TITLE PAGE

Title:

The Novel Use of Demineralised Cortical Bone Matrix and Bone-Marrow Derived

Mesenchymal Stem Cells in a Chronic Rotator Cuff Tear Model.

Running title:

Demineralised Bone and Stem Cells for Rotator Cuff Healing.

ABSTRACT

Background: The success of rotator cuff repair is primarily dependent upon tendon-bone healing. Failure is common because weak scar tissue replaces the native enthesis, rendering it prone to re-rupture. Demineralised bone matrix (DBM) consists of a network of collagen fibers that provide a sustained release of growth factors such as bone morphogenic proteins (BMPs). Previous studies have demonstrated that it can regenerate a fibrocartilaginous enthesis.

Hypothesis: The use of DBM and MSCs at the healing enthesis will result in a higher bone mineral density at the tendon insertion and enhances the regeneration of a morphologically superior enthesis when compared to acellular human dermal matrix.

Study design: Controlled Laboratory Study.

Methods: Eighteen female Wistar rats underwent unilateral detachment of the supraspinatus tendon. Three weeks later, tendon repair was carried out in animals randomized into three groups: Group 1 received augmentation of the repair with cortical allogenic DBM (n = 6); Group 2 received augmentation with non-meshed, ultra-thick acellular human dermal matrix (n = 6); and Group 3 underwent tendon-bone repair without a scaffold (n = 6). All animals received 1×10^6 MSCs delivered in fibrin glue to the repair site. Specimens were retrieved at six weeks postoperatively for histological analysis and evaluation of bone mineral density.

Results: All groups demonstrated closure of the tendon-bone gap with a fibrocartilaginous enthesis. Although there were no significant differences in the enthesis maturation and Modified Movin scores, repairs augmented with dermal matrix + MSCs exhibited a disorganised enthesis, abnormal collagen fiber arrangement, and greater cellularity compared to other MSC groups. Only repairs augmented with DBM + MSCs reached a bone mineral density not significantly lower than non-operated controls.

Conclusion: DBM enhanced with MSCs can augment rotator cuff healing at six weeks and restore bone mineral density at the enthesis to its pre-injury levels.

Clinical Relevance: Biological augmentation of rotator cuff repair with DBM and MSCs may reduce the incidence of retears, although further studies are required to determine its effectiveness.

Key Terms: demineralised bone matrix; mesenchymal stem cells; rotator cuff; tendon-bone healing.

What is known about the subject: Tears of the rotator cuff are one of the most common tendon disorders. Treatment often includes surgical repair but the rate of failure to gain or maintain healing has been reported to be as high as 94%. This has been substantially attributed to the reduced capacity of tendon to heal once damaged, particularly to bone at the enthesis. Several strategies to improve tendon-bone healing, tendon-tendon healing, and tendon regeneration have been developed. Scaffolds have received considerable attention for replacement, reconstruction, or reinforcement of

tendon defects but may not possess situation-specific or durable mechanical and biological characteristics.

Demineralised bone matrix is routinely used as a bone graft substitute material and is composed of a type 1 collagen matrix that contains multiple growth factors believed to enhance bony regeneration through endochondral ossification. Few studies have examined the effects of demineralised bone matrix on tendon healing, with those that do demonstrating that it can regenerate a histologically normal enthesis in both acute tear and tendon retraction models.

What this study adds to existing knowledge: This is the first study to investigate the effect of mesenchymal stem cells on the action of demineralised bone matrix in a small animal model of a chronic rotator cuff tear.

INTRODUCTION

Approximately 75,000 rotator cuff repairs are performed each year in the United States. [50] Up to 46% of tendons fail to heal back to bone and result in a worse functional outcome than those tendons that do go onto heal. [6] The native enthesis is a graduated structure that allows stresses to be evenly distributed between tendon and bone. [43] Following surgery, scar tissue is deposited at the healing interface and has been identified as a potential cause of the high failure rate because it possesses weak mechanical properties and does not contain mineralised fibrocartilage. [11, 38] [11, 14, 25, 38] In order to improve rotator cuff healing, biological strategies must enhance tissue regeneration in order to provide mechanically resilient tissues able to resist tearing during the crucial postoperative rehabilitation period. [29]

Mesenchymal stem cells (MSCs) are multipotent cells that are capable of differentiating into several cell types, including chondrocytes, adipocytes, and osteoblasts. [7] *In vivo* examination of the behaviour of MSCs in enthesis regeneration have reported the absence of a fibrocartilaginous interface, citing the lack of signaling as a reason for the transplanted cells not differentiating and leading to a poor outcome. [17] To enhance their effect, studies have engineered stem cells *in vitro* to express specific proteins. [18, 19] In doing so, the addition of these cells has increased fibrocartilage production and improved the strength of the repair. [18]

Demineralised bone matrix (DBM) consists of a network of collagen fibers that provide a sustained release of growth factors such as bone morphogenic proteins (BMPs). [33, 40] Wurgler-Hari et al [51] demonstrated that a slow release of growth factors

is required for tendon-bone healing, and there is evidence to indicate that BMPs are important proponents of a naturally graduated, mechanically favorable enthesis. [3, 28, 32] Differentiation of MSCs can be directed by the introduction of BMPs; a property that may be utilised to increase production of mineralised fibrocartilage at the healing enthesis. [12, 16, 26, 39] DBM has been shown to regenerate a strong enthesis with mineralised fibrocartilage in both acute and chronic tear models of tendon-bone healing. [40, 41] Similar results have additionally been demonstrated in a large animal model of severe tendon retraction. [42] Since the ultimate strength of the tendon-bone interface is dependent upon bony ingrowth and the development of fibrocartilage, we speculate that the addition of MSCs into a DBM-based repair construct will produce an enthesis resembling the native insertion site with a natural gradation between tendon, demineralised fibrocartilage, mineralised fibrocartilage, and bone.

The purpose of this study was to determine if DBM enhanced with MSCs could improve tendon-bone healing when applied to a degenerative rotator cuff tear model. We compared DBM with a commercially available human dermal matrix graft, which has been used clinically for enthesis repair (GraftJacket [Wright Medical Technology, Inc., Arlington, TN (Tennessee)]). The hypothesis was that in a rat model of chronic rotator cuff tear, the addition of DBM and MSCs to the healing enthesis would result in a higher bone mineral density at the tendon insertion and this combination would lead to regeneration of a morphologically superior enthesis characterized by greater fibrocartilage formation and improved collagen fiber organization in a rat model of a chronic rotator cuff tear when compared to acellular human dermal matrix.

Furthermore, to determine whether MSCs could enhance the structure of the healing enthesis compared to repairs without stem cells, the results of this experiment were compared to a previously published study that utilised the same animal model and scaffolds. [41] It was hypothesised that the addition of MSCs would yield a morphologically superior interface and increased bone mineral density at the tendon insertion.

MATERIALS AND METHODS

Study Design

All animal work was conducted in accordance with a Project License protocol accepted under the UK Home Office Animals (Scientific Procedures) Act 1986. Eighteen female Wistar rats underwent unilateral detachment of the supraspinatus tendon, and an additional one animal from the same genus was used to obtain bone marrow-derived MSCs. Previously published data was used to calculate the number of animals (n = 6) required to generate a power of 0.8 with significance at the 0.05 level. [40] It has demonstrated that detachment of the supraspinatus tendon in rats results in morphological changes similar to those that occur in the clinical setting. [5] After three weeks, tendon repair was carried out in animals randomised into three groups: Group 1 received augmentation of the repair with cortical allogenic DBM (n = 6); Group 2 received augmentation with non-meshed, ultra-thick acellular human dermal matrix (n = 6) (GraftJacket, Wright Medical Technology, Inc., Arlington, TN; average 1.4mm thickness); and Group 3 underwent tendon-bone repair without a scaffold (n = 6). All animals received 1×10^6 MSCs in a fibrin sealant (Tisseel® kit, Baxter, Vienna, Austria) and one animal from each study group received MSCs labeled with Quantum Dot nanoparticles (Qtracker 655, Invitrogen, Massachusetts, United States) (QDs) for cell tracking. In order to minimise the number of animals used, as per UK Home Office regulations, fibrin glue devoid of MSCs was not used as an experimental group as it has previously been shown not to enhance healing of the enthesis. [17, 37, 52]

One surgeon carried out all procedures using a standard technique. Animals were allowed to mobilise freely after surgery. Specimens were retrieved at six weeks postoperatively for histological analysis and peripheral quantitative computer tomography (pQCT) to evaluate bone mineral density (BMD) at the reattachment footprint of the tendon, reversal of degenerative changes within the tendon, and histological remodeling of the implanted augmentation material.

