The influence of different footprint preparation techniques on tissue regeneration in rotator cuff repair in an animal model

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Abstract

Introduction: Rotator cuff tears are common diseases of the upper extremity. There are no recommendations to the surgeon on how to prepare the footprint to ensure optimal tendon-to-bone healing. However, biologic augmentation using stem cells and growth factors is considered to encourage the healing process of the tendon. The aim of the study was to investigate the biomechanical and histological outcome of different footprint preparations in rotator cuff repair.
Material and methods: One hundred and eighty-nine Sprague-Dawley rats were randomly assigned to either spongialization, radiofrequency ablation or an untreated control group. Rats were killed after 1 or 7 weeks for histological evaluation or after 7 weeks for biomechanical testing.
Results: Histological evaluation showed better tissue organization in the control and spongialization group compared to the radiofrequency ablation group. The highest collagen I to collagen III quotient was found in the control group, followed closely by the spongialization group. Measured quotients showed a decrease in the values after 1 week compared to the values after 7 weeks, except in the radiofrequency ablation group, where an increase was detected. A significant difference was found in the load to failure test comparing the radiofrequency ablation group to the spongialization group ($p = 0.0409$) and control group ($p = 0.014$), but not comparing the spongialization group to the control group ($p = 0.2456$).
Conclusions: The results of this study suggest that spongialization of the footprint before attaching the torn supraspinatus tendon can lead to better structural properties and higher quality of tendon-to-bone restoration at the insertion area when compared with radiofrequency ablation.

Key words: rotator cuff repair, tissue engineering, tendon-to-bone healing.

Introduction

Rotator cuff tears are among the most common diseases of the upper extremity. According to other studies, the incidence in the population older than 60 years is up to 30% and for people over 70 years even up to 50% [1–4]. Therefore it is not surprising that conservative and especially operative treatments of rotator cuff tears have been optimized in

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the last decades. Studies investigating the outcome of arthroscopic repair showed good short-term results in pain reduction and functional outcome [5, 6]. But they also reported a high number of recurrent defects, probably due to an insufficient healing process [5–9]. Therefore attention has now shifted from biomechanical (e.g. different types of suture anchors/techniques) to biologic aspects of tendon healing in order to improve the healing process at the tendon insertion area [10–14]. Several studies describe a positive effect of biologic augmentation using stem cells, platelet-rich plasma or growth factors [15–21]. Nevertheless, still none of the new methods can be used without caution, and further studies will be necessary [10]. Based on the results of Randelli *et al.* [22], where release of stem cells and growth factors from the cancellous bone after acromioplasty could be shown, we hypothesized that spongialization of the footprint may result in enhanced tendon-to-bone healing.

To our knowledge, St Pierre *et al.* [23] were the first to try to find improvements in tendon-to-bone healing. Based on a goat model the released tendon was reattached to either cancellous or cortical bone without significant improvement in the healing process or biomechanical testing. A more recent study by Levy *et al.* [24] used cannulated humeral implants in rats in order to enable stem cells and growth factors to migrate from the cancellous bone into the insertion area of the reattached tendon. Also in this study a significant influence could not be confirmed. However, Kida *et al.* [19] demonstrated that drilling into the bone marrow in the greater tuberosity before reattaching the tendon leads to higher bone marrow cell occurrence in the rotator cuff and better outcome in biomechanical testing.

The aim of this study was to investigate the outcome after rotator cuff repair in a rat model using different footprint preparations. We obtained biomechanical and histological measurements to evaluate postoperative healing of the tendon insertion area. The hypothesis of this study was that footprint spongialization can improve the outcome of rotator cuff healing, possibly by releasing growth factors and stem cells, and that using a radiofrequency ablation device has detrimental effects on the healing process.

Material and methods

Study design

Inspired by previous recommendations about anatomical similarities [25, 26], we used a rat model. One hundred and eighty-nine female Sprague-Dawley rats at an age of 3 months and with an average weight of 250 g were obtained after approval of the Governmental Animal Care and Use Committee (permit number 55.2.1.54-3532). All animals were randomly assigned to 1 of 3 groups: spongialization, radiofrequency ablation or untreated control group. Each group consisted of 55 rats for biomechanical testing plus 8 rats for histological testing. The animals assigned to histological evaluation were split into two subgroups of 4 animals each depending on the recovery time. Euthanasia took place after 7 weeks for biomechanical testing, and after 1 or 7 weeks for histological testing (Figure 1).

