Animals were sedated with an intramuscular injection of 40 mg/kg ketamine and 4 mg/kg xylazine. Induction to anesthesia was achieved by an intravenous application of 1% propofol in the ear vein through a 24-G cannula. Epidurally 2% lidocaine was administered in a dose of 1 mL/5 kg body weight. General anesthesia was maintained by breathing a mixture of oxygen and isoflurane applied through a face mask. Antibiotic

* Correspondence: mpecin@vef.hr

cefuroxime was administered intravenously in a dose of 15 mg/kg body weight. After surgery butorphanol tartrate was administered at a dose of 0.5 mg/kg body weight subcutaneously every 6 h for 2 days. In both groups of animals a posteromedial skin incision was made with surrounding tissue resection and tendon sheath incision. Tenotomy of the Achilles tendon along the entire length of the tendon with surgical blade no. 15 with three parallel cuts was made. After longitudinal tenotomy in the IRAP group of rabbits IRAP® serum was administered at three points: the tendomuscular junction, the middle part of the tendon, and 5 mm above the insertion of the tendon to the heel bone. IRAP was administered three times in the first 48 h after surgery (0, 24, and 48 h) using the IRAP® system in a total dose of 0.6 mL per application (total: 1.8 mL) using a sterile syringe through a sterile biological filter (pore size: 0.22 µm). In the control group PBS was administered to the same places with the same doses, application technique, and time intervals. In both groups the tendon sheath was reconstructed and sutured with polyglyconate 3-0 USP continuous suture. The skin was also reconstructed with a continuous suture using nylon 4-0. Surgically treated limbs were not immobilized and therefore unrestricted movement was allowed. Four weeks following the surgical procedure animals were euthanized and Achilles tendons were sampled and subjected to histological analysis. Bonar scale comparisons were used in assessment of tendons. Interleukin-1β (IL-1β) tissue concentration was measured with the ELISA rabbit IL-1 kit by R&D Systems.

2.2. Histological tendon healing assessment

Criteria for histological assessment of Achilles tissue samples were based on the modified semiquantitative method of Movin (method by Bonar). A modified Movin (Bonar) method was used, which evaluated the shape and orientation of collagen fibers, the concentration of cellular elements, the content of glycosaminoglycans (mucopolysaccharides), the content of elastic and reticulin fibers, and blood supply with a 4-point scale (0–3).

2.3. Microscopic evaluation

In histological tissue samples of Achilles tendons in both groups the following parameters were assessed using a light microscope: cellularity; shape and size of the tenocyte nuclei; changes in the cytoplasm of cells; position, alignment, and orientation of collagen fibers and their waves in longitudinal section; the collagen bundles' organization in the longitudinal and transverse cross-section; the amount of colored glycosaminoglycans of basic substances; the presence of elastic and reticulin fibers; and the presence of blood vessels.

2.4. Statistical analysis

Statistical analysis was done using a personal computer and SPSS for Windows XP. To compare the groups

descriptive statistics (n, standard deviation, arithmetic mean, mode) were used and calculated for all parameters. The results were statistically analyzed using STATISTICA to compare the difference between the two test groups. The Mann–Whitney U test, Wilcoxon test, and t-test were used considering that there were mutually dependent and small samples. Statistically significant results were considered as those with $P < 0.01$.

2.5. Ethics

All rabbits were examined and treated according to the national law on animal care and considering all ethical requirements. The investigation was done with permission of the Ministry of Agriculture of the Republic of Croatia.

3. Results

Four weeks after the longitudinal tenotomy and administration of the IRAP serum and PBS, the concentration of IL-1β was 2.5 times lower in the IRAP group than in the control group as measured with the ELISA rabbit IL-1 kit. Lack of proinflammatory effects of IL-1β resulted in visible histological changes in fine tendon structure. Collagen fibers were more properly placed in the transverse and longitudinal cross-section in IRAP group tendons compared to the control group. Differences were evident in all areas of the tendon proximal to the distal portion (segments 1, 2, and 3). An exception was the central part of the tendons in both groups, where a great difference in relation to the intact tendon tissue was noticed. More significant changes were also observed in the central part of the tendons in the control group. Reparation of tissue was faster in the proximal part of the tendon at the muscle-tendon junction and slightly slower in the middle part and in the tendon insertion to the calcaneus.

