Title

Supraspinatus Detachment Causes Musculotendinous Degeneration and a Reduction in Bone Mineral Density at the Enthesis in a Rat Model of Chronic Rotator Cuff Degeneration.

Short title

Development of a Chronic Rotator Cuff Tear Model.

Keywords

Animal model; rotator cuff; tendon-bone healing; tendon degeneration

Abstract

Background

In order to evaluate biological strategies that enhance tendon-bone healing in humans, it is imperative that suitable animal models accurately reproduce the pathological changes observed in the clinical setting following a tear. The purpose of this study was to investigate rotator cuff degeneration in a rat, and assess the development of osteopenia at the enthesis following tendon detachment.

Materials and Methods

Eighteen female Wistar rats underwent unilateral detachment of the supraspinatus tendon. Specimens were retrieved at three (n = 6), six (n = 6), and nine weeks (n = 6) postoperatively for histological analysis and peripheral quantitative computer tomography.

Results

Three weeks following tendon detachment there was a significant increase in the modified Movin score characterized by a loss of muscle mass, fatty infiltration, an increase in musculotendinous cellularity, loss of normal collagen fiber structure/arrangement, rounded tenocyte nuclei, and an increase in the number of vascular bundles. This was accompanied by a reduction in bone mineral density at the tendon insertion site. After three weeks though, these changes were less prominent.

Conclusion

The rotator cuff tendon-muscle-bone unit in a rat model three weeks after detachment of supraspinatus represents a valid model to investigate rotator cuff degeneration.

Introduction

Rotator cuff tendon degeneration is common and can result in the development of tears in susceptible tendons, associated with degenerative changes in the relevant rotator cuff muscles and in the humeral enthesis. [1] Macroscopic structural changes include rotator cuff tendon thinning and retraction, muscle atrophy and fatty infiltration, and compensatory hypertrophic of the intra-articular biceps tendon. Ultra-structural changes include alteration of tendon cellularity, degradation of tendon matrix quality, diminution of perfusion, microcalcification, amyloid deposition, and synovial proliferation. [1; 2]

Degeneration can be initiated by a number of factors that are either intrinsic or extrinsic to the cuff itself. Accumulation of degenerative microtrauma has been proposed as the most important intrinsic factor and encompasses age-related degeneration compounded by repetitive microtrauma, eventually resulting in the development of partial, and subsequently full-thickness tears. [1] Extrinsic causes comprise environmental and anatomical influences. The former includes increasing age, shoulder overuse, smoking, and any medical condition such as diabetes mellitus that disturbs healing by microvascular impairment. [1] Abnormal acromial morphology has been postulated as the principal anatomical variant initiating the degenerative process. [1] Progressive change in the topography and shape of the undersurface of the acromion and ‘spur’ formation at its antero-inferior border with thickening of the coracoacromial ligament (the coraco-acromial arch) lead to stenosis of the subacromial space and supraspinatus ‘outlet’ deforming the supraspinatus muscle and tendon passing under the coracoacromial arch causing inflammation, physical damage

to the muscle and musculotendinous junction, and the clinical presentation of the 'impingement syndrome.'

Poor healing and recurrent tears frequently occur following repair of a degenerative rotator cuff and are associated with a poor functional outcome. [3] In order to select appropriate tendon graft materials and to determine the effect of biological augmentation on healing, it is useful to examine such strategies in a degenerative tendon model which replicates what is observed in the clinical setting. Several animal models of tendon degeneration have been developed: the rat shoulder is the most popular. [4; 5] Advantages of using the rat model as a surrogate for investigation of human rotator cuff function include the presence of an arch-like structure that encloses supraspinatus (similar to the coracoacromial arch) and the high functional loads generated in the tendon. Primate models have greater anatomical similarities to humans but due to expense and restricted use they are an impractical alternative. [6] Supraspinatus detachment has been shown, using a rat model, to lead to degenerative changes comparable to those seen in the clinical setting: tendon degeneration, inflammation, and muscle atrophy combined with a persisting defect. These were most apparent after an interval of three weeks, with longer time points associated with complete closure of the defect. [5]

The purpose of this study was to investigate rotator cuff degeneration and assess the development of osteopenia at the bony insertion of supraspinatus following tendon detachment. Osteopenia of the humeral head occurs following a rotator cuff tear in humans and compromises fixation techniques where tendon is reattached to bone. [7] It is therefore important to describe the osteopenia that develops in models of tendon

degeneration following a chronic tear. The hypothesis was that detachment of supraspinatus from the humerus would result in tendon degeneration and osteopenia of the greater tuberosity in a rat model.

