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Healing of Achilles Tendon, An Experimental Study: Part 2—Histological, Immunohistological and Ultrasonographic Analysis

H. Thermann, O. Frerichs, M. Holch*, A. Biewener*
Heidelberg and Dresden, Germany

ABSTRACT

In 105 rabbits the course of healing was examined at one, two, four, eight and 12 weeks (21 rabbits per group) after an experimental Achilles tendon rupture. The following treatment modalities were compared: A) primary functional treatment; B) operative functional treatment (resorbable suture); and C) operative functional treatment with fibrin glue. For the functional (after)-treatment a special orthosis was applied. A 7.5 MHz Ultrasound probe was used for ultrasonographic evaluation. The histological specimens were stained in Masson-Goldner and Azan technique. Collagen Type III was depicted immunhistologically. A semiquantitative fibrocyte count was performed. The histological results showed a smooth healing in the primary functional treatment group (A), reaching parallel orientation of collagen fibers at 12 weeks. In the suture group (B), a secondary gapping of the tendon stumps was detectable after one week as in all other groups. In the fibrin group (C), the fibrin was resorbed after four weeks without essential influence to the course of healing. At 12 weeks the histological evaluation in all groups showed approximately normal tendon pattern. Immunohistochemically, all groups showed cell-associated positive reactions for type-III collagen after one week with a maximum after two weeks. The semiquantitative fibrocyte count in the primary functional group showed a maximal number after one week. In the fibrin glue and suture groups the maximal number could be found after two weeks. Sonographically an increase in tendon thickness was detectable up to the fourth week in all groups. The secondary gapping of the tendon stumps in the suture group could also be detected sonographically. The echogenicity of the tendon during the course of healing showed increasing homogeneity and parallelism in all groups. At 12 weeks the echogenicity was comparable in all groups. The experiment suggests the equivalence of primary functional treatment to a combination of operative and functional therapy in Achilles tendon rupture.

Key Words: Achilles Tendon Rupture; Histology, Immunmohistology; Ultrasonography

INTRODUCTION

Functional treatment of acute Achilles tendon rupture, whether with operative or nonoperative treatment, is well established due to convincing clinical and experimental results in recent years. Ultrasonographic examinations are important for the initial therapy decision and in reassessment during regeneration.[25,28] The typical ultrasonic patterns of regeneration are defined empirically and have yet not been related to histological examinations.

The aim of this study was to evaluate different histological types of regeneration after operative therapy with tendon suture or fibrin glue and nonoperative treatment respectively. The microscopic findings were related to ultrasonic examinations during tendon healing.

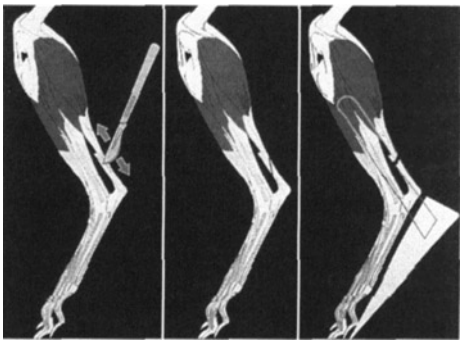


Fig. 1: Technique of tenotomy. (**a**, *left*) multiple longitudinal incisions (**b**, *middle*) oblique tenotomy (**c**, *right*) approximation of tendon stumps in orthosis.

*Gustav-Carus Universität Dresden
Klinik f. Unfall- und
Wiederherstellungschirurgie, Dresden

Corresponding Author:
Prof. Dr. H. Thermann
69115 Heidelberg
Bismarckstr 9-15
Atos — Praxisklinik, Germany
Phone: 0049-6221-983-196
Fax: 0049-6221-983-199
E-mail: thermann@atos.de