The average number of tenocytes and the number for each individual tendon segment were lower in the IRAP group. By Bonar scale score, in the IRAP group basic substances (extracellular matrix) were lower in all tendon segments in comparison to the PBS group. Arrangement and location of collagen fibers were more regularly positioned in the IRAP group with a slightly lower amount of vascular elements by Bonar scale. Differences between groups and within groups in tendon segments are shown graphically in Figure 1. The difference in tissue sample histology and IL-1 concentration between the PBS and IL-1Ra group was statistically significant at week 4 ($P < 0.01$).

The average sum for all parameters by Bonar scale for the IRAP group was less than 1.5 (1.49) while in the control group it was about 2 (1.93). Differences in the scale value were most expressed in the number of tenocytes, and slightly less in the number of basic materials and position of collagen, whereas the lowest difference was noticed in the amount of vascular elements. The mode for the tenocytes in the IRAP group was 1, while in the control

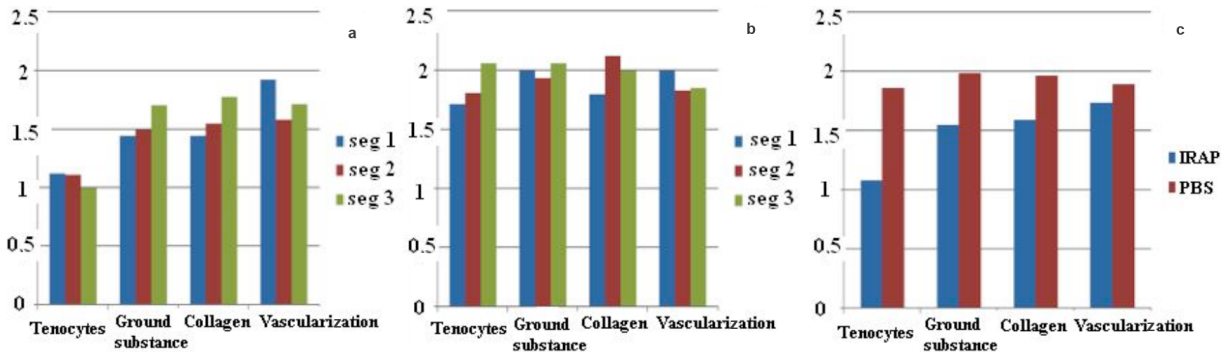


Figure 1. Overview of results of the Bonar scale for IRAP and control groups. Figure 1A shows the observed relationship of elements in each segment of the tendon (1, 2, 3) within the IRAP group, while Figure 1B shows the same relationship in the control group. Figure 1C shows the comparison of average values in tenocytes, basic substance, collagen, and vascularization between the IRAP and control groups according to Bonar scale score. The X value contains the above-mentioned parameters and the Y coordinate exhibits Bonar scale values from 0 to 3 (2.5). Segment 1- muscular part of the tendon, 2- central, 3- 0.5 mm from the calcaneus. Higher score result indicates worse healing and more pronounced changes.

group it was 2; the mode for basic substances in the IRAP group was 1.5 relative to 2 in the control group; and for the position of collagen fibers it was 1.5 in the IRAP and 2 in the control group. Vascularization mode was 2 in both groups.

3.1. Histological analysis of Achilles tendon healing

As previously mentioned the criteria for histological evaluation of tendon healing in both studied groups were based on the size and morphological features of tenocytes (fibroblasts/fibrocytes), collagen bundle shape, intercellular matrix composition, content of elastic and reticulin fibers, and angiogenesis. As the tendon healing process moved from the outside of the cut (longitudinal tenotomy line) to the interior, a difference in the organization of the newly formed tissue was observed. Consequently, for harmonization of assessment criteria, areas immediately within the cutting line (tenotomy) were analyzed. In each tissue sample at least five fields were estimated per longitudinal cut. Histological analysis of IRAP group tendon samples showed slightly increased cell numbers in comparison to intact tendons, as presented in Figure 2. In intact tendons, tenocytes had larger cell nuclei, which were oval-shaped and pale. Nucleoli were observed in the dispersed chromatin as shown in Figure 2.

The position of the tendon fibroblasts' nuclei in the healing process was largely correct and followed the natural course of the fibers, although some areas of densely laid and irregularly oriented nuclei were spotted, as shown in Figure 2. Cell cytoplasm was not observed. In most samples of IRAP tendon, groups of primary collagen fiber bundles are not clearly expressed. Following the position of the nuclei longitudinal orientation and slight fiber waves were observed, as shown in Figure 2. In some samples it seemed that the tissue remodeling process had progressed.

The healing area looked like healthy (intact) tissue tendon. Nuclei were rare, more elongated, and slightly darker. The process of formation of primary fibers bundles, which were slightly undulating and longitudinally arranged, was observed, as shown in Figure 2.