Materials and Methods

Study Design

All animal work was conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Eighteen randomly allocated (using simple randomization) female Wistar rats, who had not previously been subject to any experimentation, underwent unilateral detachment of the supraspinatus tendon. All procedures were carried out by one surgeon over several days. Using a power calculation and previously published data, an n of 6 has been shown to provide a power of 0.8, which provides significance at p =0.05. [1] Animals were allowed to freely mobilise immediately post-operatively (with cage mates and a constant supply of food and water) and specimens were retrieved after euthanasia at three (n = 6), six (n = 6), and nine weeks (n = 6) postoperatively for histological analysis and peripheral quantitative computer tomography (pQCT).

Surgical Technique

A chronic, degenerative full thickness rotator cuff tear model was developed from one that has been previously used to examine tendon degenerative changes. [2] Anaesthesia was induced and maintained using 2% Isoflurane mixed with pure oxygen via a facemask: this was undertaken by a veterinary anesthetist experienced with the technique. Continuous monitoring of vital signs (heart rat, respiratory rate, and temperature) was undertaken throughout surgery, which was performed in a dedicated operating theatre throughout the day. The right shoulder was used for tendon

detachment in all cases and the contralateral left shoulder served as a control. A 1.5 cm skin incision was made directly over the anterolateral border of the acromion. The deltoid was detached from the anterior, lateral, and posterior margins of the acromion and split caudally for 0.5 cm. The acromio-clavicular joint was divided and a traction suture was placed around the clavicle to facilitate visualization of supraspinatus (Figure 1A). The bony end of the supraspinatus tendon was marked at its musculotendinous junction with a 5'0 prolene suture to assess retraction during tissue harvest. Under tension of the suture, the tendon was detached using sharp dissection from the greater tuberosity of the humeral head and allowed to retract medially (Figures 1B and 1C). The deltoid muscle and fascia were closed with absorbable 5'0 Vicryl suture (Ethicon, Johnson & Johnson Medical Ltd., Berkshire, UK). Skin closure was achieved using absorbable 5'0 Monocryl suture (Ethicon, Johnson & Johnson Medical Ltd., Berkshire, UK) and the animals were permitted unrestricted cage activity (Figure 1D). Postoperative pain was assessed daily and analgesia (Intra-muscular buprenorphine 0.6 mg) was given every 12 hours for three days.

Macroscopic Assessment

Animals were euthanized at three (n = 6), six (n = 6), and nine weeks (n = 6). Supraspinatus tendon-bone defects were visually assessed and classified as: persistent, partial, and completely closed.

pQCT

After sacrifice pQCT scanning was performed to measure bone mineral density at the humeral head. Using an XCT 2000 Bone Scanner (Stratec Medizintechnik Gmbh, Germany) with Software version 6.20, 1 mm CT slices were taken through the humeral head and supraspinatus musculotendinous unit.

Histological Assessment

At euthanasia, the right shoulder was dissected and a specimen comprising the humerus with its attached supraspinatus musculotendinous unit was removed. The contralateral left shoulder served as a control (n = 6). Each sample was fixed in 10% formal saline and underwent decalcification in Ethylenediaminetetraacetic acid (EDTA). Decalcification was checked by radiography at weekly intervals. Following decalcification the specimens were dehydrated in ascending graded alcohol dehydration followed by defatting in chloroform, and embedding in paraffin wax. Multiple 4 micrometre sections were cut in the coronal plane through the humerus, enthesis, supraspinatus musculotendinous unit, and any scar tissue that filled the gap between tendon and bone. Sections were stained with hematoxylin and eosin (H&E).

A double blind evaluation of all sections was performed using an Olympus BH-2 light microscope (Olympus, Glasgow, UK). Using a semi-quantitative scoring system (0 = none, 1 = mild, and 2 = severe), four high-powered fields were examined in each muscle to determine the extent of fatty infiltration, cellularity, and inflammation. [5]

Tendon degeneration was assessed according to a modified Movin scale [10] and included the following variables: (1) fiber structure, (2) fiber arrangement, (3) rounding of the nuclei, (4) regional variations in cellularity, (5) increased vascularity, and (6) hyalinization. A four-point scoring system was used: 0 = normal appearance, 1 = slightly abnormal appearance, 2 = a moderately abnormal appearance, and 3 = a markedly abnormal appearance. [11] Based on this, the total score for any given slide could range from 0 (normal tendon) to 18 (the greatest level of degeneration).