Acellular human dermal matrix preparation

GraftJacket (Wright Medical Technology, Inc., Arlington, TN) is obtained from donated human cadaveric dermal tissue processed to remove its cellular components whilst retaining its extra-cellular matrix. Its acellularity has the advantage of not causing a host inflammatory reaction and it has been safely used in rats to enhance healing of a large acute rotator cuff tear. [21] Samples of dermal matrix were rehydrated at the time of surgery in normal saline for 30 minutes prior to use.

DBM Manufacture

DBM derived from cortical bone was manufactured according to Urist's protocol, with modifications. [47] The tibiae of skeletally mature female Wistar rats were harvested immediately after euthanasia; all soft tissues and periosteum were stripped from the bone surface. Bones measuring approximately 30 mm length by 3 mm width were demineralized in 0.6 N HCL at room temperature (Figure 1). Demineralization was confirmed by taking radiographs (300 seconds, 30 kV, Faxitron Corporation, Illinois, USA). This was followed by washing in phosphate-buffered saline (PBS)

until the pH was 7.4 +/- 0.1. Samples were stored at -20˚C for two hours and

transferred to a lyophiliser (Edwards Girovac Ltd, Crawley, West Sussex, UK) for

three days (Figure 2). Specimens were then sealed in individual plastic bags, sterilised

by gamma irradiation at a dose of 25 kilograys (Isotron Limited, Reading, UK), and

stored at -20˚C. Samples were rehydrated at the time of surgery in sterile saline

solution for 30 minutes prior to use.

Figure 1. Rat tibiae following demineralisation.

Figure 2. Demineralised rat tibiae in lyophiliser.

Bone Marrow-Derived Mesenchymal Stem Cell Harvest and Culture

One six-month-old female Wistar rat was euthanised by carbon dioxide inhalation and bilateral femurs were harvested under sterile conditions. MSCs were harvested by lavaging the intramedullary canals with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS) (HyClone, Logan, Utah, USA) and 1% penicillin/streptomycin solution (ThermoFisher Scientific, United States), to obtain a bone marrow aspirate, which was plated onto T-75 culture flasks. Cells were cultured at 37°C in a 5% carbon dioxide incubator. After five days, the contents of the flask were removed and washed with media, leaving the MSCs that adhered to the bottom of the flask. Afterwards, media was changed twice a week for 14 days. Once confluent, the MSCs were detached with trypsin and serially subcultured. Third passage cells were used for implantation. Previous studies have

shown that this protocol is a reliable method of obtaining MSCs, and that their pluripotency is maintained up to the third passage. [1, 17, 18, 34, 46] No immunogenic reaction was anticipated with stem cell implantation because the rats from which cells were harvested were the same strain as those receiving them.

Surgical Technique

Two surgeries were performed on each animal: full-thickness supraspinatus tendon detachment and complete tendon reattachment. Anaesthesia was induced and maintained using 2% isoflurane mixed with pure oxygen via a facemask for both procedures. The right shoulder was operated on in all cases. A 1.5 cm skin incision was made directly over the anterolateral border of the acromion. The deltoid was detached from the acromion and split caudally for 0.5 cm, in order to identify the tendon of supraspinatus. The supraspinatus tendon was completely detached from its bony insertion on the humeral head, marked with a 5'0 Prolene suture (Ethicon, Johnson & Johnson Medical Ltd., Berkshire, UK) at the musculotendinous junction (MTJ), and allowed to retract medially. Deltoid muscle, superficial fascia, and skin were closed with 5'0 Vicryl suture (Ethicon, Johnson & Johnson Medical Ltd., Berkshire, UK). Animals were allowed unrestricted cage activity and received analgesia (subcutaneous buprenorphine) every 12 hours for three days. The second surgery to reattach the tendon was undertaken three weeks after the first procedure. Prior to making the skin incision, the DBM or dermal matrix was rehydrated for 30 minutes in sterile normal saline at the operating table. [4]

A 2 cm skin incision was made in line with the supraspinatus muscle belly, ending anterior to the lateral end of the clavicle. This approach was perpendicular to the incision used for tendon detachment in order to make use of a virgin anatomical plane devoid of scar tissue. The muscle belly of supraspinatus was identified and followed distally to reveal the tendon stump with the suture marker in the MTJ. Scar tissue between the tendon stump and its insertion was excised and the tendon was grasped with a double-armed 5'0 prolene using a modified Mason-Allen technique. [44] Despite traction on the tendon stump, it could not be directly brought back to the humeral head in any of the cases. The bare tendon-bone insertion footprint was decorticated using a #11 surgical blade until bleeding was seen. A custom-made dental drill was used to drill a 1 mm hole from the neck of the humerus to the bony insertion of the detached supraspinatus.

MSCs were incorporated into the repair construct using a fibrin sealant [Tisseel® (Baxter, Vienna, Austria)] that contained two parts: a vapor-heated Tisseel® powder dissolved in aprotinin solution and a vapor-heated thrombin powder, dissolved in calcium chloride solution. One milliliter of the Tisseel® solution contained 100–130 mg of total protein, of which 75–115 mg was fibrinogen. One milliliter of thrombin solution contained 45–55 mg of total protein, of which 500 International Units (IU) [2] was thrombin. [24] Preoperatively, MSCs were implanted into the thrombin component *in vitro* at a concentration of 1×10^6 cells/100 μl and mixed with the Tisseel® solution. The fibrin glue was injected into a custom-made square-shaped stainless steel mold measuring 1 cm by 1cm containing DMEM, which provided nutrition to the cells up to the point at which they were implanted (Figure 3).
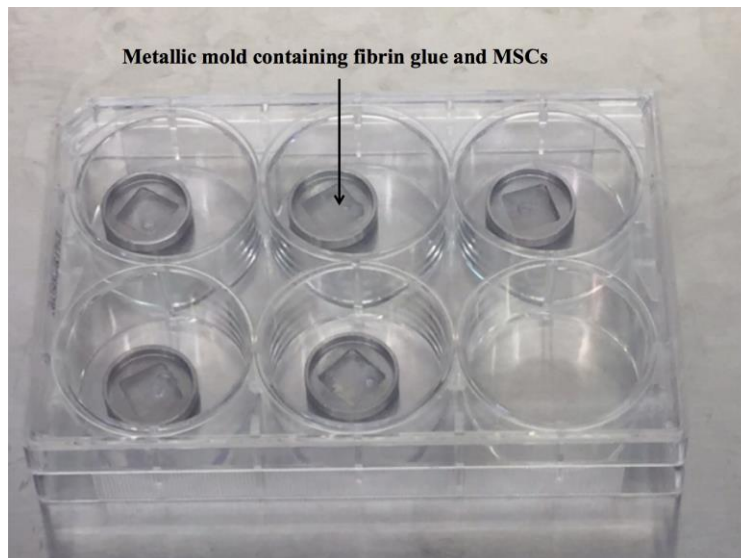
Figure 3. Metallic molds containing fibrin glue and MSCs *in vitro*.

Each limb of the suture was passed through the scaffold to secure it in position. One suture limb was passed through the hole in the prepared tendon stump and the other suture limb was passed through the hole on the neck of the humerus. The supraspinatus tendon-scaffold complex was attached to the insertion site, with the graft in contact with both the tendon stump proximally and decorticated bone surface distally. In the control group the sutures were inserted directly into the drill holes, leaving a 5 mm gap between the tendon and bone in all cases.