IRAP group tendon tissue samples dyed with alcian blue (pH 1.0)-Kernechtrot stain did not show any reaction while dyeing tissue with alcian blue (pH 2.5) gave a slight red and blue discoloration as evidence of acid sulfate mucopolysaccharides absence and a small amount of polysaccharides and mucin with glycosidic groups.

The maximum level of elastic fibers was estimated using several specific staining methods. In the IRAP group samples stained by Gomori method with aldehyde fuchsin, a clearly purplish discoloration of the area of healing was noticed, as shown in Figure 2. Individual elastic fibers were very rare, as shown in Figure 3, and a small amount of reticulin fibers was present, as shown in Figure 3. In the IRAP group tendons, within healing areas blood capillary clusters were present with fat cells near them. These changes were not expressed in all preparations and are best shown in Figure 3.

Histological analysis of control tendon group samples showed a large number of cells present. The fibroblasts' nuclei were large, round to oval, and usually randomly distributed. In some cells slightly bluish cytoplasm was seen, as shown in Figure 3.

In the histological samples of control group tendons primary bundles of collagen fibers were not clearly organized. Collagen fibers were oriented incorrectly and did not follow the normal horizontal flow and waviness of fiber. In some places stronger or weaker ripple was noticed that did not correspond to the correct fiber position as in intact tendon, as shown in Figure 3.