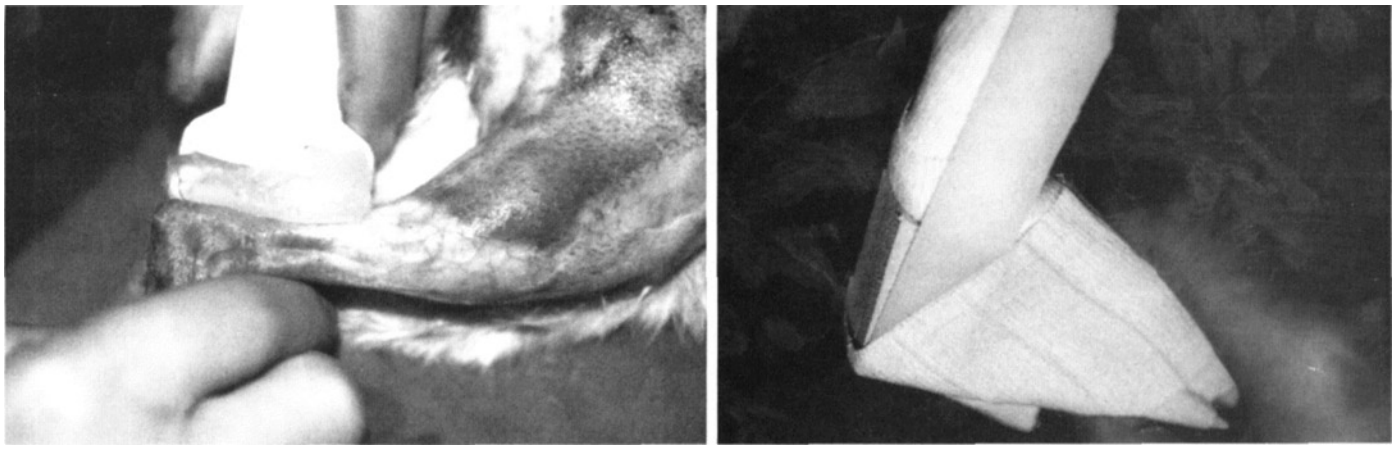


Fig. 2: Special orthosis for functional aftertreatment.

MATERIAL AND METHODS

One hundred and five female chinchilla rabbits, weighing 2,900-3,200g, were divided in three groups:

A. nonoperative functional treatment

B. absorbable tendon suture and functional treatment

C. fibrin glue and functional treatment

Experimental Achilles tendon rupture was produced under IV anesthesia in a standardized manner using a paratendineal incision of cutis, subcutis and fascia. After resection of the musculus plantaris, the Achilles tendon was sliced five times in longitudinal direction 1 cm proximal to the insertion. With an oblique tenotomy a realistic mob-end tear with fringed tendon stumps was achieved (Fig. 1).

In group A the wound was then closed with a running suture of the fascia and interrupted skin sutures. After ultrasound examination of the rupture site a sterile dressing was applied.

The tendon stumps in Group B were additionally sutured with 5x0 PDS (Ethicon, Hamburg, Germany) in plantarflexion.

In Group C animals received 1 mm of fibrin glue (Tissucol, Immuno, Wien, Austria) in the tenotomy site before wound closure.

All animals received a special orthosis that was taped to the limb (Fig. 2). In this device the ankle was held in 20 to 30° plantarflexion by a cuneiform rubber sole and torsion was prevented by two side-splint reinforcements. Thus the orthosis resembled an Achilles tendon boot for functional treatment in humans.

The rabbits were kept in groups of 21 in hutches of 25 m^2 in area and were allowed to move freely. Dressings and the orthosis were changed regularly during wound healing and for ultrasound examinations.

Seven animals in each group were killed after one, two, four, eight and 12 weeks, followed by immediate ultrasound examination and tendon explantation.

Ultrasound examinations were performed using a 7.5-MHz linear array transducer (Picker 9200) (Fig 3).

Tendon tissue was immediately fixed after harvesting and stained in a standard Masson-Goldner or Azan technique or with anti-human collagen type-III antibodies, respectively. Stained specimens were analyzed with a 25-250-fold magnification.

A semiquantitative fibrocyte count was performed on specimens from the central area of the regenerating tendon tissue. One hundred chambers were counted with an 80-fold magnification.

Statistical analysis was performed with an ANOVA-test and the Tukey-HSD-test with a significance level of p 0.05.

RESULTS

Histology

One week: An equal gap between the tendon stumps is observed in all groups.

In group A the gap is filled with granulation tissue that consists mainly of longitudinally aligned fibroblasts. Collagen fibers can be observed in the extracellular matrix near the peritenon. The tendon stumps seem to be necrotic without a sign of increased metabolism.

In group B the granulation tissue consists mainly of fibroblasts that are not aligned.

In group C the gap is mainly filled with fibrin glue. Around the fibrin are cells of the lymphatic system, granulocytes and macrophages. Fibroblasts and extracellular fibers are found near the peritenon and seldom inside the fibrin clot (Figs. 4a-c).

Two weeks: In group A the gap is filled with longitudinally aligned granulation tissue. Young fibroblasts with light cytoplasm are dominating in central areas. Beginning angiogenesis enters the still necrotic tendon stumps from the peritenon.