Surgical technique

The right shoulders of the rats were operated on under anesthesia with isoflurane and intramuscular injection of 50 mg/kg ketamine. During surgery anesthesia was maintained by isoflurane and oxygen via a nose cone. In addition, every rat received a subcutaneous injection of enrofloxacin 2.5 mg/kg for antibiosis. To avoid variation in the surgical procedure all operations were performed by a single surgeon (AF). After shaving the shoulder region the animals were operated on in a lat-
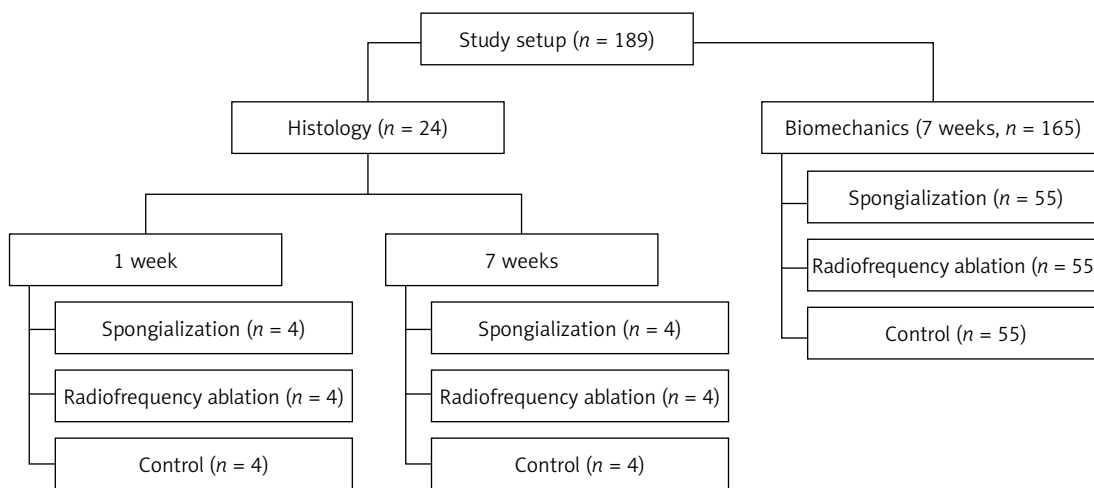

Figure 1. Study setup. Listing of the experimental groups used in the study

eral position on a heating pad and under sterile conditions. All animals underwent only unilateral detachment and repair of the right supraspinatus tendon. After skin incision the deltoid was split and the acromioclavicular joint was divided to reveal the rotator cuff. The supraspinatus tendon was identified and then cut off at the insertion to the greater tuberosity. The footprint was now prepared in three different ways, depending on group membership. In one group ($n = 63$) the cortical bone was removed by a fine burr (PROXXON Micromot 50/EF, Proxxon, Föhren, Germany) to expose cancellous bone. In the other group ($n = 63$) a monopolar radiofrequency ablation device (Cool-Cut 45 SJ, Arthrex, Germany) was used to ablate all soft tissue. In the last group ($n = 63$) the supraspinatus tendon was only reattached without any further treatment of the insertion area. For the reattachment of the tendon we used a 5-0 double armed Prolene suture (Ethicon, Somerville, NJ), which was passed through the tendon to perform a Mason-Allen suture. On the greater tuberosity, 2 mm from the articular surface, two tunnels were drilled, each 0.5 mm, to pass the suture ends through the bone and tie them over the humeral cortex to reconnect both ends of the supraspinatus tendon. The deltoid split was closed with a 4-0 Etibond and the skin with 4-0 Monocryl absorbable suture (Ethicon). Postoperative animal care was performed according to the guidelines provided by the Institutional Animal Care and Use Committee for discomfort, distress and pain. Pain was managed with buprenorphine 0.05 mg/kg body weight subcutaneously directly after surgery and additionally given after 6 and 12 h.

