1 TITLE PAGE

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3 Title:

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5 ==The Effectiveness of Demineralised Cortical Bone Matrix in a Chronic Rotator Cuff==

6 ==Tear Model.==

7

8 Conflict of Interest:

9

10 No benefits in any form have been received or will be received from a commercial

11 party related directly or indirectly to the subject of this article.

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14

15   ABSTRACT

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17   Background:

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19   The purpose of this study was to assess the effect of demineralised bone matrix

20   (DBM) on rotator cuff tendon-bone healing. The hypothesis was that compared to a

21   commercially-available dermal matrix scaffold, DBM would result in a higher bone

22   mineral density and regenerate a morphologically superior enthesis, in a rat model of

23   chronic rotator cuff degeneration.

24

25   Methods:

26

27   Eighteen female Wistar rats underwent unilateral detachment of the supraspinatus

28   tendon. Three weeks later, tendon repair was carried out in animals randomized into

29   three groups: Group 1 were repaired with DBM (n = 6); Group 2 received

30   augmentation with the dermal scaffold (n = 6); and Group 3 (controls) underwent

31   non-augmented tendon-bone repair (n = 6). Specimens were retrieved at six weeks

32   postoperatively for histological analysis and evaluation of bone mineral density.

33

34   Results:

35

36   No failures of tendon-bone healing were noted throughout the study. All groups

37   demonstrated closure of the tendon-bone gap with a fibrocartilaginous interface.

38   Dermal collagen specimens exhibited a disorganized structure with significantly more

39   abnormal collagen fiber arrangement and cellularity than the DBM-based repairs.

40 Non-augmented repairs exhibited a significantly higher bone mineral density than

41 DBM and the dermal collagen specimens, and were not significantly different to non-

42 operated control limbs.

43

44 Conclusion:

45

46 The application of DBM to a rat model of chronic rotator cuff degeneration did not

47 improve the composition of the healing enthesis compared to non-augmented controls

48 and a commercially-available scaffold. ==However, perhaps the most important finding==

49 ==of this study was that the control group demonstrated a similar outcome to augmented==

50 ==repairs.==

51

52 Level of evidence: Basic science study.

53

54 Keywords: animal model; enthesis healing; rotator cuff; scaffold; shoulder; tendon

55 bone; tissue engineering

56   INTRODUCTION

57

58   Tendon-bone healing is an important factor affecting the outcome of rotator cuff

59   repair. [6] Anatomical reattachment of the rotator cuff to its bony insertion is crucial,

60   but tendon degeneration and poor bone quality at the enthesis compromises the

61   quality, healing capacity, and durability of the performed repair. [14] In order to absorb

62   the energy of loading between tendon and bone, the native enthesis comprises a

63   natural gradation of four histological zones (tendon, demineralized fibrocartilage,

64   mineralized fibrocartilage, and bone). [18] Following injury, this is replaced by a weak

65   fibrovascular bridge with inferior biomechanical properties. [4; 7; 10] Osteopenia at the

66   greater tuberosity often accompanies this change in structure, leading to a reduction in

67   the pullout strength of suture anchors. [1; 3; 15; 21]

68

69   Demineralised bone matrix (DBM) is an osteoinductive agent that consists of a

70   collagen scaffold containing several growth factors. It has been demonstrated *in vivo*

71   to regenerate a fibrocartilaginous enthesis capable of resisting physiological forces,

72   but has not been investigated in a degenerative model of tendon-bone healing. [12; 16; 17]

73   The purpose of this study was to assess the effect of DBM on regeneration of an

74   enthesis following repair of a degenerative rotator cuff tear. We compared DBM with

75   another commercially available augmentation product with clinically-investigated

76   profiles of activity (GraftJacket [Wright Medical Technology, Inc., Arlington, TN

77   (Tennessee)]). GraftJacket (Wright Medical Technology, Inc., Arlington, TN) is

78   obtained from donated human cadaveric dermal tissue processed to remove its cellular

79   components whilst retaining its extra-cellular matrix. Its acellularity has the advantage

80   of not causing a host inflammatory reaction and it has been safely used in rats to

81   enhance healing of a large acute rotator cuff tear. [8] The hypothesis was that

82   augmentation with DBM will result in a higher bone mineral density in the recipient

83   footprint and regenerate a morphologically superior enthesis characterized by greater

84   fibrocartilage formation and improved collagen fiber organization in a rat model of

85   chronic rotator cuff degeneration when compared to accellular human dermal matrix,

86   after repair of a tendon tear.

87

88

89 MATERIALS AND METHODS

90

91 Study Design

92

93 In this basic science study, all animal work was conducted in accordance with a

94 Project License protocol accepted under the UK Home Office Animals (Scientific

95 Procedures) Act 1986. Eighteen female Wistar rats underwent unilateral detachment

96 of the supraspinatus tendon. Previously published data was used to calculate the

97 number of animals (n = 6) required to generate a power of 0.8 with significance at the

98 0.05 level. [16] Three weeks later, tendon repair was carried out in animals randomized

99 into three groups : Group 1 received augmentation of the repair with cortical allogenic

100 DBM (n = 6); Group 2 received augmentation with non-meshed, ultra-thick accellular

101 human dermal matrix ((n = 6) (GraftJacket, Wright Medical Technology, Inc.,

102 Arlington, TN; average 1.4mm thickness); and Group 3 underwent direct tendon-

103 bone repair without augmentation (n = 6). One surgeon carried out all procedures

104 using a standard technique. Animals were allowed to mobilise freely after surgery.