Group B shows a non-homogeneous picture of regeneration. A partial longitudinal alignment appears

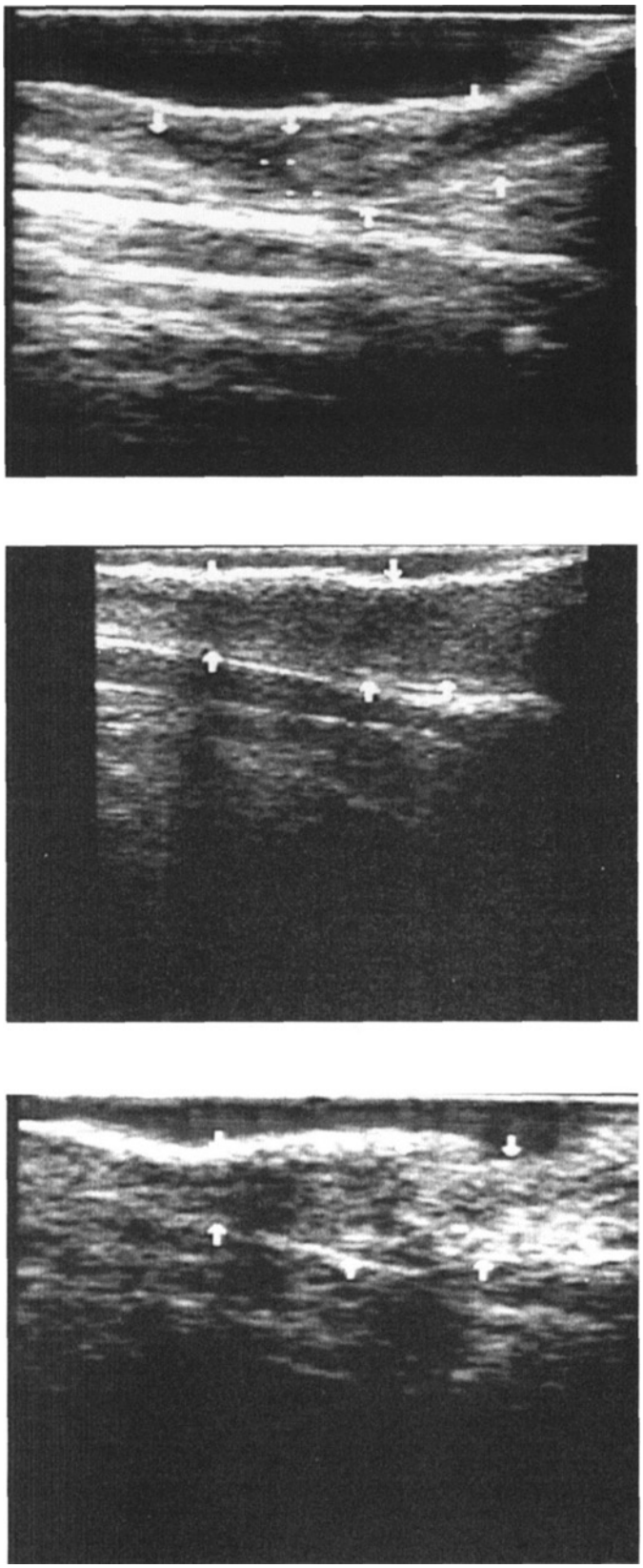


Fig. 3: Technique of ultrasound examination. Typical results after two weeks of regeneration. (**a**, *top*) suture (**b**, *middle*) fibrin glue (**c**, *bottom*) nonoperative treatment.

between the stumps. The suture material is partially resorbed.

In Group C the fibrin clot is invaded by lymphatic cells and clearly reduced in size. The tissue alignment is not as organized as in group A.

Four weeks: Group A shows homogeneous and intensively stained scar tissue with parallel fibers. Numerous fibroblasts lie around the borders of the scar tissue. Lymphatic cells and resorptions can be seen within the tendon stumps.

In group B the inhomogeneous picture remains partially. Between parallel and longitudinal fibers are regions of immature granulation tissue where fibers run at angles to the surrounding tissue (Figs. 4d-f).

The fibrin clot in group C is totally resorbed. The tissue is less organized than in group A.

Eight weeks: In group A tendon tissue fills the gap that is largely connected to the tendon stumps. Macrophages appear rarely in connective tissue between the collagen fibers.

The new tendon tissue in group B is inhomogeneous and shows signs of resorption.

Group C is less homogeneous than Group A. In some areas the fibers do not run parallel. Macrophages can be found between the old and new tendon tissue.

Twelve weeks: In all three groups the regenerated tissue differs only marginally from the original tendon under microscopic aspects. The number of fibrocytes is increased in all groups and in group B and C the fibers are slightly less parallel (Figs. 4g-l).