Prior to securing the graft material (cortical DBM or dermal matrix) during tendon reattachment surgery, the sheet of fibrin glue containing MSCs was placed on the decorticated humeral head whilst lying in contact with the proximal tendon stump (Figure 4). All grafts used to augment healing measured approximately 1.5 cm in length and 3-5 mm in width.
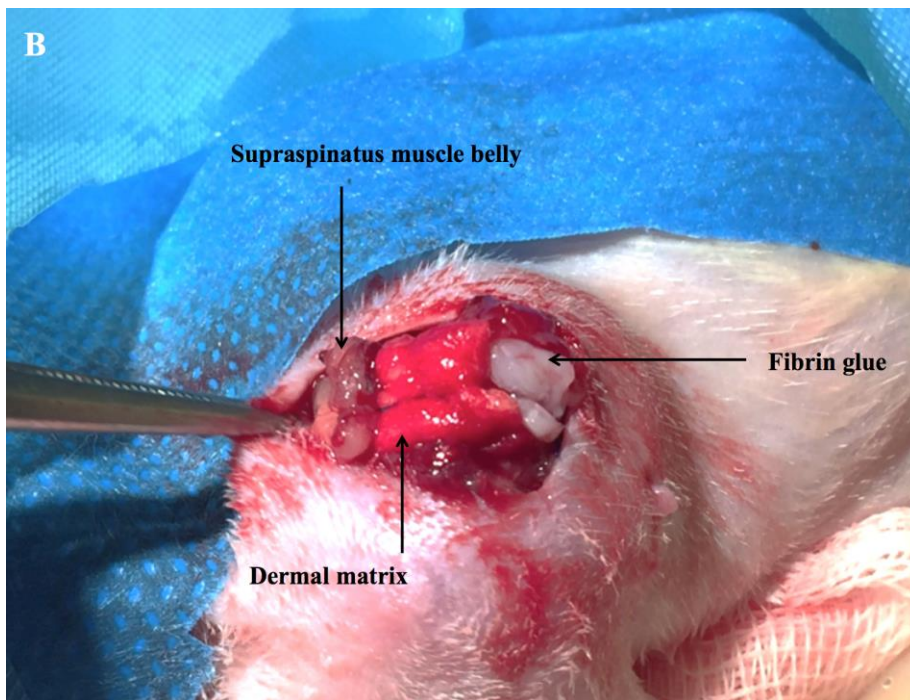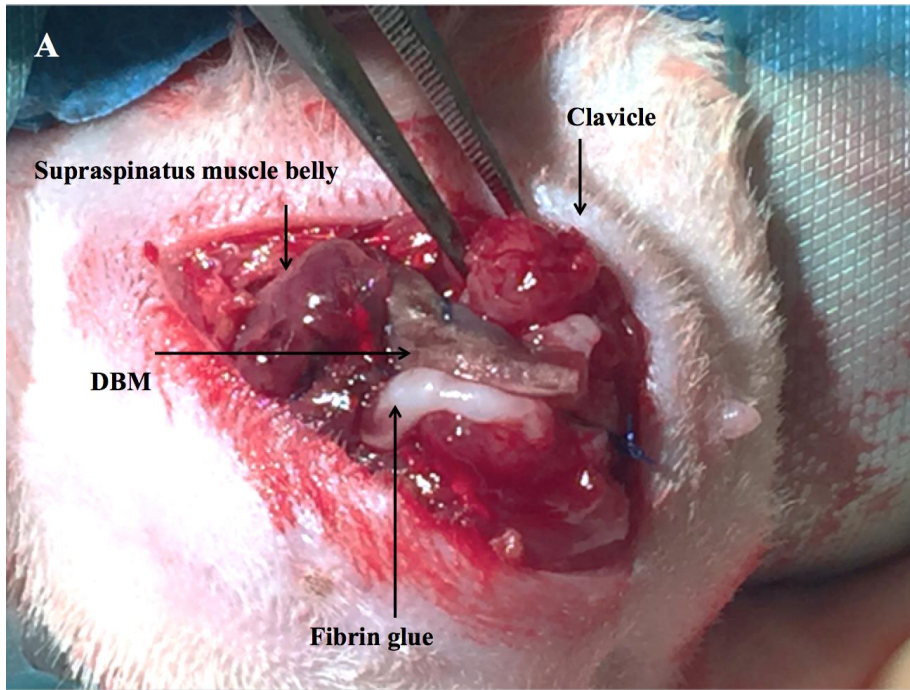
Figure 4. Supraspinatus tendon-bone interface with cortical DBM + MSCs (A) and dermal matrix + MSCs (B).

In the group where MSCs were used in isolation, the fibrin glue bridged the gap between the retracted supraspinatus musculotendinous unit and its bony insertion. The repair construct resembled a laminated structure: superficial layer – cortical DBM/dermal matrix, central layer – fibrin glue with MSCs, and deep layer – humeral head with tendon footprint (Figure 5).
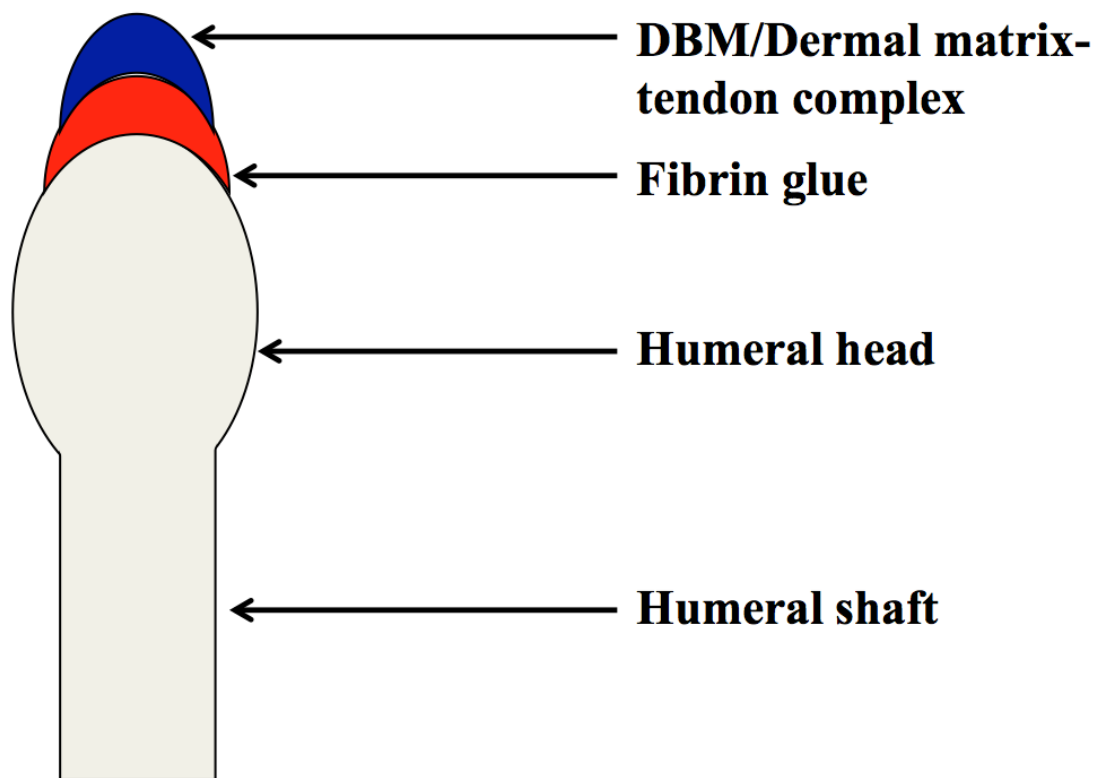

Figure 5. Lateral view of the tendon-bone repair construct comprising superficial layer – cortical DBM/dermal matrix, central layer – fibrin glue with MSCs, and deep layer – humeral head with tendon footprint.