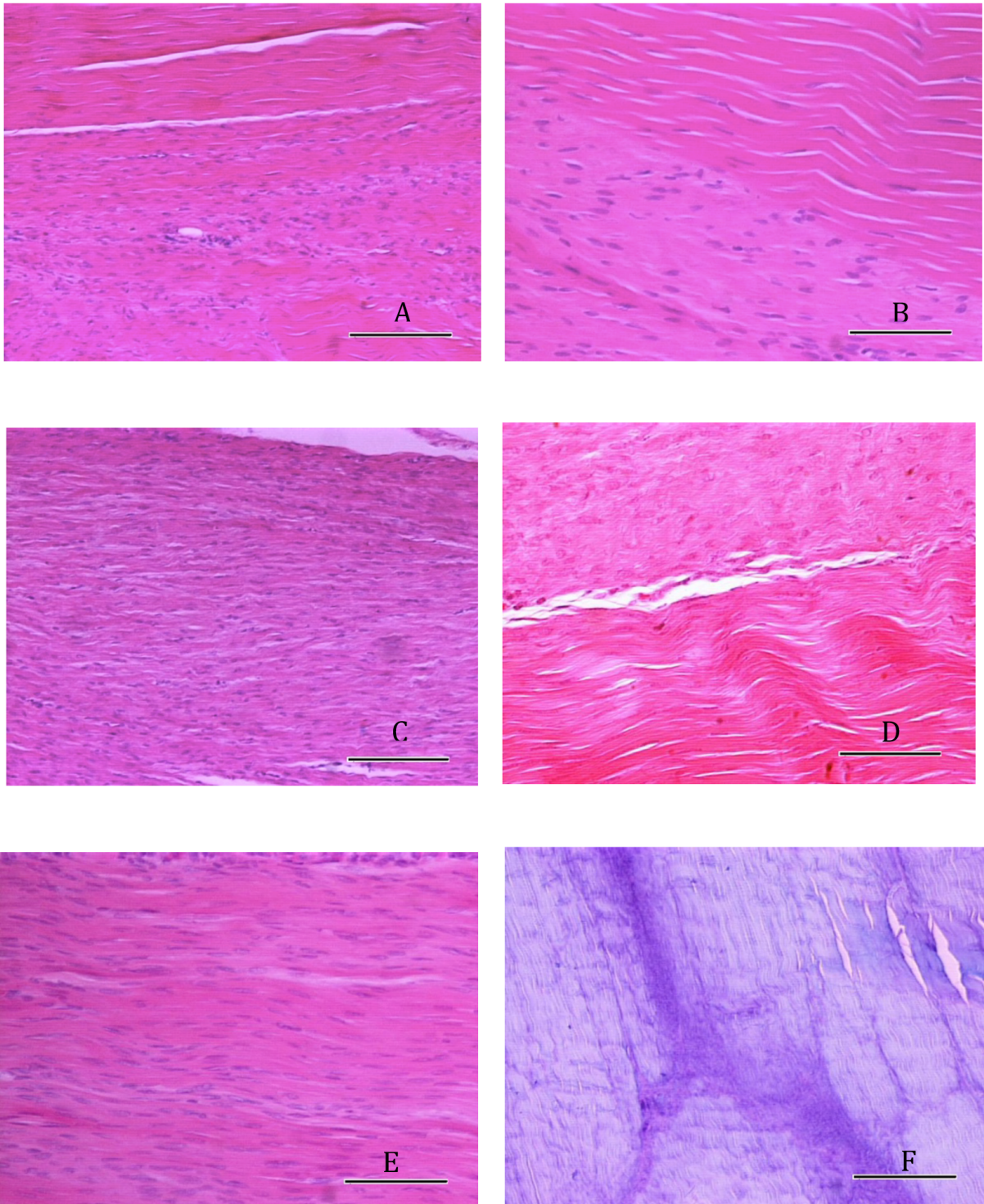


Figure 2. A) IRAP group. Intact tendons and tendon healing area. H&E stained, lens 10 \times , bar scale of 200 μ m. B) IRAP group. Picture presents typical oval fibroblasts nucleus. Stained by H&E, 20 \times lens, scale bar 100 μ m. C) IRAP group. The position of fibroblasts' nuclei. H&E, 10 \times lens, scale bar 200 μ m. D) IRAP group. Longitudinal direction and a mild ripple of collagen fibers was displayed. Stained by H&E, 20 \times lens, scale bar 100 μ m. E) IRAP group. Advanced tendon healing process. Stained by H&E, 20 \times lens, scale bar 100 μ m. F) IRAP group. Purple discoloration of the areas of healing, stained by Gomori method with aldehyde fuchsin, lens 4 \times , scale bar 500 μ m.