Immunhistochemistry

In specimens of a control group there was no reaction to collagen type III antibodies in the tendon tissue.

One week after the operation many positive reactions can be seen in the granulation tissue around the fibrocytes. Only a weak reaction can be found in the tendon stumps around the areas of the beginning angiogenesis. This picture is similar in all three treatment groups. In group A a high number of positive cell-associated reactions around extracellular fibers can be found near the stumps and in the center of regeneration. A similar reaction with a lesser number is found in the fibrin group. The number of reactions in the suture group is, especially in the center of regeneration, considerably less than in the other groups.

After two weeks, the number of positive reactions has grown in all groups. The positive reactions are concentrated in the areas where the regeneration is already parallel aligned.

The number of reactions after four weeks is declining in all groups. This comes together with a further alignment and condensation of the regenerating tissue and is therefore less obvious in the suture group. The tendon

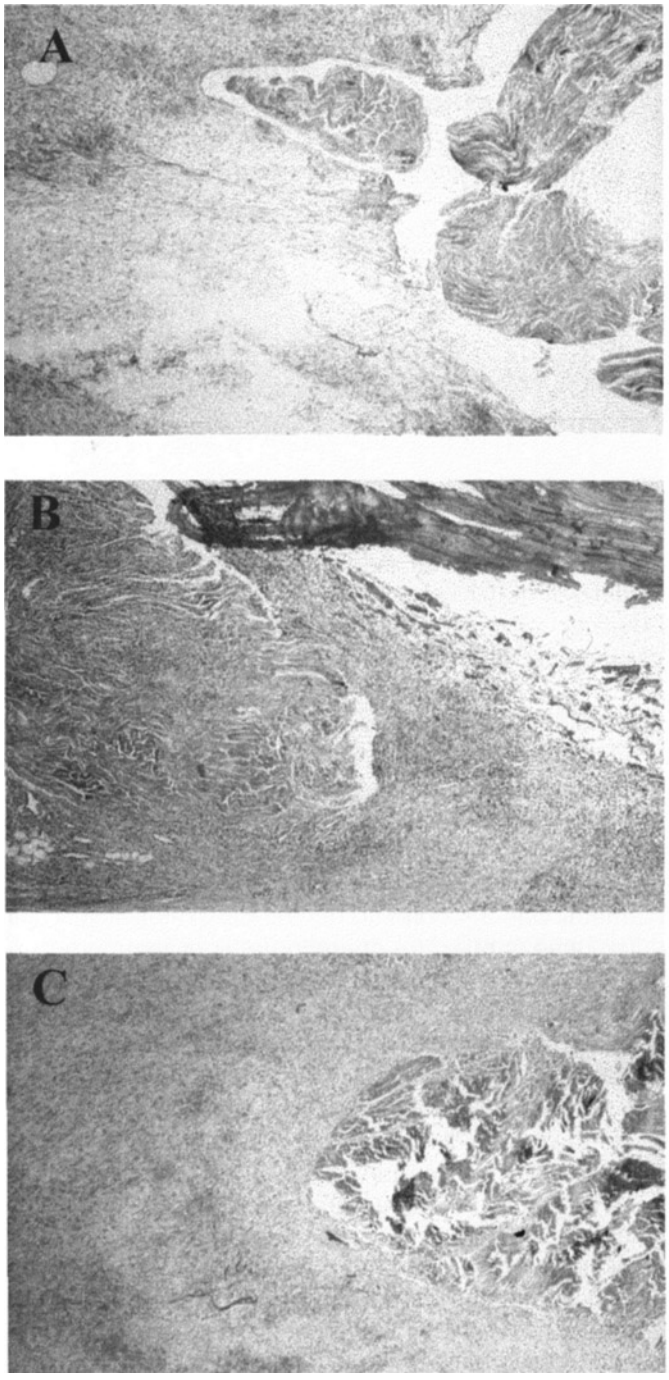


Fig. 4a-c: Histology after one week. **(a)** nonoperative **(b)** suture **(c)** fibrin glue.