A layered wound closure was undertaken in a similar manner to the first surgery, and the animals were permitted unrestricted cage activity. Postoperative analgesia (Intra-muscular Buprenorphine 0.6 mg) was given every 12 hours for three days.

Mesenchymal Stem Cell Tracking

QDs are semiconductor nanoparticles that are phagocytosed or taken up by pinocytosis into cells and distributed within cytoplasmic vesicles, causing the cells to intensely fluoresce. [31] MSCs were able to be tracked using this method. Properties that make QDs suitable for cell tracking include their compatibility with MSCs without effecting proliferation and differentiation, and their resistance to chemical and metabolic degradation. [2, 8, 10, 13, 22, 31]

In this study, three animals (one from each experimental group) received MSCs labeled with a Q-Tracker 655 Cell Labeling kit (Thermo Fisher Scientific, Massachusetts, United States). QDs were applied to cells at a concentration of 300,000 particle units/1×10^{6} MSCs. On the morning of surgery transduced MSCs were suspended in the fibrin sealant, stored in plain DMEM, and implanted at the repair site as described above.

Histological Assessment

At euthanasia, the right shoulder was dissected and a specimen comprising the humerus with its attached supraspinatus musculotendinous unit was removed. Each sample was fixed in 10% formal saline and underwent decalcification in EDTA, ascending graded alcohol dehydration, defatting in chloroform, and embedding in paraffin. Using a Reichert-Jung Model 1130 microtome (Reichert-Jung, Vienna, Austria), multiple 4 μm thick slides were cut in the coronal plane through the

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humerus, enthesis, and supraspinatus musculotendinous unit before staining with H&E.

Two blinded observers evaluated all sections using an Olympus BH-2 light microscope (Olympus, Glasgow, UK). Tendon degeneration was assessed according to a modified Movin scale that was initially conceived to define microstructural features of Achilles tendinopathy. [30] It has subsequently been used as a semi-quantitative scoring system to assess tendon degeneration and includes the following variables: (1) fiber structure, (2) fiber arrangement, (3) rounding of the nuclei, (4) regional variations in cellularity, (5) increased vascularity, and (6) hyalinisation. [4] 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. [27] Based on this, the total score for any given slide could range from 0 (normal tendon) to 18 (the greatest level of degeneration).

Maturation of the enthesis was assessed according to the scoring system developed by Ide et al that was initially used to characterise the histological features of the tendon-bone interface of rats in which supraspinatus fixation was achieved using a dermal matrix graft. [21] It is a semi-quantitative scoring system ranging from 1-4 (1 being a poorly defined enthesis and 4 being a well defined enthesis): score 1 – the insertion had continuity without fibrous tissue or bone ingrowth, score 2 – the insertion had continuity with fibrous tissue ingrowth but no fibrocartilage cells, score 3 – the insertion had continuity with fibrous tissue ingrowth and fibrocartilage cells but no tidemark, and score 4 – the insertion had continuity with fibrous tissue ingrowth, fibrocartilage cells, and a tidemark.

Presence of red fluorescence using microscopy was used to confirm the position and distribution of the MSCs containing QDs using a fluorescent microscope (Zeiss, Cambridge, UK).

pQCT

Changes in bone mineral density at the humeral head were assessed using pQCT scanning. One millimeter slices were obtained through the humeral head and supraspinatus musculotendinous unit using an XCT 2000 Bone Scanner (Stratec Medizintechnik Gmbh, Germany) with Software version 6.20. Controls were obtained from the contralateral (non-operated) shoulder in six animals subjected to the same rehabilitation conditions as the study groups.

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). Mann Whitney U tests were used to compare data between groups. Results were considered significant at the $p < 0.05$ level. In order to determine whether there was any benefit in using MSCs the data from this study was compared to a previously published one that evaluated the effect of DBM and dermal matrix on enthesis healing in the same chronic rotator cuff tear model without the addition of MSCs. [41]

RESULTS

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

Mesenchymal Stem Cell Tracking

QDs were observed using fluorescent microscopy in all specimens where they were implanted, suggesting that the MSCs remained at the tendon-bone interface (Figure 6).
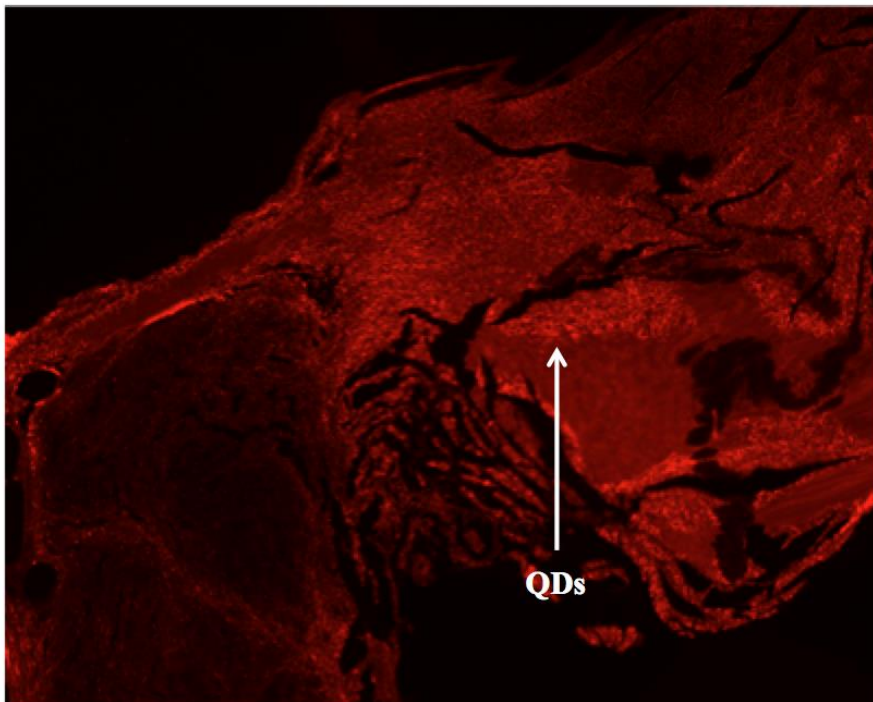

Figure 6. QDs at the tendon-bone interface denoted by red florescent cells; when viewed under a fluorescent microscope.

Macroscopic Findings

At the time of euthanasia there was continuity between the repaired tendon and the bone in all groups. There were no infections and none of the repairs had failed as illustrated by the intact sutures. Remodeling of the graft material occurred to a greater extent in DBM group, whereby the scaffold could not be discerned from other tissue-types in the regenerated tendon-bone interface. In contrast, the dermal matrix group was clearly visible at necropsy. MSC-only specimens demonstrated complete closure of the enthesis.