Statistical Analysis

Nonparametric statistical methods were used for all analyses because of the non-normality of the data in the groups being compared. Numerical data were inputted into SPSS software package, version 23 (SPSS Inc, an IBM Company, Chicago, Illinois). The data are presented as median values (with 95% confidence intervals) unless otherwise stated. Mann Whitney U tests were used to compare between data sets for each group. Results were considered significant at the $p < 0.05$ level.

Results

All animals survived the duration of the study and none had post-operative infection. **Limping was noted for all animals for the first three to five postoperative days but a normal gait pattern returned afterwards.**

Macroscopic Findings

Scar tissue was noted in all animals. Based on the position of the suture marker, the supraspinatus tendon had retracted approximately 5 mm in all cases. The muscle belly of supraspinatus was atrophic and was pale in appearance (Figure 2). Some degree of tendon-bone defect closure occurred in all animals at all time points. At three weeks, partial defect closure was evident in all cases. At six weeks, two animals had partial closure of the defect and four animals had complete closure. All animals in the nine-week group had complete closure of the tendon-bone defect (Figure 2).

pQCT Scans

The contralateral shoulder in which the supraspinatus had not been detached represented control specimens. Median total bone mineral density significantly decreased three ($p = 0.006$), six ($p = 0.004$), and nine weeks ($p = 0.025$) following tendon detachment **(Table 1)** (Figure 3). No significant change in bone mineral density occurred between three, six, and nine weeks (Table 2).

Histological Findings

Muscle Evaluation

A loss of muscle mass was observed at all time points, and was accompanied by degenerative changes (characterized by increased amounts of fibrotic tissue) that were most prominent three weeks after detachment and less evident by nine weeks. No inflammatory changes were present in any of the animals. All groups demonstrated a degree of fatty infiltration, which peaked at three weeks **(Table 3)** (Figure 4). Compared to controls (where there was no fatty infiltration present) fatty infiltration significantly increased ($p = 0.002$) at three weeks but reduced at six- ($p = 0.140$) and nine weeks ($p = 0.138$).

Cellularity significantly increased at three weeks ($p = 0.001$), at six weeks ($p = 0.002$), and at nine weeks ($p = 0.002$), compared to controls **(Table 3)**. Furthermore, cellularity was significantly greater in the three-week group than in the six- and nine-week groups ($p = 0.006$ and 0.007 respectively).

Tendon Evaluation

Modified Movin Score

The modified Movin score was significantly higher (indicating degeneration) in the

three experimental groups compared to the controls ($p = 0.003$: three, six, and nine weeks after supraspinatus tendon detachment) **(Table 3)** (Figure 5). There were no significant inter-group differences (Table 4).

Fiber Structure

In control specimens, collagen fibers were close together and arranged in parallel. Abnormal specimens lost this uniform structure (increased waviness and distance between fibers) to differing degrees (Figure 6) **(Table 3)**. Fiber structure was significantly more abnormal in the nine-week group compared to the three- ($p = 0.003$) and six-week groups ($p = 0.007$).

Fiber Arrangement

In control specimens, the fibers were arranged in parallel. Abnormal specimens lost this arrangement to differing degrees (Figure 6) **(Table 3)**. Fiber arrangement was significantly more abnormal in the three-week group compared to the controls ($p = 0.002$), the six-week group ($p = 0.001$), and the nine-week group ($p = 0.002$).

Tenocyte Nuclei

Tenocyte nuclei were flattened and spindle-shaped in control specimens, but following tendon detachment became more rounded (Figure 7) **(Table 3)**. Tenocyte

nuclei were significantly more abnormal than controls following tendon detachment ($p = 0.002$ at three-, $p = 0.003$ at six-, and $p = 0.002$ at nine weeks), with the three-week group demonstrating more abnormal rounded nuclei than the six- and nine-week groups.

Cellularity

Specimens were evaluated for an increase in cellularity. There was a significant increase in cellularity following tendon detachment ($p = 0.003$ at three, $p = 0.003$ at six, and $p = 0.002$ at nine-weeks), however there were no significant differences between experimental groups (**Tables 3** and 5).