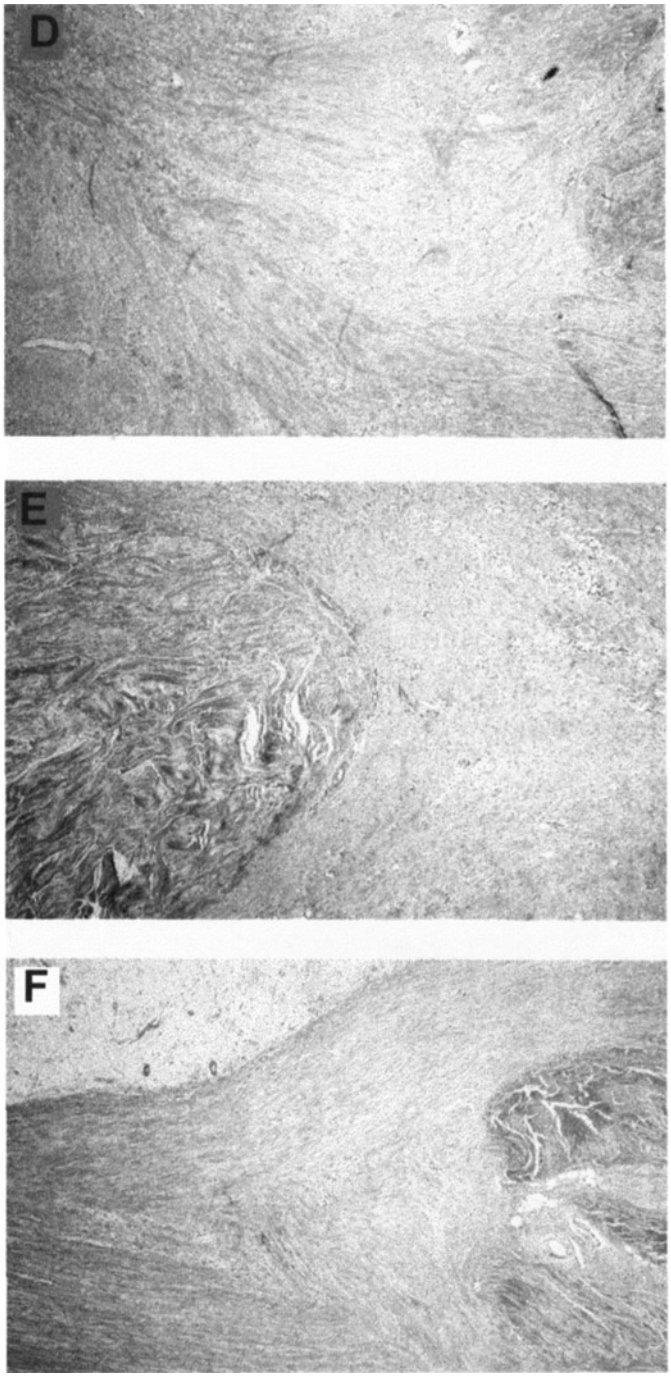


Fig. 4d-f: Histology after four weeks. **(d)** nonoperative **(e)** suture **(f)** fibrin glue.

stumps can be differentiated from the surrounding tissue by a number of positive reactions inside the stumps.

After eight weeks the picture is similar in all groups with a further decline in positive reactions.

Twelve weeks after the operation the neotendons in all groups can still be differentiated from the original tendon by a small number of positive reactions and by the higher number of fibrocytes.

Semiquantitative Fibrocyte Count

In control-group specimens, a mean number of 54.9 fibrocytes per counting chamber was found.

After one week group A showed 279.4 cells and group B 169.9. This difference was of statistical significance with $p<0.05$. A cell count in group C was not possible because the area of interest was filled with the fibrin clot.