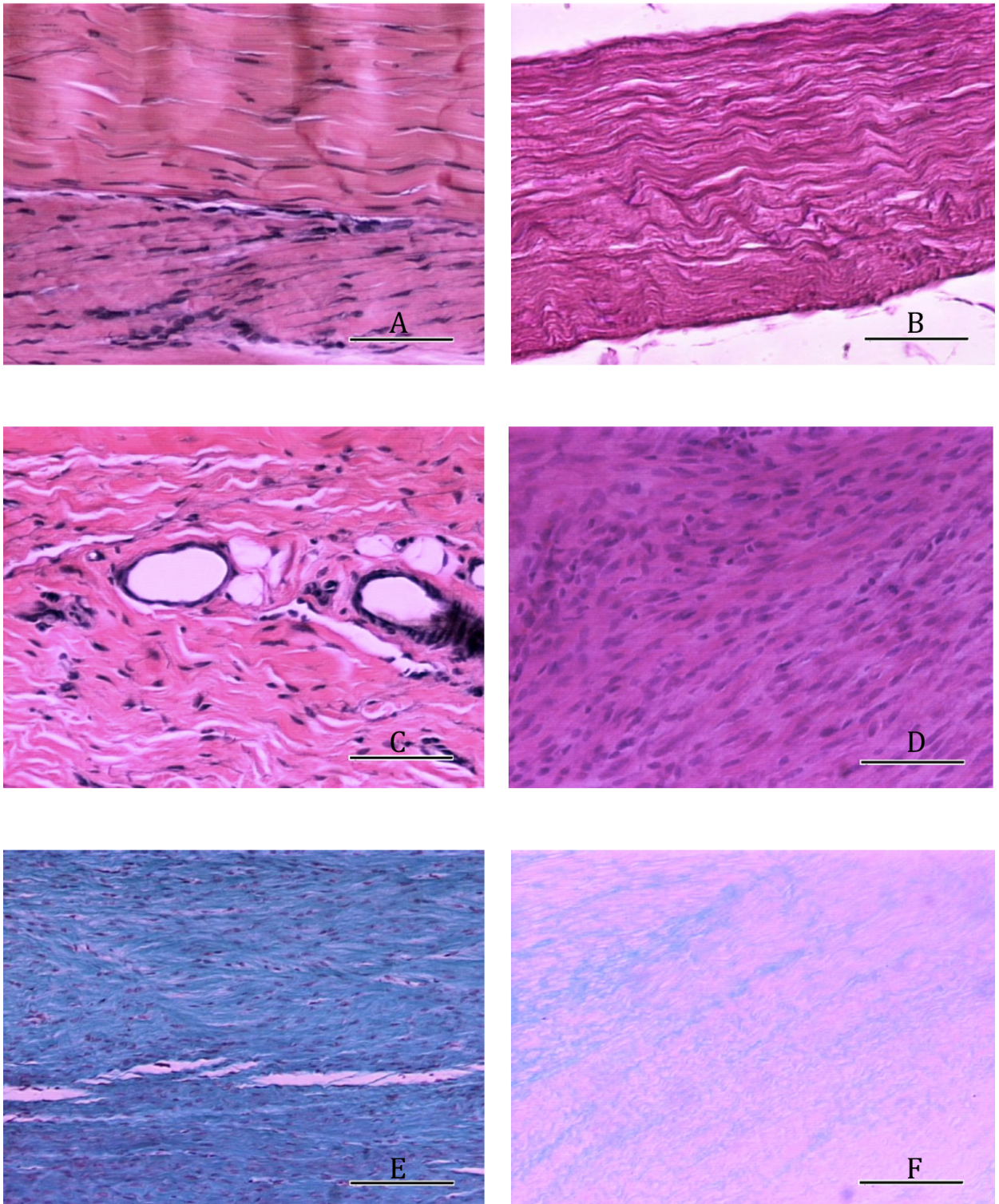


Figure 3. A) IRAP group. Displaying rare elastic fibers. Stained by Verhoeff-van Gieson method, lens 20×, scale bar 100 μm . B) IRAP group. Displaying reticulin fibers. Stained by Gridley method, lens 40×, scale bar 50 μm . C) IRAP group. Blood vessels and fat cells presented. Stained by Verhoeff-van Gieson method, lens 20×, scale bar 100 μm . D) Control group. Displaying randomly distributed fibroblasts nuclei. Stained by H&E, 20× lens, scale bar 100 μm . E) Control group. Visible incorrect orientation of the fibers. Stained with Masson trichrome, 10× lens, scale bar 200 μm . F) Control group. Presents a lot of colored mucopolysaccharides (GAG), stained with alcian blue (pH 2.5)-PAS, lens 10×, scale bar 200 μm .

Similarly as in the above-described IRAP fiber samples, alcian blue staining (pH 1.0)-Kernechtrot stain showed no effect in the control group. However, control tendon samples stained by alcian blue (pH 2.5)-PAS method provided a clear red and blue discoloration as evidenced by a larger amount of sulfate acid mucopolysaccharides and neutral mucopolysaccharides, which were colored blue, as shown in Figure 3.

Control group tendon samples had more purplish discoloration for displaying elastic fibers using the Gomori method with aldehyde fuchsin staining. There were also individual elastic fibers present, in some places very dense, as shown in Figure 4. There were slightly more reticulin fibers than in the IRAP tendons and they were also more pronounced, as shown in Figure 4. Very dense networks of blood capillaries were present in histological samples of the control group Achilles tendons, as shown in Figure 4.

4. Discussion

Longitudinal tenotomy with surgical blade no. 15 in three parallel cuts along the entire length and thickness of the tendons caused histologically visible changes that were more expressed in the control group. On the other hand, healing patterns were more visible in IRAP group tendon samples. The selected technique for achieving inflammation of the Achilles tendon with longitudinal tenotomy yielded satisfactory results, and the differences between the experimental tendon group were significant. Similar inflammatory reactions after longitudinal tenotomy in rabbit tendons were observed in a previously published study by Friedrich et al. (1). Research has shown that such scarification and tenotomy tendon can cause inflammation of the Achilles tendon in rabbits.

In the current study noticeable changes within the control group tendons such as the degeneration of collagen, irregular fiber orientation, fiber attenuation, scattered blood vessels, and an increased amount of GAG between fibers were observed by the semiquantitative Movin method (Bonar scale). These two methods are very similar and give almost the same results (2), except that the Bonar method estimates fewer histological elements in tendon tissue. The Movin method estimates 8 elements (3): structure and position of the collagen fibers, the appearance of nuclei within the cells, changes in cellularity, increased vascularity, reduced staining of collagen, hyalinization, and amount of GAGs. The same method was used by Tallon et al. (4) in the assessment of histological differences between the rupture and inflammation of tendons.

IRAP autologous serum prevented the progression of the inflammation of tendons and the changes in the IRAP group were much less pronounced than in the control group. Many authors noticed that histologically largest lesions are evident in tendon degenerative changes

without or with rarely present inflammatory cells with reduced healing reaction (5) with noninflammatory degeneration of collagen, an incorrect position of collagen fibers that are thinner, dispersed vascularization, increased amount of cellular elements, and increased amount of glycosaminoglycans (5–9). Such changes were observed in the current histological samples. Tendon tissue that healed after administration of IL-1Ra histologically appeared to be better structured, with characteristic appearance of fiber and more properly arranged bundles with little ground substance and cellular elements. Collagen fibers were more mature with highly indicated places of defect repair. It seems that the IRAP group tendons had fewer defects in composition in relation to the control group tendons that spontaneously healed.