Histology

After dissection of the humerus with the attached supraspinatus tendon and the affiliated muscle the specimens were directly fixed in 4% neutral-buffered formalin (Micros GmbH, Garching, Germany) for 48 h. For decalcification we used EDTA-4Na 20% citric acid for at least 21 days. Dehydration and paraffin embedding were performed by a tissue processor (Hypercenter XP, Thermo Scientific Fisher, Schwerte, Germany). Sections were cut at a thickness of three micrometers in the coronal plane and dried overnight at 50°C. Besides hematoxylin-eosin, immunohistochemical staining for type I and type III collagen positive areas (Novus Biologicals, Littleton, CO) was performed. Sections stained with hematoxylin-eosin were qualitatively evaluated for vascularity, inflammation and organization of the collagen tissue at the tendon-bone interface as well as integrity of the epiphysis and the tendon. From each specimen, 5 different sections were obtained and evaluated by 3 investigators (MS, MP and AF)

who were blinded regarding the specimen group assignment. For staining of collagen type I and III positive areas we used pairs of sequential sections, allowing for a direct comparison between the differently labeled sections. Digital images were taken with an Axio Cam MRc5 camera (Zeiss, Göttingen, Germany), attached to a Zeiss Axioskop 40 light microscope (Zeiss, Göttingen, Germany). To prevent variations in illumination and magnification, all pictures were recorded by the same person (MS). From each pair of recorded sections an identical excerpt of the tendon-to-bone interface was made. Therefore an area of 500 × 500 pixels was chosen. These excerpts underwent further evaluation using computerized image analysis software (Image J, National Institutes of Health, Bethesda, Maryland, USA). Color intensity (i.e. below and above the threshold) and color amount (i.e. positively stained area) were measured by the analysis program by converting the excerpts into 8-bit black-and-white pictures after using color filters. Measurement of the amount of black pixels showed the area of collagen type I or III in the 500 × 500 pixel frame. Using the same three different filter settings for every specimen, an unbiased objective evaluation was achieved. The measured areas of a pair were used to determine the ratio between collagen I and III in the different groups of footprint preparation.

Biomechanical testing

All specimens selected for biomechanical testing were stored in a freezer at –30°C directly after the dissection. Before testing they were thawed at room temperature. The testing was performed blinded to the groups. The humerus with affiliated supraspinatus tendon and muscle was carefully dissected from surrounding tissue. Afterwards the supraspinatus muscle was stripped from the tendon. The diameter of the exposed tendon was measured with a micrometer and cross-sectional area was calculated. Inspired by previous studies [27–29] the biomechanical testing followed. The humerus was embedded in a custom-made aluminum cylinder using polymethylmethacrylate. The specimens were then placed in a Zwick Universal Testing Machine (model Z010/TN2A, Zwick, Ulm, Germany). Using sandpaper and cyanoacrylate (Pattex Ultra Gel; Henkel, Düsseldorf, Germany) the tendon was placed in between two aluminum clamps, which were screwed together. Afterwards the force was measured by a transducer with a measurement range of up to 100 N and a measurement uncertainty of 0.2%. All tests were performed with the shoulder in a simulated position of 90° abduction. To ensure that all samples start from the same "zero load" state, a preload to 0.2 N was performed first, followed by five cycles of

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preconditioning at 5% grip-to-strain at a rate of 0.1%/s. Thereby a consistent load history was given for each sample. A constant strain rate test to failure was performed at the end.

Statistical analysis

Visco-elastic parameters were compared with the nonparametric Mann-Whitney U test (Graph-Pad Prism software, version 5.02 for Windows; GraphPad Software, San Diego, CA). Strong control for multiple comparisons of groups regarding the primary endpoint at the two-sided 0.05 level of significance was pre-planned by closed testing (principle of Randelli *et al.* [22]); this enhanced nonparametric Dunnett-type testing of the footprint preparation groups versus the control group (Steel test) for all pair-wise comparisons (subordinately including the comparison of the footprint preparation groups). Experience regarding the load of failure in the control group from a former experiment yielded estimated means (15.7 N/mm^2 for operative specimens (SD, 4.0 N/mm^2) and 20.3 N/mm^2 for nonoperative specimens) but with suspicion of relevant deviations from the normal distribution. Originally, 3 footprint preparations with 60 rats calculated per group were planned in the protocol. Feasibility forced us to reduce the number of preparations to 2, and the number of rats was recalculated to be 55 per group. The sample size aimed to detect a relevant difference of 2.5 N/mm^2 between at least 1 of the footprint preparations and the control group with a power of 80%, assuming normal distributions with an SD of 4 N/mm^2. The calculations were based on the closed testing procedure using the correlated Wilcoxon-Mann-Whitney tests but with slightly conservative Bonferroni correction for 2 tests instead of laborious calculations of the Steel critical values. For histologic analysis, the type I and III collagen-positive areas were statistically compared using the t test; all other parameters were qualitative in nature. The number of animals per histologic group (4 per group) was chosen in accordance with similar histologic investigations in several other publications [16, 20, 21].