Vascularity

Vascular bundles ran with collagen fibers and increased in number with tendon degeneration. [11] The number of vascular bundles significantly increased at three- ($p = 0.002$), six- ($p = 0.002$), and nine-weeks ($p = 0.006$) following supraspinatus detachment **(Table 3)**. A significant reduction in vascularity was noted between three- and nine-weeks ($p = 0.030$).

Hyalinisation

Hyalinisation was not observed in any of the specimens.

Discussion

This study presents a rat model for the investigation of chronic rotator cuff tears. Following detachment of supraspinatus there was a significant rise in the modified Movin score characterized by a loss of muscle mass, fatty infiltration, an increase in musculotendinous cellularity, loss of normal collagen fiber structure/arrangement, rounded tenocyte nuclei, and an increase in the number of vascular bundles. These results, in conjunction with those from the pQCT evaluation, support our hypothesis that tendon detachment induces supraspinatus musculotendinous degeneration and a reduction in bone mineral density at the enthesis. These changes occurred acutely, after three weeks duration. However after this time defect closure occurs with complete closure of the defect seen at nine weeks, and there appears to be no further degradation of the tendon or muscle. Contrary to previous reports, fatty infiltration was present in muscle specimens at three-weeks but were no longer evident during the latter stages of the study. [3, 4] These transient changes in fatty infiltration suggest that with time, there is gradual reconstitution of the tendon-bone interface with fibrous tissue that permits the transfer of load and subsequent remodeling of this neo-enthesis into a tendon-like structure. [4, 5]

Chronic rotator cuff tears are characterized by retraction, muscle atrophy, reduced/increased cellularity, reduced/increased vascularity, fatty infiltration, calcification, and degeneration of the muscle. [6, 7] In humans fatty infiltration into the rotator cuff is irreversible and represents an important predisposing factor to repair failure and poor functional outcomes. [8] Current rodent models have been unable to establish a significant amount of fat accumulation following tendon detachment,

306 making it difficult to specifically examine hypotheses related to it. [3, 4] In this study,
307 there was a significant amount of fatty accumulation into the muscle belly of
308 supraspinatus compared with controls, peaking at three-weeks following tendon
309 detachment and subsiding thereafter. This novel finding may be associated with
310 fundamental inter-species differences between the Wistar rats used in this study and
311 the Sprague-Dawley rats used in others. [3, 4] Lipoprotein lipase catalyses the hydrolysis
312 of triglycerides and is highly expressed in skeletal tissues. It is regulated differently
313 between Wistar and Sprague-Dawley rats and may account for the lack of fat
314 accumulation in otherwise degenerative muscle tissue in some studies. [9]
315
316 Rotator cuff tears can cause osteopenia at the enthesis due to a loss of physical
317 stimuli. [10, 11] During surgery, suture anchors are inserted into the greater tuberosity
318 and therefore any reduction in bone mineral density may cause loosening or pullout
319 before adequate tendon-bone healing can occur. [12] Accordingly, this has been
320 recognised as an independent risk factor predictive of healing, with a higher bone
321 mineral density resulting in better outcomes. [13, 14] The majority of studies ascribe this
322 alteration in bone mineral density to attritional changes secondary to tendon damage,
323 but it is plausible that they may precede the tear and be causative in nature. [15] In order
324 to examine biological strategies that specifically address bone quality, relevant animal
325 models are required. While the anatomical similarities between the rat and human
326 rotator cuff have been extensively described, to date, there are no studies evaluating
327 the onset of osteopenia in the rat. In this study, supraspinatus detachment caused a
328 reduction in bone mineral density at three-, six-, and nine-weeks with no significant
329 change between successive time-points. During a chronic rotator cuff tear the forces
330 borne by the greater tuberosity reduce and therefore cause an imbalance in bone

331 turnover, favoring bone resorption over bone formation: a principle governed by
332 Wolff's law. [13]
333
334 Limitations of this study include those associated with using the contralateral shoulder
335 as a control given that its mechanical and histological properties may have altered
336 during the few days that the animals were limping and therefore placing more weight
337 through the non-operated limb. Additional time points (two and 12 weeks) would
338 have been beneficial to evaluate the progression and further resolution of degenerative
339 musculotendinous changes and alterations in bone mineral density.
340
341 In conclusion, this study has shown that three weeks following detachment, the
342 supraspinatus musculotendinous unit in a rat undergoes degeneration, and the greater
343 tuberosity exhibits a reduction in bone mineral density. These changes are similar to
344 those that occur in the clinical setting following a chronic rotator cuff tear, with the
345 difference that scar tissue bridges the defect in a rat whereas in a human the tendon-
346 bone gap is largely maintained. These findings suggest that the detached rat
347 supraspinatus tendon, after three weeks, could represent a suitable model for
348 investigating biological strategies targeted towards improving tendon-bone healing in
349 chronic rotator cuff tears.