Two weeks after the operation 233.3 cells were found

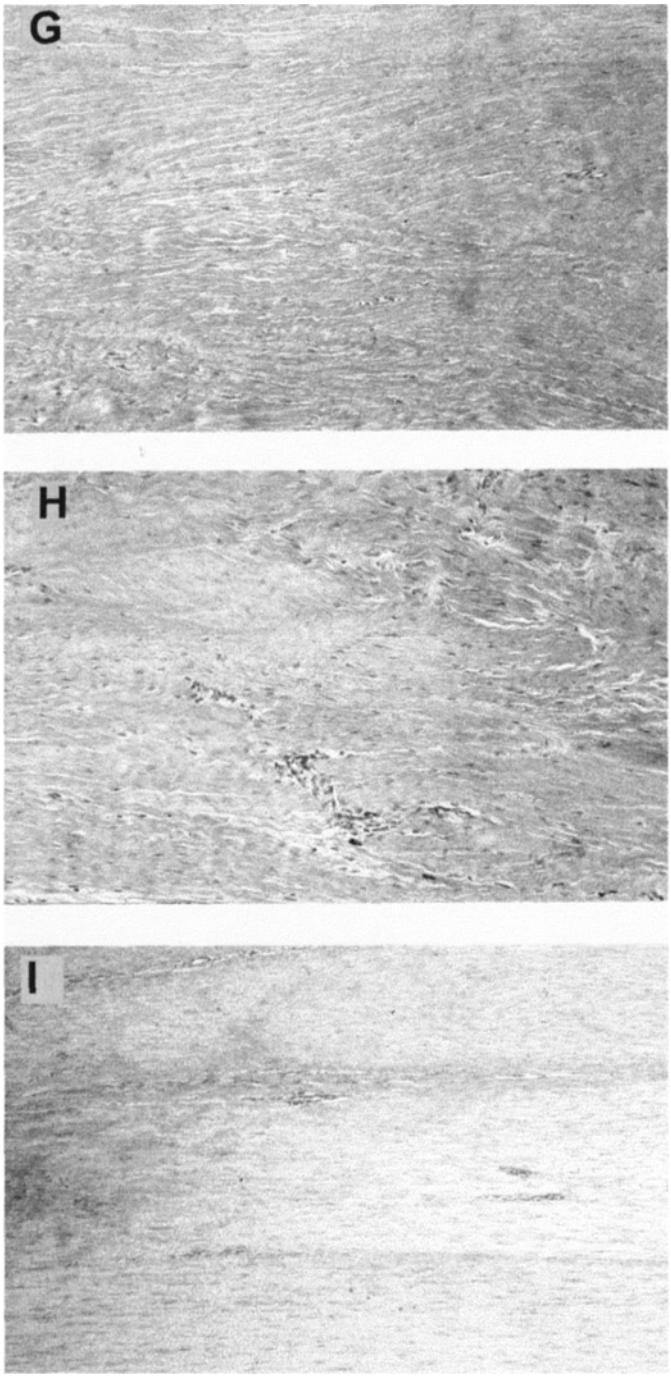


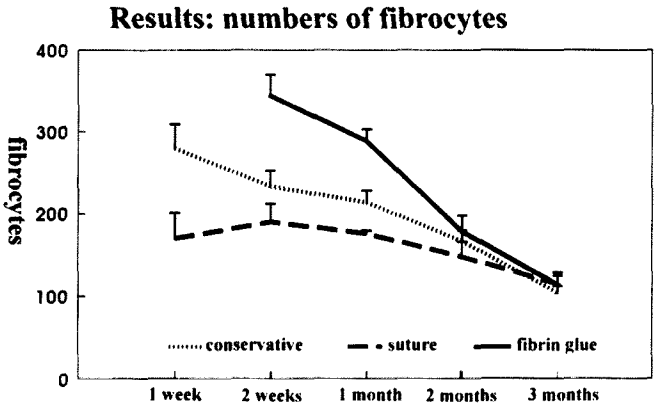
Fig. 4g-i: Histology after 12 weeks **(g)** nonoperative **(h)** suture **(i)** fibrin glue.

in group A, 189.6 in group B and 343.9 in group C. These differences were all significant ($p<0.05$).

The mean number of fibrocytes declined after four weeks in all groups. 213.6 were counted in group A, 175.4 in group B and 288.4 in group C. Differences were all statistically significant with $p<0.05$.

After eight weeks the cell number declined further in all groups. The mean number of cells in group A was

Table 1: Semiquantitative fibrocyte count



166.3, in group B 147.3 and in group C 178.1. The difference between the suture and the fibrin group was significant ($p<0.05$).

No significant differences were found between the groups after 12 weeks. In group A 104.4 fibrocytes were found per chamber, in group B 114.7 and in group C 113.3 (Table 1).

Ultrasound Examinations: Tendon Diameter

The diameter of the tendon one centimeter proximal to the insertion was measured with ultrasound examination in all animals prior to the operation. The mean diameter was 3.42 mm.

Two weeks after the operation a significant increase in tendon diameter was found in all groups. Mean diameter in group A was 4.75 mm, in group B 4.7 mm and in group C 4.6 mm. The differences were not significant.

A further thickening was observed after four weeks when the mean diameter in group A was 5.14 mm, in group B 4.89 mm and in group C 5.2 mm. The difference between group A and B was significant ($p<0.05$).

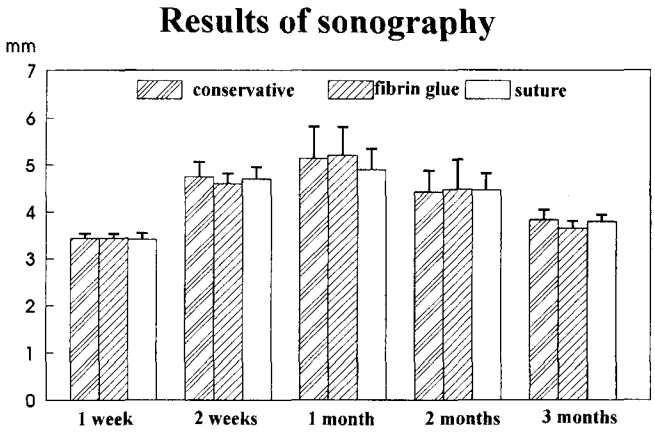
At eight weeks the diameter decreased and no significant differences were found with mean values of 4.43 mm in group A, 4.47 mm in group B and 4.48 mm in group C.