The parameters of hematoxylin-eosin staining were qualitative in nature and not statistically compared.

Results

No animal was lost during surgery or post-operatively. All animals returned quickly to a normal level of activity, food intake and gait with full use of the involved forelimb. We could not find any significant difference in body weight between the groups at the time of euthanasia.

Histological analysis

The epiphysis was intact in all specimens and not damaged by spongialization. After 1 week the hematoxylin-eosin staining qualitatively showed less vascularity in the control group than in the spongialization group and on the other hand the spongialization group less than the radiofrequency ablation group. The same was seen after 7 weeks. All groups showed higher cellularity after 1 week than after 7 weeks consisting mainly of fibroblasts and inflammatory cells. The radiofrequency ablation group presented the highest amount of cells both after 1 week and after 7 weeks, whereas the spongialization group showed after 1 week only slightly higher cellularity than the control group and equal after 7 weeks. The interface tissue and fiber orientation showed better organization in control and spongialization groups compared to the radiofrequency ablation group. All groups became progressively more organized after 7 weeks. After 1 week the decorticalized area of the spongialization group was highly filled with connective tissue, which regressed after 7 weeks. Some sections of the radiofrequency ablation group showed an unorganized and heterogeneous insertion area with uncertainty of fully recovered and grown on tendon (Figure 2).

Quantitative analysis of immunohistochemical labeling for type I and type III collagen positive areas revealed that the spongialization and the control group had significantly more type I collagen in relation to collagen III than the radiofrequency ablation group ($p < 0.05$) after 1 week. There was no statistically significant difference between the control and the spongialization group. After 7 weeks we did not observe any statistically significant difference between the groups (Figures 3 and 4).

Biomechanical analysis

Three specimens of the control group and two specimens of each of the spongialization and the radiofrequency ablation group were lost to biomechanical testing because of a ruptured supraspinatus tendon while stripping off the muscle. Another 8 specimens of the control group, 9 of the spongialization and 2 of the radiofrequency ablation group were lost due to a broken humeral head while testing force at failure. The cross-sectional area of the tendon was significantly smaller in the radiofrequency ablation group (0.07 ±0.03 mm²) compared to each of the other two groups (spongialization group 0.14 ±0.08 mm², control group 0.12 ±0.08 mm², each $p < 0.0001$). The spongialization group compared to the control group showed no significant difference ($p = 0.1178$).

Similar results were obtained in the load to failure test. We found a significant difference
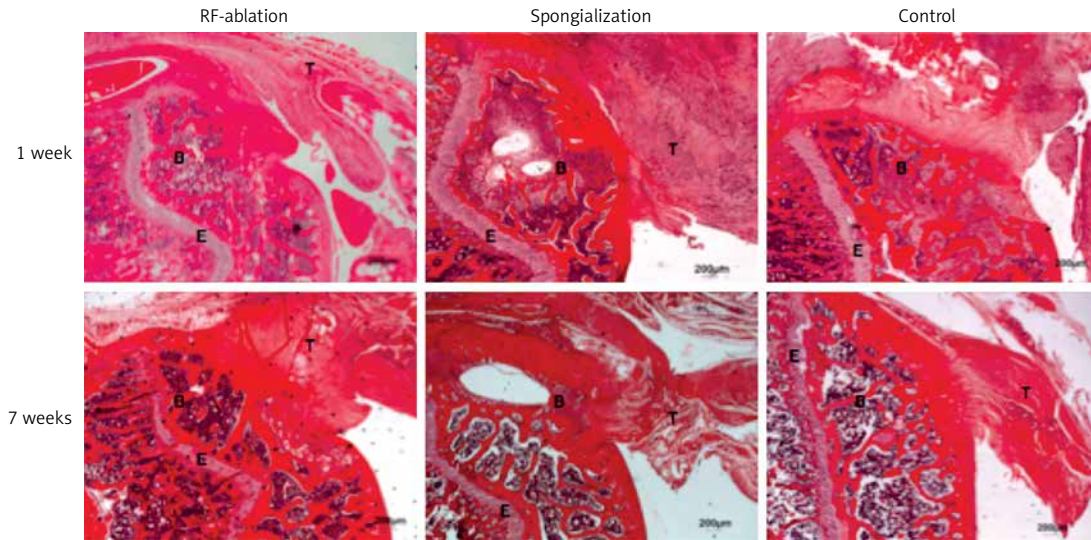
Figure 2. Hematoxylin-eosin staining of radiofrequency ablation, spongialization and control groups after 1 and 7 weeks. After 1 week, specimens from the control group showed less cellularity and less vascularity than the other groups. There were fewer cells and less vascularity in the spongialization group than in the radiofrequency ablation (RFA) group. The transition tissue in the control and spongialization groups was better organized and more inter-digitated than that in the RFA group. In the spongialization group, it could be observed that the decorticated area filled with blood and connective tissue. There were still more cells after 7 weeks in the RFA group. Fiber orientation was disorganized and heterogeneous