Quantitative Histology

Supraspinatus muscle-scaffold interface

Both scaffolds had remodeled completely and were characterised by a well cellularised interface, similar to controls only repaired with MSCs (Figure 7).
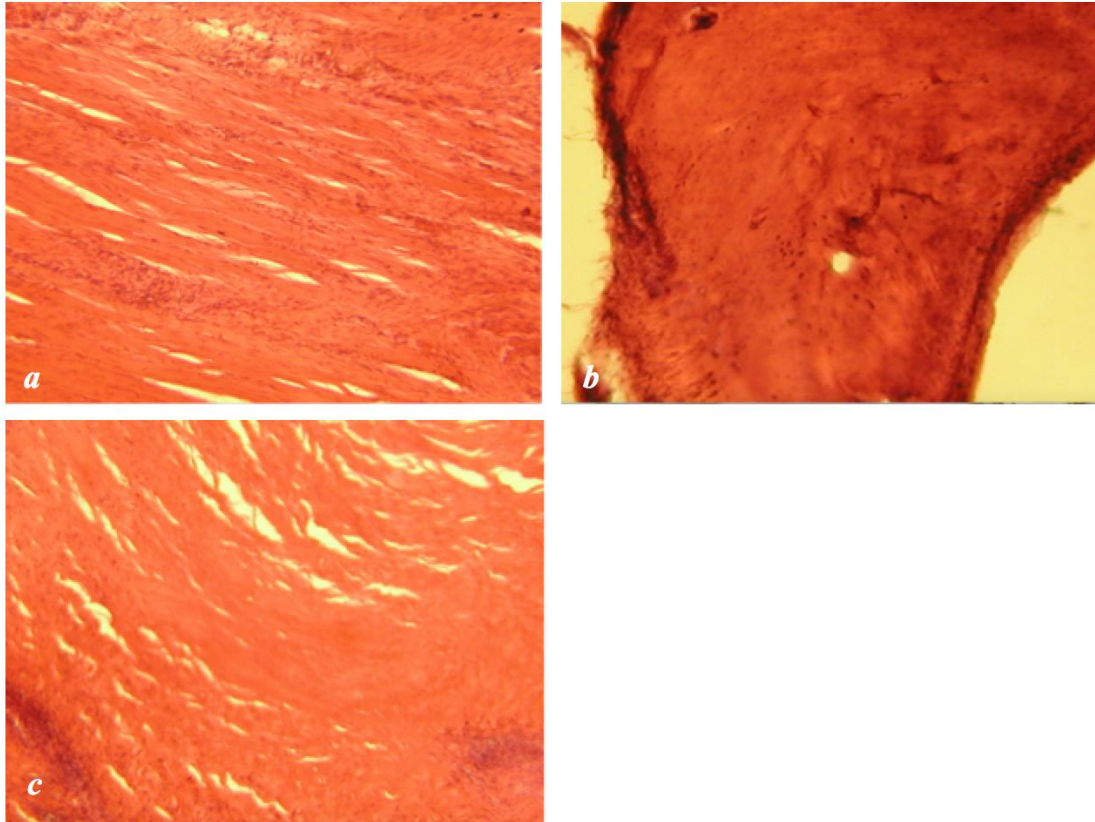
Enthesis Maturation Score

No significant differences were observed in the enthesis maturation scores between experimental groups (Figure 8). DBM had remodeled to a greater extent than the dermal matrix, although not completely in all specimens. The median enthesis maturation score was 3 (95% CI 2.47 to 3.70) in the MSC group, 3 (95% CI 3 to 3) in the DBM + MSC group, and 2.5 (95% CI 1.88 to 3.46) in the dermal matrix + MSC group.
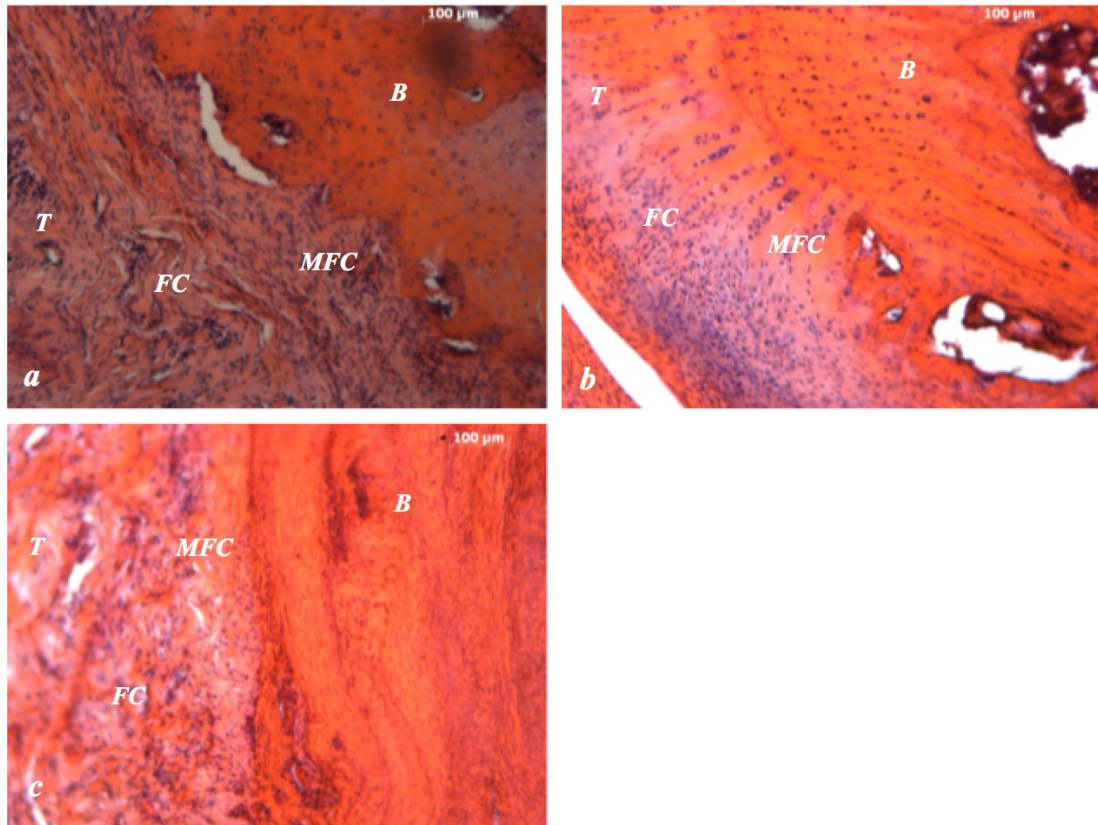
Figure 8. Photomicrograph of the enthesis at six weeks. Specimens stained with H&E.

(a) Control: Tendon-bone repair with MSCs, characterised by a graded enthesis comprising tendon (T), fibrocartilage (FC), mineralised fibrocartilage, and bone (B).

(b) DBM + MSCs: DBM neo enthesis comprising a well-organised, graded enthesis.

(c) Dermal matrix + MSCs: Dermal matrix neo enthesis with a disorganised structure.

Modified Movin Score

No significant differences in the modified Movin scores (indicating degeneration) were demonstrated between experimental groups. The median modified Movin score was 4.25 (95% CI 2.19 to 5.48) in the MSC group, 6.75 (95% CI 3.46 to 9.04) in the

DBM + MSC group, and 5.75 (95% CI 4.55 to 7.62) in the dermal matrix + MSC group.

Fiber Structure

All groups exhibited increased waviness and distance between collagen fibers. The median score was 1 (95% CI 0.12 to 1.55) in the MSC group, 1.75 (95% CI 1.03 to 2.30) in the DBM + MSC group, and 1.25 (95% CI 0.34 to 1.66) in the dermal matrix + MSC group (Figure 9). No significant inter-group differences were identified.
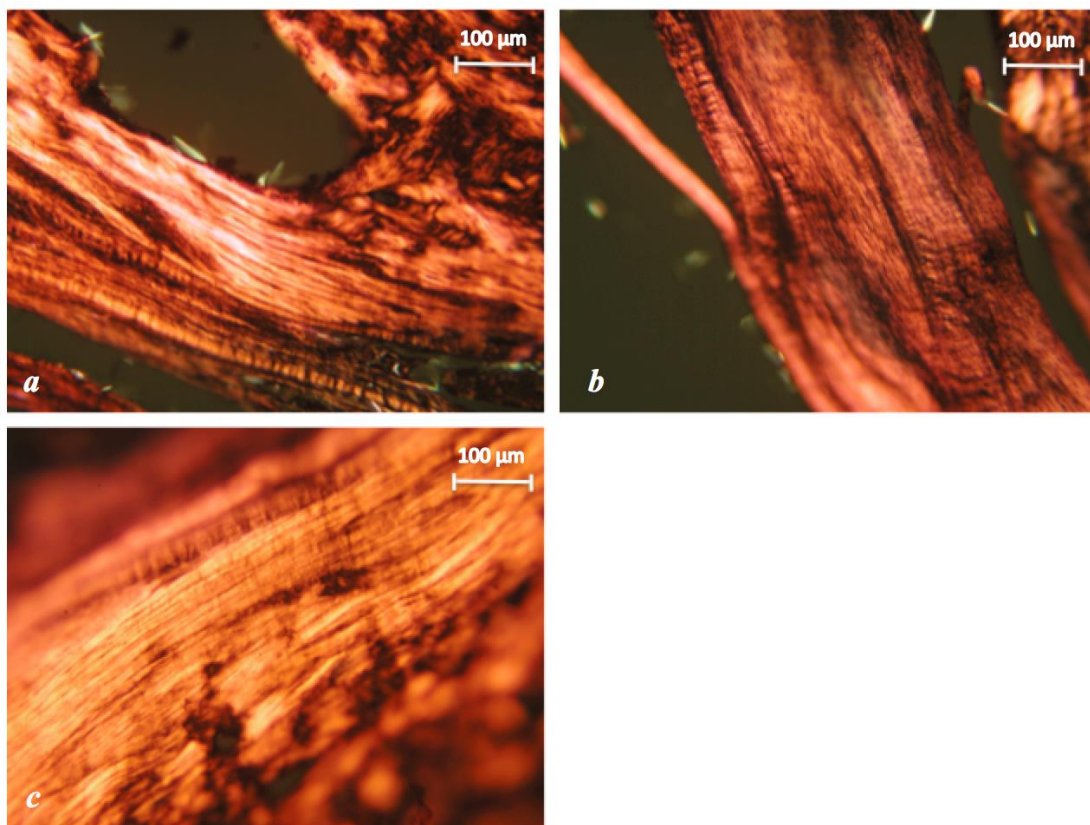
Figure 9. Photomicrograph (under polarised light) showing collagen fiber structure: (a) tendon-bone repair using MSCs alone, (b) DBM + MSCs, and (c) Dermal matrix + MSCs.