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Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

Table 1: Median total bone mineral density at the supraspinatus tendon-bone insertion three, six, and nine weeks following tendon detachment.

	Control (non-operated shoulder) group (n = 6)	3 week group (n = 6)	6 week group (n = 6)	9 week group (n = 6)
Median total bone mineral density (mg/ccm)	793.25 (95% CI 754.24 to 844.70)	684.70 (95% CI 639.21 to 739.82)	642.85 (CI 610.74 to 711.33)	665.20 (CI 594.01 to 763.62)

Table 2: Statistical significance (p-values) between total bone mineral density at the supraspinatus tendon-bone insertion three, six, and nine weeks following tendon detachment.

	Control (non-operated shoulder) group (n = 6)	3 week group (n = 6)	6 week group (n = 6)	9 week group (n = 6)
Control (non-operated shoulder) group (n = 6)	-	0.006	0.004	0.025
3 week group (n = 6)	0.006	-	0.200	0.749
6 week group (n = 6)	0.004	0.200	-	0.631
9 week group (n = 6)	0.025	0.749	0.631	-

Table 3: Muscle and tendon histological outcome scores three, six, and nine weeks following tendon detachment.

	Control (non-operated shoulder) group (n = 6)	3 week group (n = 6)	6 week group (n = 6)	9 week group (n = 6)
Muscle: fatty infiltration	0 (95% CI 0 to 0)	0.5 (95% CI 0.40 to 0.94)	0 (95% CI -0.19 to 0.69)	0 (95% CI -0.10 to 0.44)
Muscle: cellularity	0 (95% CI 0 to 0)	2 (95% CI 2 to 2)	1 (95% CI 0.91 to 1.69)	1.5 (95% CI 1.02 to 1.81)
Modified Movin score	0 (95% CI -0.27 to 0.60)	8.75 (95% CI 7.08 to 11.26)	7.75 (95% CI 6.45 to 9.38)	8 (95% CI 7.53 to 9.87)
Tendon: Fiber structure	0 (95% CI 0 to 0)	2 (95% CI 1.56 to 2.10)	1.75 (95% CI 1.40 to 2.26)	2.5 (95% CI 2.40 to 2.94)
Tendon: Fiber arrangement	0 (95% CI 0 to 0)	2 (95% CI 1.52 to 2.31)	1.5 (95% CI 1.20 to 1.63)	1.5 (95% CI 1.02 to 1.81)
Tendon: Tenocyte nuclei	0 (95% CI – 0.13 to 0.30)	2.50 (95% CI 2.06 to 2.60)	1.75 (95% CI 1.40 to 2.26)	2 (95% CI 1.56 to 2.10)
Tendon: Cellularity	0 (95% CI -0.13 to 0.30)	1.25 (95% CI 0.84 to 2.16)	1.75 (95% CI 1.40 to 2.26)	1.75 (95% CI 1.46 to 2.03)
Tendon: Vascularity	0 (95% CI 0 to 0)	1.5 (95% CI 0.68 to 2.49)	1 (95% CI 0.53 to 1.47)	0.5 (95% CI 0.67 to 1.10)

Table 4: Statistical significance (p-values) between modified Movin scores three, six, and nine weeks following tendon detachment.

	Control (non-operated shoulder) group (n = 6)	3 week group (n = 6)	6 week group (n = 6)	9 week group (n = 6)
Control (non-operated shoulder) group (n = 6)	-	0.003	0.003	0.003
3 week group (n = 6)	0.003	-	0.256	0.326
6 week group (n = 6)	0.003	0.256	-	0.513
9 week group (n = 6)	0.003	0.326	0.513	-

Table 5: Statistical significance (p-values) between cellularity three, six, and nine weeks following tendon detachment.

	Control (non-operated shoulder) group (n = 6)	3 week group (n = 6)	6 week group (n = 6)	9 week group (n = 6)
Control (non-operated shoulder) group (n = 6)	-	0.003	0.003	0.002
3 week group (n = 6)	0.003	-	0.246	0.315
6 week group (n = 6)	0.003	0.246	-	0.789
9 week group (n = 6)	0.002	0.315	0.789	-

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