A small number of tenocytes with less pronounced amount of extracellular matrix mucopolysaccharides in the IRAP group indicated rapid restoration of certain parts of the tendon in relation to the findings in the control group. Increased cellularity and vascularization in the control group could have been the start of angiofibroblastic hyperplasia (10). Newly formed blood vessels do not necessarily mean increased healing, but they were to a greater extent present in the control group in some parts of the tendon. The vessels were placed in different directions, even perpendicular to the fiber direction. Such vessels have mostly nutritional function, like real blood vessels (11). Therefore, lower blood vessel count by Bonar scale in the IRAP group does not necessarily mean slower healing.

The Bonar scale proved to be a good choice in the assessment of histologically visible differences between groups in earlier research (4), and within each group of tendons. Although in the current study differences presented by Bonar scale are small, the scale is from 0 to 3 and therefore even small average differences from 0.25 to 0.8 can make a significant difference. The mode as used in the current study proved to be a good method choice because in a scale of 4 points (0–3) it was much easier to assess the differences between the groups. Unfortunately, the mode does not give good insight into the small differences that are possible within the Bonar scale and could have statistical significance. Therefore, differences were calculated by average value.

Reconstruction of tissue was faster in the proximal part of the tendon at the start with muscle and the insertion on the heel bone, which may suit better blood circulation by tendon intrinsic capacity overgrowth in relation to the central part, which is poorly vascularized mostly by a fine extrinsic system. Thirty-five percent of the central tendon part's blood supply goes through the paratendon (12,13), which was dissected and compromised during tenotomy. The reason for this lies in the fact that at the muscle tendon junction capillaries and arterioles located between muscle