Fiber Arrangement

All groups exhibited a loss of the parallel arrangement observed in an intact tendon-bone interface). The median score was 1 (95% CI 0.52 to 1.31) in the MSC group, 1 (95% CI 0.81 to 1.69) in the DBM + MSC group, and 2 (95% CI 1.70 to 2.13) in the dermal matrix + MSC group. Fiber arrangement was significantly more abnormal in the dermal matrix group than in the DBM group (p = 0.014) and the MSC group (p = 0.003). No other significant inter-group differences were identified.

Tenocyte Nuclei

Rounding of nuclei (indicating persistent degeneration) was identified in all groups following tendon reattachment. The median score was 1 (95% CI 0.74 to 1.60) in the MSC group, 1.50 (95% CI 0.48 to 2.19) in the DBM + MSC group, and 1 (95% CI 0.79 to 1.88) in the dermal matrix + MSC group. No significant inter-group differences were identified.

Cellularity

Specimens were evaluated for an increase in cellularity, indicating persistent degeneration. The median score was 1 (95% CI 0.56 to 1.10) in the MSC group, 1.50

(95% CI 0.72 to 2.11) in the DBM + MSC group, and 1.25 (95% CI 0.90 to 1.93) in the dermal matrix + MSC group. Cellularity was significantly less (indicating less noticeable degeneration) in the MSC group than in the dermal matrix group (p = 0.031). No other significant inter-group differences were identified.

Vascularity

Specimens were evaluated for an increase in vascularity, indicating persistent degeneration. [27] The median score was 0 (95% CI -0.13 to 0.30) in the MSC group, 0.25 (95% CI -0.26 to 1.42) in the DBM + MSC group, and 0.5 (95% CI 0.02 to 0.81) in the dermal matrix + MSC group. No significant inter-group differences were identified.

Hyalinisation

Hyalinisation was not observed in any of the specimens.

pQCT

Total bone mineral density

Control specimens comprised the contralateral shoulder of animals that had undergone unilateral tendon detachment three weeks earlier. In this group (n = 6), the

median total bone mineral density at the supraspinatus tendon-bone insertion was 793.25 mg/ccm (95% CI 754.24 to 844.70). This significantly decreased six weeks following repair with dermal matrix + MSCs and repair with MSCs alone to a median of 717.25 mg/ccm (95% CI 582.54 to 782.39) (p = 0.010) and 719.65 (95% CI 666.80 to 765.27) (p = 0.010) respectively. In repairs with DBM + MSCs, median bone mineral density was 701.20 mg/ccm (95% CI 621.27 to 794.73). This was not significantly different to controls (p = 0.055) (Figure 10).
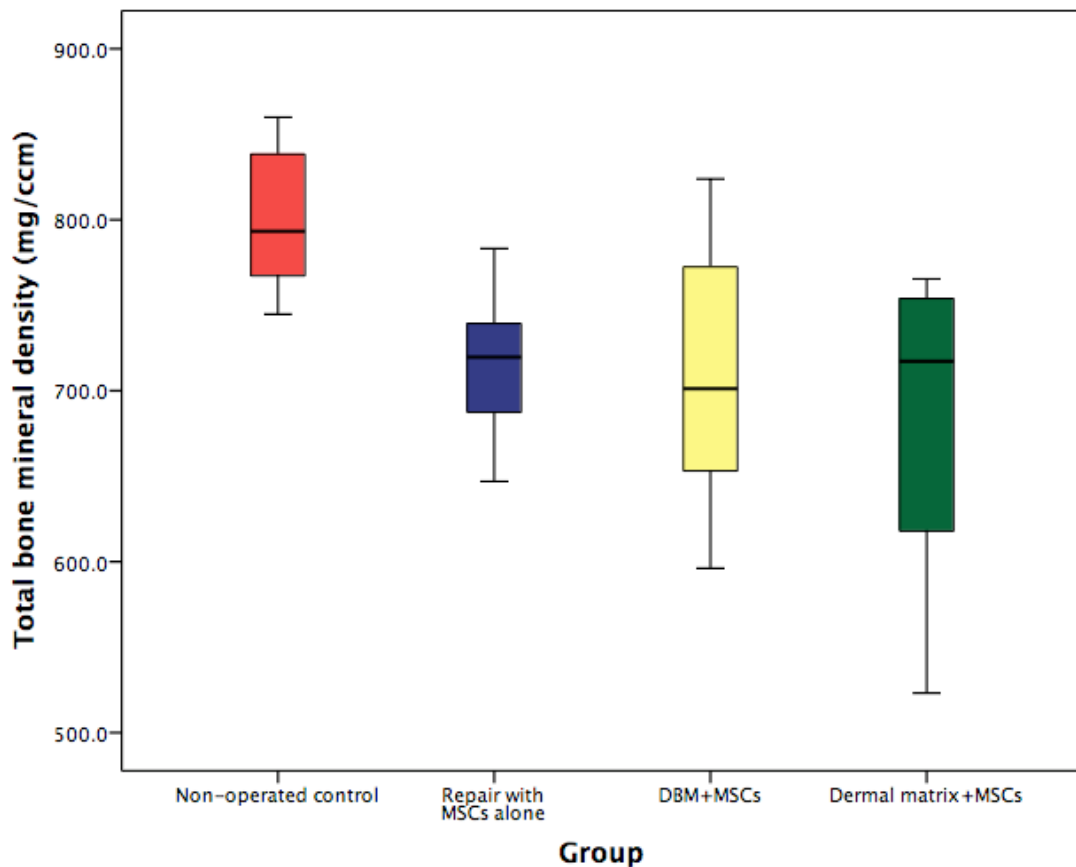

Figure 10. Box and whisker plot showing total bone mineral density at the supraspinatus tendon-bone insertion six weeks following repair with MSCs alone, cortical DBM and MSCs, and dermal matrix and MSCs.

Trabecular bone mineral density

In controls, the median trabecular bone mineral density at the supraspinatus tendon-bone insertion was 767.05 mg/ccm (95% CI 711.44 to 811.87). This did not significantly change following repair with MSCs alone [median of 751.60 mg/ccm (95% CI 691.33 to 810.10) (p = 0.52)], with DBM + MSCs [median of 743.65 mg/ccm (95% CI 653.87 to 877.30) (p = 0.69)], and with dermal matrix + MSCs [median of 723.15 mg/ccm (95% CI 598.97 to 823.86) (p = 0.63)].