B – bone, E – epiphysis, T – tendon.
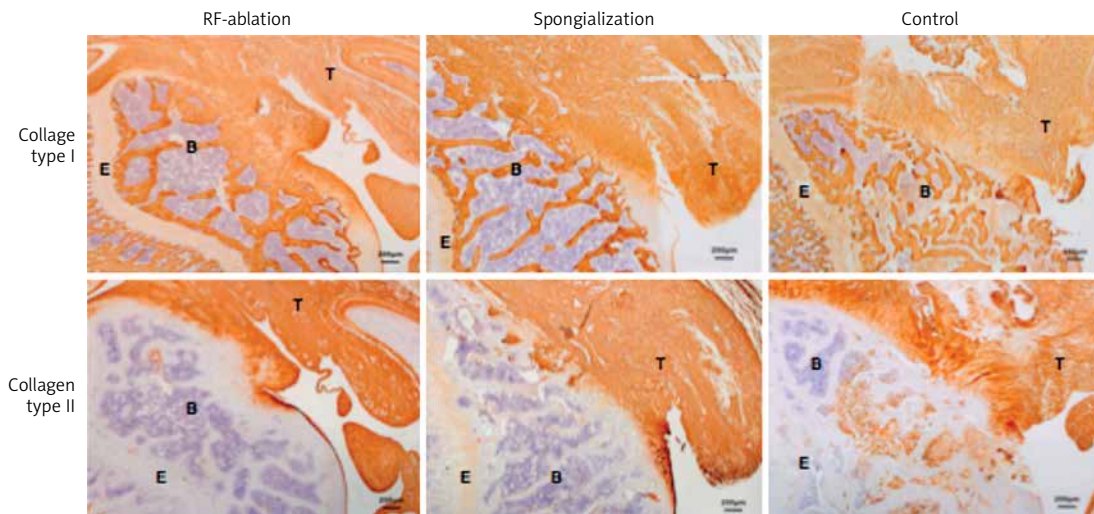
Figure 3. Immunohistochemistry for type I and type III collagen in radiofrequency ablation, spongialization and control groups after 1 and 7 weeks. More collagen type I is present in the spongialization and control groups when compared with the radiofrequency ablation group

B – bone, E – epiphysis, T – tendon.

between the radiofrequency ablation group (15.56 ±4.85 N/mm²) and both the spongialization group (17.51 ±4.46 N/mm², $p = 0.0409$) and control group (19.21 ±5.19 N/mm², $p = 0.0014$). Again there was no significant difference between the control group and spongialization group ($p = 0.2456$; Figure 5).
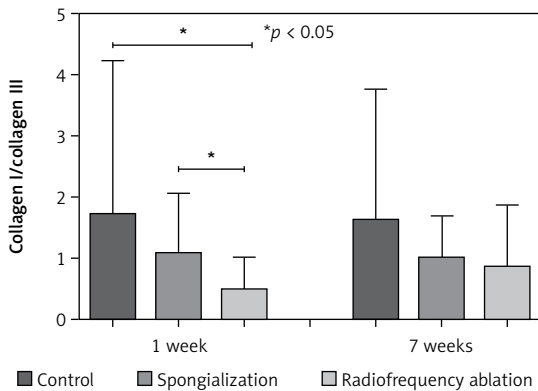
Testing tendon stiffness, the radiofrequency ablation group (52 ±54 N/mm²) and control group (60 ±210 N/mm²) both showed a significant difference compared to the spongialization group (40 ±37 N/mm²), with *p*-values of 0.0437 (control vs. spongialization) and 0.0373 (radiofrequency vs. spongialization). No significant difference was found between the control group and radiofrequency group ($p = 0.2824$; Figure 6).