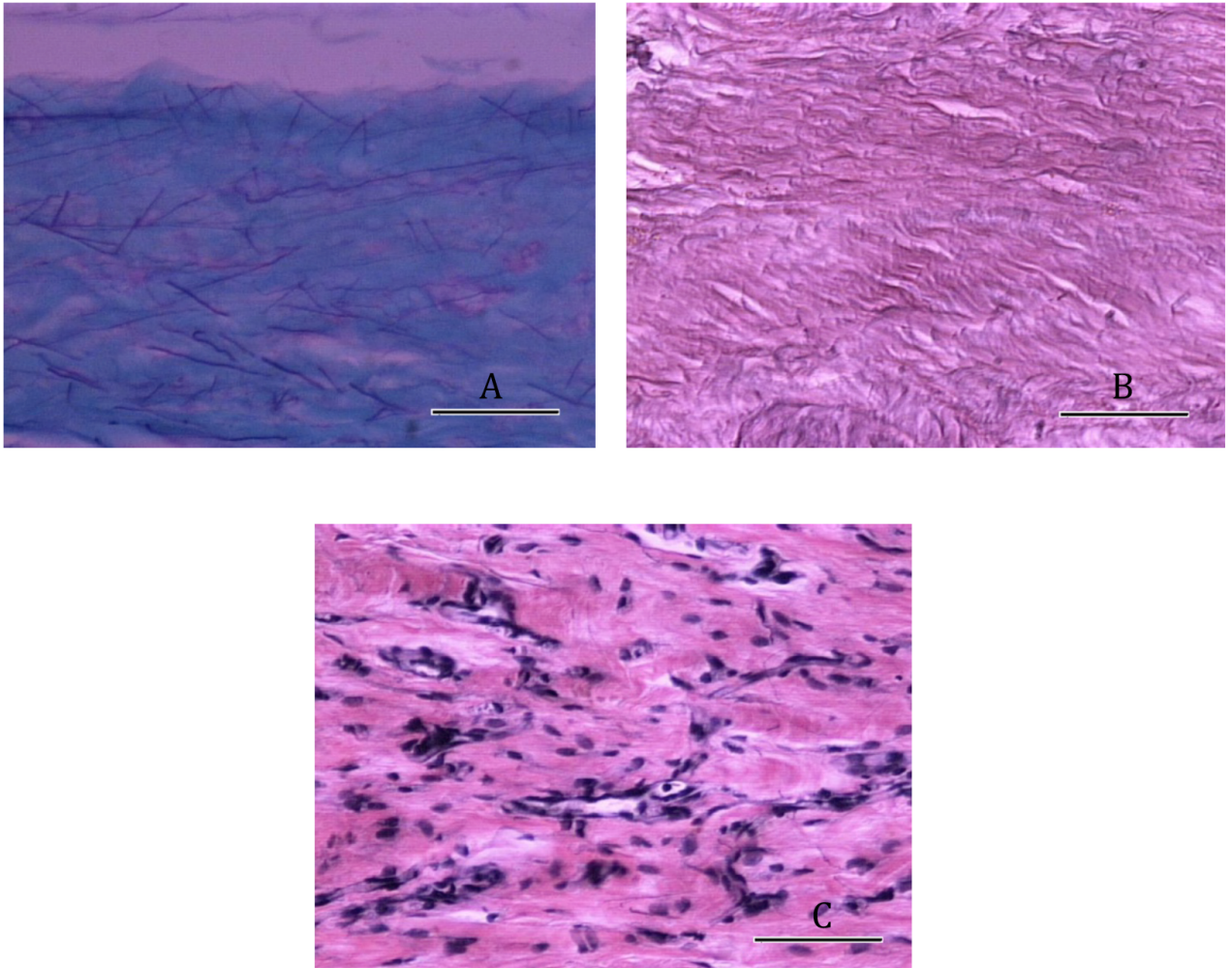


Figure 4. A) Control group, visible elastic fibers. Stained by Gomori method with aldehyde fuchsin, lens 40×, scale bar 50 μm. B) Control group. Displaying reticulin fibers. Stained by Gridley method, lens 40×, scale bar 50 μm. C) Dense network of blood capillaries. Stained by Verhoeff-van Gieson method, lens 20×, scale bar 100 μm.

fibers are entering between the secondary tendon fibers bundles (fascicles) and do not pass deeper than the first third of the tendon (14). In a study of the microvascular anatomy of the Achilles tendon using coloring of the tendon's bloodstream, Carr and Norris (14) came to the conclusion that the blood supply was the weakest in the middle part of the tendon. Reduced amount of blood vessels and blood elements was also noticed. The middle part of the Achilles tendon becomes a place of reduced healing and therefore differences between the groups were most visible within this area. Achilles tendon healing in rabbits depends on the location where the injury occurred (15). Injuries in the proximal part near the muscle had the best healing properties and this was slightly lower in the central part of the tendon. Healing is slowest when injury occurs near the tendon insertion to calcaneus in relation to the proximal part (15). Similar results were obtained in

the current study, where the proximal part in both groups had an average lowest Bonar scale score and therefore had the best healing properties, while the middle and distal were the worse. As mentioned, the biggest differences between the groups were observed by Bonar scale in the middle part of the tendon. The distal part of the tendon in both groups had the highest score by Bonar scale with more pronounced changes in the control group, which means that it healed worse than other parts.

There are 6 different collagen degenerations (16), but in the Achilles tendon only lipoidal and mucoidal ones are present (17). Lipoid degeneration is characterized by the deposition of lipids in the fiber bundles with a loss of characteristic hierarchical structure of collagen fibers (18). In the control group bundles, fat cells were found between the collagen fibers with abnormal positioning of collagen fibers, which corresponded to lipoidal degeneration (19).

In some histological samples of IRAP group tendons, clusters of blood capillaries were found with only a few fat cells present. This finding is characteristic for the tendon healing phase. Collagen type III creates thinner reticulin fibers, which do not have the mechanical properties of mature collagen type I fibers. Thinner fibers, particularly elastic or reticulin, are typical for tendon healing phases. Reticulin fibers were equally present in both groups of tendons but the amount of elastic fibers was greater in the control group with a pronounced characteristic mesh structure of elastic fibers. Reticulin fibers in the tendon healing phase are used as a primary provisional matrix and granulation tissue later to be replaced by type I collagen, which has improved biomechanical properties. Elastic and reticulin fibers found in the samples indicated tendon healing phases in both groups. A small amount of the aforementioned fibers was not necessarily a bad sign, because part of these fibers may be replaced by type I collagen, as seen in IRAP group tendons with pronounced healing progress.

Clinical studies have demonstrated that inhibition of IL-1 decreased osteoclast activity with consequent reduced production of enzymes metalloproteinases. Since IL-1 is secreted from almost all cells, including fibroblasts and tenocytes/tenoblasts, blocking the secretion of IL-1 by preventing the binding of IL-1 to the target cell receptors can reduce the metalloproteinase enzyme activity in the tendon tissue. Consequently, tendon healing would be more efficient and faster, and the effect of MMP on tendon degradation would be highly reduced.