Cortical bone mineral density

In controls, the median cortical bone mineral density at the supraspinatus tendon-bone insertion was 836.55 mg/ccm (95% CI 753.33 to 907.40). This significantly decreased six weeks following repair with MSCs alone [median of 702.50 mg/ccm (95% CI 632.51 to 742.86) (p = 0.025)], with DBM + MSCs [median of 679.25 mg/ccm (95% CI 557.87 to 764.70) (p = 0.016)], and with dermal matrix + MSCs [median of 691.35 mg/ccm (95% CI 563.44 to 754.26) (p = 0.010)].

Comparison of Histology: MSCs vs No MSCs

Enthesis Maturation Score

Repairs with MSCs alone and DBM + MSCs resulted in a significantly more mature enthesis than repairs augmented with dermal matrix alone (p = 0.044 and 0.007

Modified Movin Score

tendon that was significantly less degenerative (p = 0.025). There were no significant differences between other experimental groups (Figure 12).
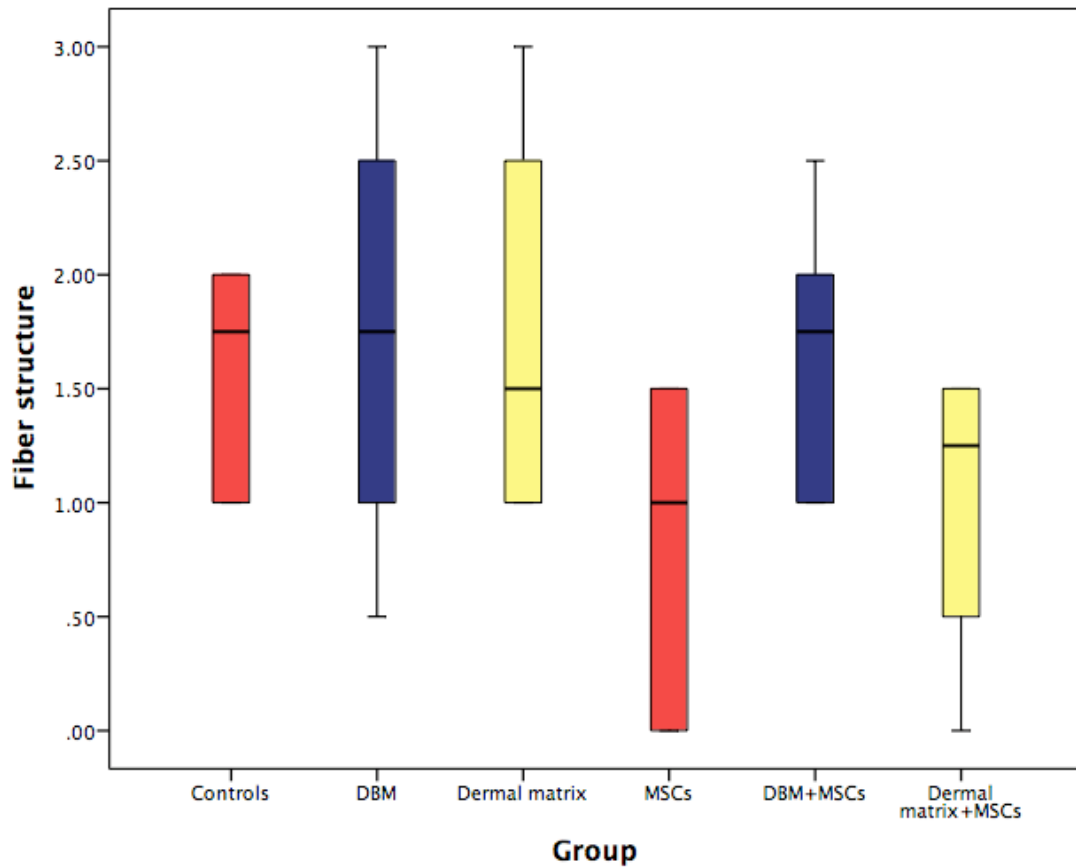

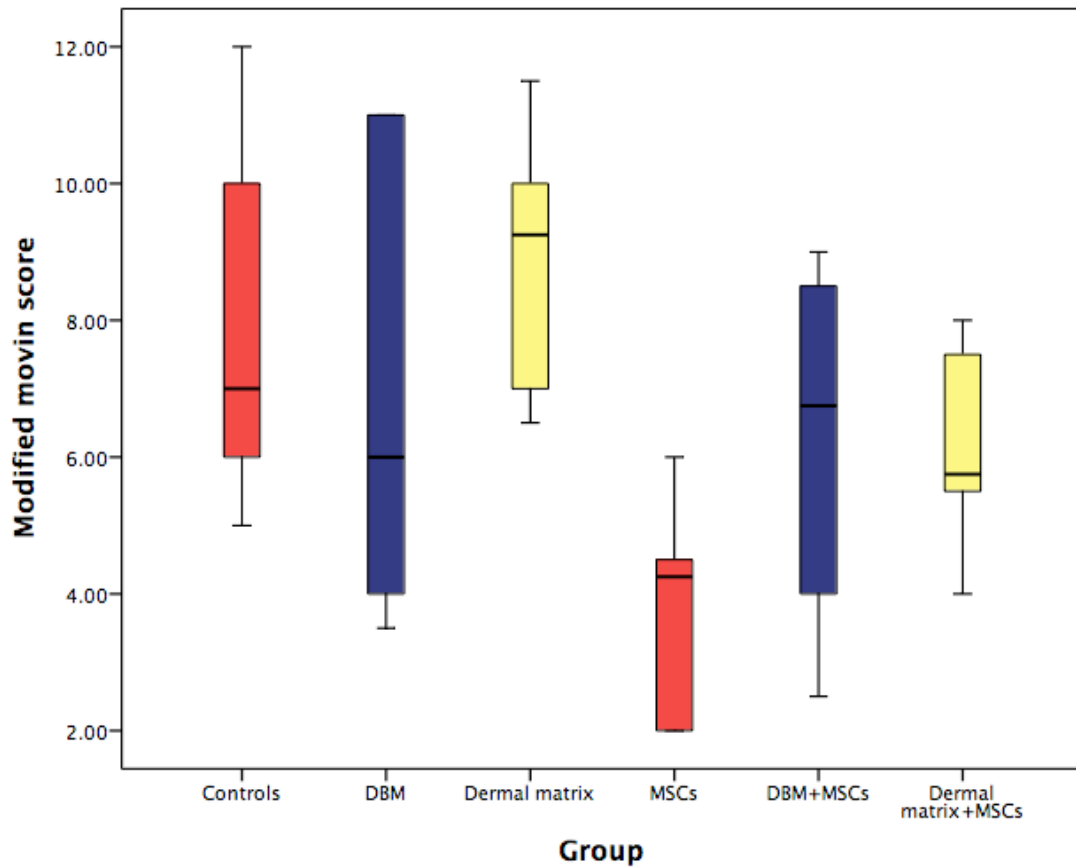Figure 12. Box and whisker plot illustrating the modified Movin scores following tendon reattachment between MSC and non-MSC groups.