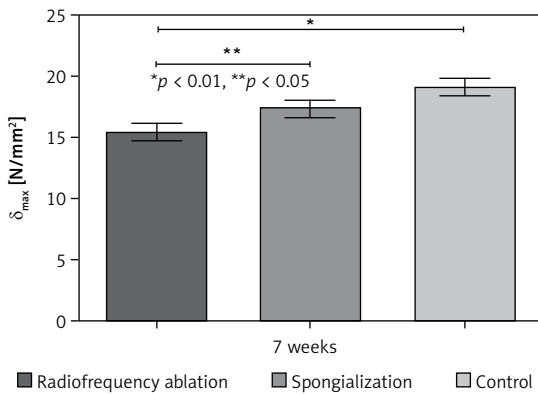The specimens undergoing viscoelastic testing showed significant differences between the radiofrequency group (0.75 ±0.56 N/mm²) and spongialization group (0.43 ±0.32 N/mm², $p < 0.0001$), as well as between the control group (0.6 ±1.8 N/mm²) and spongialization group ($p = 0.0296$). Control and radiofrequency groups did not differ significantly ($p = 0.9791$).

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Discussion

The main goal of this study was to investigate the outcome of different footprint preparations for

Figure 4. Collagen type I to III ratio. The ratio between type I and type III collagen positive areas is significantly higher in favor of type I collagen after 1 week in the control and spongialization groups when compared to the radiofrequency ablation group

Figure 5. Load to failure. In failure testing the probes of the spongialization and control groups showed a significantly higher load to failure when compared with the radiofrequency ablation group. There was no statistically significant difference between spongialization and control groups
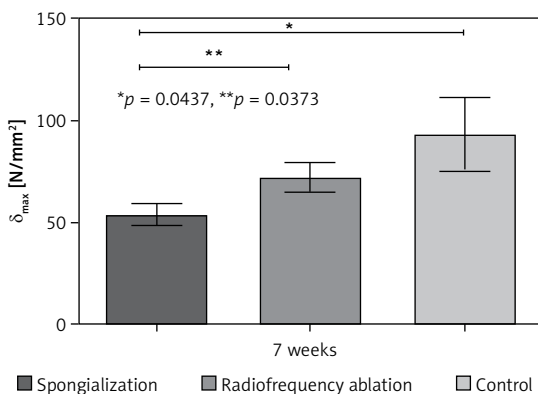
Figure 6. Viscoelastic testing. The specimens undergoing viscoelastic testing showed significant differences between radiofrequency and spongialization groups ($p < 0.0001$), as well as between control and spongialization groups ($p = 0.0296$). Control and radiofrequency groups did not differ

rotator cuff repair in a rat model. In the literature there are no recommendations given to the surgeon on how to prepare the insertion area before reattaching the torn tendon [30–32]. Therefore we evaluated the outcome and healing process after spongialization and radiofrequency ablation compared to a control group with no further treatment. In our study we confirmed the hypothesis that spongialization of the footprint alters the process of tendon-to-bone healing in a positive way when compared to radiofrequency debridement. Both histological and biomechanical analyses showed better results in the spongialization group than in the radiofrequency ablation group.

A previous study by St Pierre et al. [23] analyzed tendon healing to cancellous bone versus healing to cortical bone. Unlike our study they used a goat model with 16 goats for biomechanical testing and 4 goats for histological analysis and bilateral tenotomy of the infraspinatus tendon. With a burr they prepared a cancellous bed before attaching the tendon. At 6 and 12 weeks after surgery the healing processes appeared similar, and in biomechanical testing no significant differences between the groups were found.