Longitudinal tenotomy caused microscopically visible inflammation of the Achilles tendon. Histological examination found differences between the groups in cellularity, position of the collagen fibers, the amount of GAG, vascularization, and the amount of elastic and reticulin fibers. These changes were typical for inflammation and tendon healing phases.

IRAP experimental group tendons had less pronounced histological changes compared to control group tendons after 4 weeks. This was confirmed with the 4-point Bonar scale score gradation. Different parts of the tendon showed uneven healing properties. The proximal part of the tendon by the tendomuscular junction healed faster and better than the middle and distal parts.

Both tendon groups showed histologically visible changes compared to the intact tissue with more pronounced changes in the control group.

Lower concentration of IL-1 β resulted in a reduction of iatrogenic inflammation, which resulted in lower degeneration of the fine structure of collagen fibers with minor changes in the molecular and cellular composition of the tendons in the IRAP experimental group.

This work supports the theory that the inflammation of the tendon starts with activation of the cascade of inflammatory factors by proinflammatory cytokine IL-1 β . By blocking the activation and activity of this cytokine in early stages of tendon injury or inflammation, prevention of chronic inflammation can be achieved with accelerated tendon healing.

References

- Friedrich T, Schmidt W, Jugmichel D, Horn LC, Josten CH. Histopathology in rabbit Achilles tendon after operative tenolysis (longitudinal fiber incision). *Scand J Med Sci Sports* 2011; 11: 4-8.
- Maffulli N, Longo UG, Denaro V. Movin and Bonar scores assess the same characteristics of tendon histology. *Clin Orthop Relat Res* 2008; 466: 1605-1611.
- Maffulli N, Barrass V, Ewen SW. Light microscopic histology of Achilles tendon ruptures. A comparison with unruptured tendons. *Am J Sports Med* 2000; 28: 857-863.
- Tallon C, Maffulli N, Ewen SW. Ruptured Achilles tendons are significantly more degenerated than tendinopathic tendons. *Med Sci Sports Exerc* 2011; 33: 1983-1990.
- Astrom M, Rausing A. Chronic Achilles tendinopathy: a survey of surgical and histopathologic findings. *Clin Orthop* 1995; 346: 151-164.
- Leadbetter WB. Cell-matrix response in tendon injury. *Clin Sports Med* 1992; 11: 533-578.
- Movin T, Gad A, Reinhold FP, Rolf C. Tendon pathology in longstanding achillodynia: biopsy findings in 40 patients. *Acta Orthop Scand* 1997; 68: 170-175.
- Jozsa LG, Kannus P. Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 1997; 7: 113-118.
- Khan KM, Maffulli N. Tendinopathy: an Achilles heel for athletes and clinicians. *Clin J Sport Med* 1998; 8: 151-154.
- Nirschl RP, Pettrone F. Tennis elbow: the surgical treatment of lateral epicondylitis. *J Bone Surg* 1979; 61: 832-839.
- Kraushaar B, Nirschl RP. Tendinosis of the elbow (tennis elbow). *J Bone Joint Surg Am* 1999; 81: 259-278.
- Naito M, Ogata K. The blood supply of the tendon with a paratendon. An experimental study using hydrogen washout technique. *Hand* 1983; 15: 9-14.
- Kvist M, Jozsa L, Jarvinen M. Vascular changes in the ruptured Achilles tendon and paratendon. *Int Orthop* 1992; 16: 377-382.
- Carr AJ, Norris SH. The blood supply of the calcaneal tendon. *J Bone Joint Surg Br* 1989; 71: 100-101.
- Kuschner SH, Orlando CA, Mckellop HA, Sarmiento A. A comparison of the healing properties of rabbit Achilles tendon injuries at different levels. *Clin Orthop Relat Res* 1991; 272: 268-273.

16. Movin T. Aspects of aetiology, pathoanatomy and diagnostic methods in chronic mid-portion achillodynia. PhD, Karolinska Institute, Stockholm, Sweden, 1998.
17. Kvist M. Achilles tendon overuse injuries. *Ann Univ Turku* 1991; 87: 1-121.
18. Maffulli N, Ewen SW, Waterston S, Reaper J, Barrass V. Tenocytes from ruptured and tendinopathic Achilles tendons produce greater quantities of type III collagen than tenocytes from normal Achilles tendons. An in vitro model of human tendon healing. *Am J Sports Med* 2000; 28: 499-505.
19. Khan KM, Cook JL, Bonar F, Harcourt P, Astrom M. Histopathology of common tendinopathies. Update and implications for clinical management. *Sports Med* 1999; 27: 393-408.