105 Specimens were retrieved at six weeks postoperatively for histological analysis and

106 peripheral quantitative computer tomography (pQCT) to evaluate bone mineral

107 density (BMD) at the reattachment footprint of the tendon, reversal of degenerative

108 changes within the tendon, and histological remodeling of the implanted

109 augmentation material.

110

111 DBM Manufacture

112

113   DBM derived from cortical bone was manufactured according to Urist's protocol,

114   with modifications. [20] The tibiae of skeletally mature female Wistar rats were

115   harvested immediately after euthanasia; all soft tissues and periosteum were stripped

116   from the bone surface. Bones measuring approximately 30 mm length by 3 mm width

117   were demineralized in 0.6 N HCL at room temperature. Demineralization was

118   confirmed by taking radiographs (300 seconds, 30 kV, Faxitron Corporation, Illinois,

119   USA). This was followed by washing in phosphate-buffered saline until the pH was

120   7.4 +/- 0.1. Samples were stored at -20˚C for two hours and transferred to a

121   lyophiliser (Edwards Girovac Ltd, Crawley, West Sussex, UK) for three days.

122   Specimens were then sealed in individual plastic bags, sterilised by gamma irradiation

123   at a dose of 25 kilograys (Isotron Limited, Reading, UK), and stored at -20˚C.

124   Samples were rehydrated at the time of surgery in normal saline for 30 minutes prior

125   to use.

126

127   Surgical Technique

128

129   Two surgeries were performed on each animal: full-thickness supraspinatus tendon

130   detachment and complete tendon reattachment. Anaesthesia was induced and

131   maintained using 2% isoflurane mixed with pure oxygen via a facemask for both

132   procedures. The right shoulder was operated on in all cases. A 1.5 cm skin incision

133   was made directly over the anterolateral border of the acromion. The deltoid was

134   detached from the acromion and split caudally for 0.5 cm, in order to identify the

135   tendon of supraspinatus. The supraspinatus tendon was completely detached from its

136   bony insertion on the humeral head, marked with a 5'0 prolene suture (Ethicon,

137   Johnson & Johnson Medical Ltd., Berkshire, UK) at the musculotendinous junction

138 (MTJ), and allowed to retract medially. Deltoid muscle, superficial fascia, and skin

139 were closed with 5'0 Vicryl suture (Ethicon, Johnson & Johnson Medical Ltd.,

140 Berkshire, UK). Animals were allowed unrestricted cage activity and received

141 analgesia (subcutaneous buprenorphine) every 12 hours for three days. The second

142 surgery to reattach the tendon was undertaken three weeks after the first procedure.

143 Prior to making the skin incision, the DBM or GraftJacket (Wright Medical

144 Technology, Inc., Arlington, TN) was rehydrated for 30 minutes in sterile normal

145 saline at the operating table.

146

147 A 2 cm skin incision was made in line with the supraspinatus muscle belly, ending

148 anterior to the lateral end of the clavicle. This approach was perpendicular to the

149 incision used for tendon detachment in order to make use of a virgin anatomical plane

150 devoid of scar tissue. The muscle belly of supraspinatus was identified and followed

151 distally to reveal the tendon stump with the suture marker in the MTJ. Scar tissue

152 between the tendon stump and its insertion was excised and the tendon was grasped

153 with a double-armed 5'0 prolene using a modified Mason-Allen technique. [19] Despite

154 traction on the tendon stump, it could not be directly brought back to the humeral

155 head in any of the cases. The bare tendon-bone insertion footprint was decorticated

156 using a #11 surgical blade until bleeding was seen. A custom-made dental drill was

157 used to drill a 1 mm hole from the neck of the humerus to the bony insertion of the

158 detached supraspinatus.

159

160 The scaffold (DBM or GraftJacket) was cut into a strip 10 mm long and 3 mm wide.

161 Each limb of the suture was passed through the scaffold to secure it in position. One

162 suture limb was passed through the hole in the prepared tendon stump and the other

163 suture limb was passed through the hole on the neck of the humerus. The

164 supraspinatus tendon-scaffold complex was attached to the insertion site, with the

165 graft in contact with both the tendon stump proximally and decorticated bone surface

166 distally (Figure 1). In the control group the sutures were inserted directly into the drill

167 holes, leaving a 5 mm gap between the tendon and bone in all cases.

168

169 A layered wound closure was undertaken in a similar manner to the first surgery, and

170 the animals were permitted unrestricted cage activity. Postoperative analgesia (Intra-

171 muscular Buprenorphine 0.6 mg) was given every 12 hours for three days.