After 12 weeks the mean tendon diameter was 3.83 mm in group A, 3.8 mm in group B and 3.65 mm in group C. These values differed significantly from the preoperative values in all groups ($p<0.05$) (Table 2).

Qualitative Analysis of the Ultrasound Examination

The preoperative picture of all tendons showed the typical picture of healthy tendon tissue with parallel internal echo structure and echogenic peritenon. The serial ultrasound examination after the operation showed patterns comparable to those already described in human Achilles tendon healing.[25]

Table 2: Tendon diameter measured by ultrasound examination.



The initial postoperative examination showed a hypoechoic region at the tenotomy site in group A and C, whereas a thickening of the tendon with an irregular echo pattern appeared in group B.

After two weeks fluid-rich tissue with poor internal echo structures was found in a gap zone between the tendon stumps in all groups.

After four weeks the gap zone was thickening with an increase in irregular echo patterns consisting of short and nondirect parallel echo structures. The most irregular echo pattern appeared in group B.

At eight weeks the tendon thickness was decreasing in all groups and the echo structures became longer and developed a more parallel pattern despite some less echogenic areas especially in group B.

Twelve weeks after the operation the ultrasound examination showed in all groups results comparable to the preoperative examination.

DISCUSSION

Histology

The histological picture of tendon regeneration in our study resembles the already known phases with initial capillarization, inflammatory response and formation of cell-rich granulation tissue.[6,7,29]

Comparison of treatment modalities after one week revealed gross differences between the groups. Nonoperative treatment resulted in typical regeneration with a harmonic impression of aligned fibroblasts. In the sutured tendons the dehiscence with intersected tendon stumps contrasted this picture and in the fibrin treated group the fibrin clot prevented a direct contact of the stumps.

After two weeks the groups started to give a similar appearance, but the nonoperated group showed the most regular texture of regeneration. In the fibrin group the positive effect of fibrin glue on the fibrocyte cell count was noted as described by Haas et al.[11] The regenerating tissue in this group grew around the clot and so the fibrin did not have, as seen by Bösch et al.,[3] a positive influence of direct regeneration between the stumps.

Bösch et al.[3] described a complete regeneration of fibrin-treated rabbit tendon ruptures after four weeks. This results have not been retraced in our study. After four weeks the tissue in our fibrin group showed partially aligned fibers and immature areas with many fibroblasts. In the suture group the dehiscence is filled with regeneration tissue, and a similar picture with mature and immature areas was seen. The superiority of fibrin glue compared to tendon suture concerning fiber alignment, fiber and cell count as found by Haiböck et al.[12] was only seen for the cell count in our study. Rather showed the conservative group the most homogen and matured regeneration tissue.

After eight weeks the superiority of the nonoperated tendons concerning fiber alignment was still observed, but after 12 weeks all three groups had a similar appearance and resembled intact tendon tissue with a slightly higher cell count. Thus the end product in all groups seemed to be of identical quality. This supports the steady state of connective tissue regeneration as postulated by Vidiik et al.[29]

In the late stadium of regeneration, at eight to 12 weeks, the tendons in our study seem to be more matured than described in previous studies. In contrast to Mohr,[17] who described the tissue at this time as scar tissue with irregularly shaped elastic fibers, the regenerated tendon in our study resembles original tendon tissue. The time needed to reach the histological stadium seen in our study at 12 weeks is said to be up to one year.[29] This acceleration cannot only be explained with the faster regeneration in rodents. We believe that functional aftertreatment is the major influence for early alignment of tendon fibers as seen in previous studies.[30] Brown et al.[4] reported an incomplete collagen fiber formation and low fiber counts after nonoperated tendon ruptures and 10 weeks of plaster cast immobilization. Roberts et al.[22] found nonorganized granulation tissue with weak fiber orientation in rabbit tendon ruptures after 10 weeks of immobilization and further 10 weeks of mobilization. Thus our study proved the positive influence of functional aftertreatment on tendon regeneration as also shown by Schatzker,[23] Lipscomp[14] and Muneta.[18]

Collagen Type III

According to Fleischmajer et al.[8] collagen type III consists of thin fibrils with a diameter of up to 60 nm, whereas collagen type I has a diameter of more than 100 nm. Bailey et al.[1] found a high number of type III

fibers in the initial regeneration tissue in tendon healing as was seen in our study. This finding was confirmed in immunhistochemical and biochemical studies.[10,30] While the control tendons in our study showed a weak positive reaction, the ruptured tendons had a strong reaction throughout the experiment. Postacchini et al.[21] found a high number of small-diameter fibrils 30 weeks after a tenotomy. These findings underline that collagen type III is the typical sign for regenerated tendon tissue. Our observations, as those made by Vidiik et al.,[29] show that the quality of the original tendon tissue is not reached after trauma and regeneration.