Recently Levy et al. [24] published another study based on the same hypothesis. They used cannulated humeral implants with the idea to deliver local bone marrow stem cells into the insertion area to promote a better healing response. Based on a rat model with supraspinatus injury and repair, 10 rats were used for biomechanical testing and 4 rats for histological analysis each after 4 and 8 weeks. They did not observe significant differences between the groups. Levy et al. postulated that the insufficient amount of bone marrow emigration can be explained by the diminutive size of the implants. Indeed, this could have been the problem, as Kida et al. [19] noted a higher amount of stem cells in the drilling group of their study. They used "bone marrow chimeric rats that express green fluorescent protein in bone marrow- and circulation-derived cells". Nine rats were examined each after 2, 4 and 8 weeks following surgery. In the drilling group 6 drill holes were created on the greater tuberosity prior to supraspinatus tendon repair. Biomechanical testing showed a significant higher ultimate force-to-failure in the drilling group than in the control group, and a higher amount of GFP-positive cells was detected in the drilling group.

In our study the whole footprint was spongialized and the cortical bone penetrated. Therefore a direct contact between the supraspinatus tendon and humeral bone marrow was guaranteed. Histologically we could demonstrate better tissue restoration in the spongialization and control groups compared to the radiofrequency ablation group and almost equal regeneration in the spongialization and control groups.

The calculated values of the collagen I to collagen III quotient were lowest in the radiofrequency ablation group and almost equal in the control and spongialization groups. Uninjured tendons of the rotator cuff are believed to contain mainly collagen type 1 and only a reduced amount of collagen III [33], whereas collagen III is largely synthesized in scar tissue of degenerative or ruptured tendons [34]. The calculated averages of the collagen type I/collagen type III quotient showed a decrease in the spongialization and control groups from week 1 to week 7, which means higher occurrence of collagen III in relation to collagen I after a longer healing period. Only in the radiofrequency ablation group did the quotient of collagen I to III increase in the time from 1 week to 7 weeks after surgery. As the radiofrequency ablation group showed poor results in all other evaluations, whether histological or biomechanical, we assume that a higher quotient after 7 weeks correlates with a delayed healing process. This thesis is supported by other studies, where collagen III has been detected in an early stage of ligament and tendon healing [29, 34, 35].

Literature about the use of radiofrequency devices for tendon repair is sparse and differs in recommendations. A study by Tibor *et al.* [36] asserts that "RF-treated tendons showed faster return to mechanical integrity", allowing earlier rehabilitation. However, other studies evidenced thermal complications [37, 38]. The high temperature caused by radiofrequency energy leads to cell death. The use of fluid flow during the procedure, like in arthroscopic treatments, leads to even higher temperatures with corresponding greater damage [37]. Due to our results we agree with these studies and do not recommend using a radiofrequency device for footprint preparation.

From a biomechanical view, we observed a significantly higher average load to failure in the spongialization and control groups compared to the radiofrequency ablation group. Between the spongialization group and control group a significant superior load to failure could not be demonstrated. We suppose that the rats' high intrinsic healing potential could be responsible for that. As we found in both histological and biomechanical analysis almost equal results in the spongialization and control groups, and the rats' healing potential is noticeable, the positive effects of stem cells and growth factors due to spongialization could be much more pronounced in humans.

This study has a few limitations. Our study is based on an acute injury model, whereas most rotator cuff tears are chronic [39] and caused by degeneration [40]. Further acute tears treated immediately have a higher potential of healing than chronic tears have [41, 42], and the additional fact that rats have a high intrinsic healing potential [25] impedes the transferability to humans concerning the short-term outcome. We did not analyze directly whether stem cells and/or growth factors were released after spongialization. Contrary to expectations, no animal suffered from re-tearing of the tendon in the radiofrequency ablation group. This again might be explained by the rat's high healing potential. Another limitation might be the relation between drill holes and rat shoulder size. Apart from the fact that drill holes that large compared to the bone would not be acceptable for humans, it is possible that the holes were large enough for mesenchymal cells to migrate, causing the same effect as spongialization. According to that, the third limitation of this study is the missing evaluation of existing mesenchymal stem cells in the restoration area.

In conclusion, the results of this study support the hypothesis that spongialization of the footprint before attaching the torn supraspinatus tendon can lead to better structural properties and higher tensile quality of tendon-to-bone restoration at the insertion area. Further studies are needed to investigate the potential of released growth factors and stem cells from the bone marrow. However, using radiofrequency ablation leads to poor results in biomechanical strength and causes a delayed healing process and cannot be advised for rotator cuff repair.

Conflict of interest

The authors declare no conflict of interest.

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