pQCT

No significant increase in bone mineral density was conferred by the addition of MSCs to the healing interface (Figure 13).
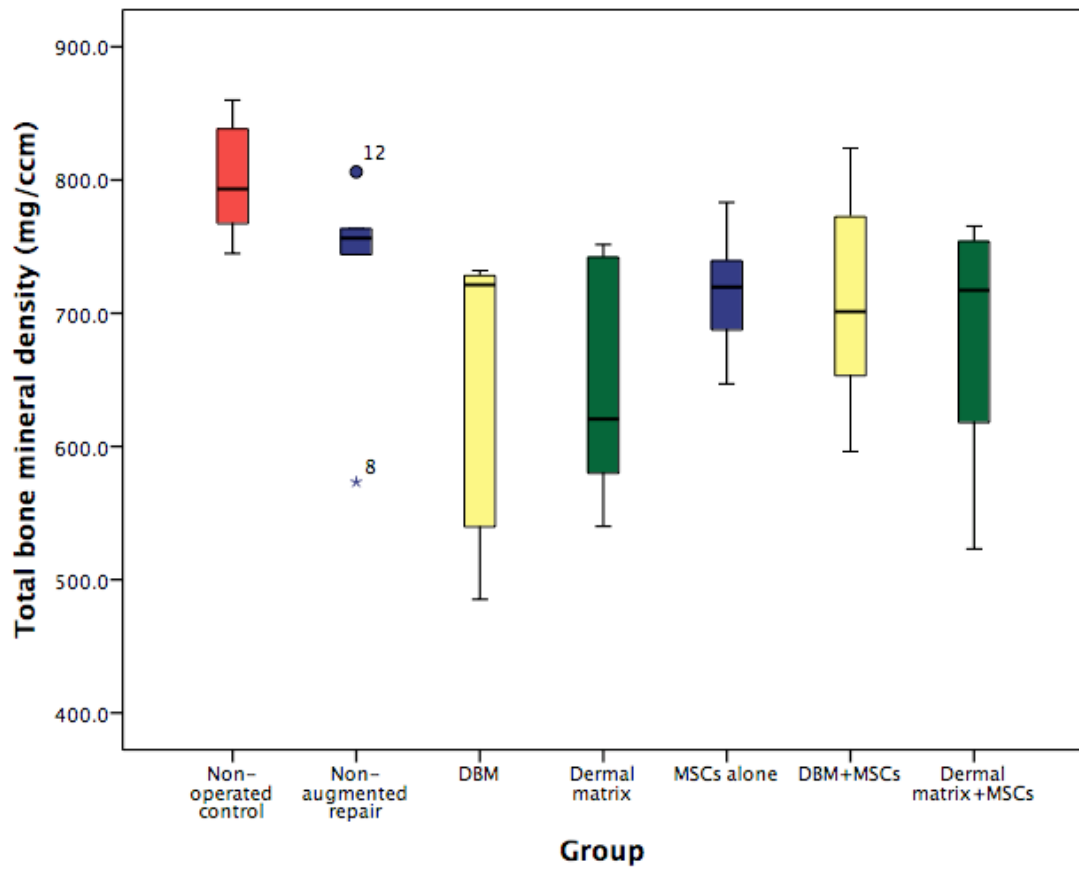
Figure 13. Box and whisker plot showing total bone mineral density at the supraspinatus tendon-bone insertion six weeks following repair with and without MSCs.

DISCUSSION

In this study, we hypothesised that compared to MSCs alone, DBM + MSCs would regenerate a morphologically superior enthesis characterised by greater fibrocartilage formation and improved collagen fiber organisation in a rat model of a chronic rotator cuff tear. We further hypothesised that DBM would result in a higher bone mineral density at the insertion site. These hypotheses though, are only partially supported by the results.

Using a rat model of a chronic rotator cuff tear, tendons were reattached to their bony insertion with DBM + MSCs, dermal matrix+ MSCs, and MSCs alone (controls), and analysed after six weeks. MSCs were confined to the healing enthesis as illustrated by the presence of QDs on fluorescent microscopy. All groups demonstrated closure of the tendon-bone gap with a fibrocartilaginous enthesis, but the degenerative process could not be reversed (as illustrated by a persistently high Modified Movin score). Although there were no significant differences in the enthesis maturation and Modified Movin scores, repairs augmented with dermal matrix + MSCs exhibited a disorganised enthesis, abnormal collagen fiber arrangement, and greater cellularity (indicating persistent degeneration) compared to other MSC groups. In addition to these histological parameters, bone mineral density at the enthesis was examined. This showed that only repairs augmented with DBM + MSCs reached a total bone mineral density not significantly lower than non-operated controls, although the absolute values were similar to the other experimental groups. This may be due to the upper range of the data set skewing the statistical analysis and we speculate that this difference would become more apparent with a larger sample size. Furthermore, this

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was not noted in a previous study where DBM was applied to a chronic rotator cuff tear model without MSCs. [41] We postulate that this may be due to endogenous growth factors within DBM (e.g. BMPs) causing MSCs to differentiate into osteoblasts and subsequently lead to new bone formation by activating SMAD proteins and increasing expression of RUNX2. [23] Low bone mineral density has been identified as an independent risk factor for postoperative rotator cuff healing and so it is plausible that the small improvement demonstrated in this study may improve tendon healing in *vivo* through its effect on increasing the pullout strength of suture anchors. However this does need to be investigated more comprehensively to determine whether this difference is maintained at later time points (to withstand the high forces exerted on the repair construct during rehabilitation) and to define the precise cellular mechanism responsible for it. [9, 45]

To determine whether MSCs could enhance the structure of the healing enthesis compared to repairs without stem cells, the results of this experiment were compared to a previously published study that utilised the same animal model and scaffolds. [41] It was hypothesised that the addition of MSCs would yield a morphologically superior interface. This was partially supported by the results, in that groups treated with MSCs alone and DBM + MSCs resulted in a more mature enthesis (characterised by a significantly higher enthesis maturation score) than those repaired with dermal matrix. The addition of MSCs to dermal matrix produced a tendon that was significantly less degenerative (characterised by a lower Modified Movin score). Furthermore, repairs with MSCs alone produced a tendon that was significantly less degenerative than non-augmented controls and repairs enhanced with dermal matrix. This highlights a

Several *in vivo* studies have evaluated the use of MSCs in tendon-bone healing. [17-20] In a severe model of acute tendon retraction, Thangarajah et al [42] applied DBM incorporated with MSCs (derived from the mononuclear fraction of a bone marrow aspirate) to the healing enthesis. At 12 weeks, there was reconstitution of the tendon-bone interface with a predominance of mineralised fibrocartilage, and an improvement in functional weight bearing. In a non-degenerative rotator cuff tear model, Gulotta et al [17] applied MSCs to an acutely detached supraspinatus tendon. At four weeks, the biomechanical strengths of the repairs were equal between MSC and control groups, and there was no difference in the amount of new cartilage formation or collagen fiber organisation. This was attributed to a lack of growth/transcription factors and so further studies evaluated the effect of membrane type 1 matrix metalloproteinase (MT1-MMP) on MSCs and MSCs transduced with adenoviral-mediated scleraxis (Ad-Scx). [18, 19] The results suggested that MSCs used in this context could result in more fibrocartilage, a higher ultimate load to failure, and a higher ultimate stress to failure.

The current study could not reproduce the results of MSC-induced tendon-bone healing observed in other animal models, even in the presence of DBM. [18, 19, 42] This is most likely due to the limited tendon-bone surface area in a rat model not presenting an environment that is as conducive to healing as large animal models and those that utilise a tendon-bone tunnel. [15, 17] DBM contains multiple growth factors such as BMPs and transforming growth factors (TGFs). [35, 36, 47-49] We speculate that

these were released slowly over time and were therefore unable to completely exert their effect on the MSCs during the six weeks where enthesis healing was permitted to take place.

Limitations of this study are principally due to the short time-period (six weeks) over which the healing enthesis was allowed to develop. It is feasible that with a longer study protocol, the new bony ingrowth in the DBM group that led to a preservation of bone mineral density compared to other experimental groups where bone mineral density had decreased, could have developed into fibrocartilage and mineralised fibrocartilage. A larger number of animals within the groups may have resulted in more significant values and a clearer indication of the benefits or drawbacks of the treatments. The lack of biomechanical testing precludes discussion about functional recovery of the enthesis, which is a particularly important consideration in the clinical setting as this may affect the type and success of postoperative rehabilitation following rotator cuff repair.

CONCLUSION

In conclusion, this study demonstrated that when DBM and MSCs were applied to the healing enthesis in a chronic rotator cuff tear model, a fibrocartilaginous-based structure was produced, albeit not significantly more mature than other experimental groups. Aside from these important histological findings, pQCT analysis showed that it was only in the DBM + MSC specimens where bone mineral density was not significantly different to non-operated controls.

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