Ultrasound Examination

The results of our animal experiment confirm the results of clinical studies performed by our study group[26,27,28] when the faster regeneration of rodent tendons is considered. The maximum tendon diameter in rabbit tendons was found after four weeks whereas the human tendon has its maximum at about 12 weeks. This growth of tendon diameter was also found in other studies.[2,5,9,13] The value of tendon diameter measurement is controversially discussed in the literature[9,13,15] because the results are dependent on the examiner. The examination plane has to be exactly at a right angle to the tendon, otherwise the diameter appears to be larger.[9,16,25,27] For this reason the ultrasound was performed in a standardized way by only one examiner in our study.

When the time of maximum tendon diameter is seen as the peak of tendon regeneration, the sutured tendons lag behind the two other groups. This confirmed the problem of secondary dehiscence of the stumps with disturbance of regeneration.[20] The decrease in diameter after eight weeks in all groups showed the organization of the new tissue to tendon tissue. This conversion has, to our knowledge, not been observed at this early point of regeneration in other studies. This indicates the positive influence of functional treatment on tendon regeneration.

In accordance with the course of tendon diameter the examination after two and four weeks showed tissue with low echogenity as a sign of connective tissue with a low number of collagen fibers. Few thin echo patterns can be seen in the early stadium of regeneration and in the later examination the pattern shows broader and more longitudinally aligned echoes. After three months the picture resembles intact tendon tissue.

The ultrasound findings in this study can be correlated to the classification of tendon healing phases in humans.[26,28] After two weeks the findings corresponded to grade four of this classification with a lack of oriented echoes (Fig. 3). Few oriented and parallel echoes as described as a grade three were found after four weeks. Thin and aligned echoes which lay apart were observed after eight weeks and can be compared to grade two in humans. The echo pattern after 12 weeks with the picture of intact tendons and many broad and parallel echoes is described as grade one.

The harmonic course of tendon healing observed in the conservative group when compared to the other groups may be an evidence for the superiority of this treatment in our study.

Correlation of Histological Results and Ultrasound Examinations

The comparison of histological phases of regeneration with the according ultrasound examination is of importance for clinical monitoring of regenerating tendons.

After two weeks the poor internal echo structures with single echo reflexes in the conservative group corresponded to the histological picture of pale stained granulation tissue with partially longitudinally aligned fibroblasts. The histological difference to the fibrin group was not observed in ultrasound examination. In contrast to this, the histological differences of this groups to the suture group was found in ultrasound examinations. The non-homogenous histological picture with secondary dehiscence of the stumps corresponded to the irregular internal echo pattern of the dehiscent tendons.

The beginning condensation of collagen fibers with building of parallel fiber bundles after four weeks correlated with an increase in short and nondirect parallel echo structures.

The further maturation of the regenerating tissue after eight weeks with histologically seen parallel fibers and a decrease of fibrocytes corresponded to a further increase of echogenity and a more parallel pattern. Scattered areas of resorption as seen in the fibrin and the suture group resulted in regions of poor internal echo structures.

After 12 weeks the histological picture and the ultrasound examination showed almost intact tendon tissue.

According to the mentioned correlations between histological and ultrasonographic examinations, the following conclusions are resulting for clinical application:

1. Poor internal echo structures correspond with immature granulation tissue
2. An increase of echogenity is induced by maturing of functionally adapted scar tissue
3. Longer and more parallel echo structures indicate mature tissue with parallel aligned fiber bundles

As a conclusion of the experimental results, the functional aftertreatment of Achilles tendon ruptures can be seen as an important stimulus to a fast and secure regeneration of tendon tissue. The comparison of the healing course after different treatment schemes shows a slight advantage of the conservative functional treat-

ment during the early phases of regeneration which diminishes in the end of observation time in this study. A further conclusion is that tendon suture and fibrin glue seem unnecessary when functional bracing is used to treat Achilles ruptures after ultrasonic confirmation of well adapted tendon stumps.

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