172

173 Histological Assessment

174

175 After euthanasia with carbon dioxide, the right shoulder was dissected and a specimen

176 comprising the humerus with its attached supraspinatus musculotendinous unit was

177 removed. Each sample was fixed in 10% formal saline and underwent decalcification

178 in EDTA, ascending graded alcohol dehydration, defatting in chloroform, and

179 embedding in paraffin. Multiple sections, 4μm thick, were cut in the coronal plane

180 through the humerus, enthesis, and supraspinatus musculotendinous unit using a

181 microtome. Sections were attached to glass slides and stained with hematoxylin and

182 eosin (H&E).

183

184 Two blinded observers evaluated all sections using an Olympus BH-2 light

185 microscope (Olympus, Glasgow, UK). Tendon degeneration was assessed according

186 to a modified Movin scale [13] and included the following variables: (1) fiber structure,

187 (2) fiber arrangement, (3) rounding of the nuclei, (4) regional variations in cellularity,

188 (5) increased vascularity, and (6) hyalinization. A four-point scoring system was used:

189 0 = normal appearance, 1 = slightly abnormal appearance, 2 = a moderately abnormal

190 appearance, and 3 = a markedly abnormal appearance. [11] Based on this, the total score

191 for any given slide could range from 0 (normal tendon) to 18 (the greatest level of

192 degeneration).

193

194 Maturation of the enthesis was assessed according to the scoring system developed by

195 Ide et al [8]: score 1 – the insertion had continuity without fibrous tissue or bone

196 ingrowth, score 2 – the insertion had continuity with fibrous tissue ingrowth but no

197 fibrocartilage cells, score 3 – the insertion had continuity with fibrous tissue ingrowth

198 and fibrocartilage cells but no tidemark, and score 4 – the insertion had continuity

199 with fibrous tissue ingrowth, fibrocartilage cells, and a tidemark.

200

201 Measurement of Bone Mineral Density

202

203 Changes in bone mineral density at the humeral head were assessed using pQCT

204 scanning. One millimeter slices were obtained through the humeral head and

205 supraspinatus musculotendinous unit using an XCT 2000 Bone Scanner (Stratec

206 Medizintechnik Gmbh, Germany) with Software version 6.20. Controls were obtained

207 from the contralateral (non-operated) shoulder in six animals subjected to the same

208 rehabilitation conditions as the study groups.

209

210 Statistical Analysis

211

212   Numerical data were analysed using SPSS software package, version 23 (SPSS Inc,

213   an IBM Company, Chicago, Illinois). Mann Whitney U tests were used to compare

214   data between groups. Results were considered significant at the $p < 0.05$ level.

215   RESULTS

216

217   All animals survived the duration of the study and none had post-operative infection.

218   Limping was noted for the first three to five postoperative days but a normal gait

219   pattern returned thereafter.

220

221   Macroscopic Findings

222

223   At the time of euthanasia there was continuity between the repaired tendon and the

224   bone in all groups (Figure 2). No signs of infection were noted in any specimen, none

225   of the repairs had failed, and all the sutures were intact. Remodeling of the graft

226   material occurred to a greater extent in the DBM group, whereby the scaffold could

227   not be discerned from other tissues in the regenerated tendon-bone interface. In

228   contrast, accellular human dermal matrix was clearly visible at necropsy. Control

229   group specimens demonstrated complete closure of the enthesis.

230

231   Quantitative Histology

232

233   Enthesis Maturation Score

234

235   No significant difference was observed in the enthesis maturation score between

236   experimental groups. However, the dermal matrix specimens exhibited a more

237   disorganized enthesis than control and DBM groups, which were characterized by a

238   well organised, graded structure (Figures 3 and 4). The median enthesis maturation

239   score was 2.5 (95% CI 1.88 to 3.46) in the controls, 3 (95% CI 2 to 4) in the DBM

240   group, and 2.5 (95% CI 2.02 to 2.81) in the accellular human dermal matrix group.

241

242   Modified Movin Score

243

244   No significant difference in the modified Movin score (indicating degeneration) was

245   demonstrated between experimental groups (Figure 5). The median modified Movin

246   score was 7 (95% CI 5.04 to 10.62) in the controls, 6 (95% CI 3.37 to 10.47) in the

247   DBM group, and 9.25 (95% CI 6.94 to 10.89) in the dermal matrix group.

248

249   Fiber Structure

250

251   All groups exhibited increased waviness and distance between collagen fibers (Figure

252   6). The median score was 1.75 (95% CI 1.07 to 2.10) in the controls, 1.75 (95% CI

253   0.66 to 2.83) in the DBM group, and 1.5 (95% CI 0.83 to 2.67) in the dermal matrix

254   group. There were no significant inter-group differences.

255

256   Fiber Arrangement

257

258   All groups exhibited a loss of the parallel arrangement that typically characterises the

259   fiber arrangement in tendons (Figure 6). The median score was 1.5 (95% CI 0.88 to

260   2.46) in the controls, 1.25 (95% CI 0.84 to 2.16) in the DBM group, and 2.25 (95% CI

261   1.90 to 2.76) in the accellular human dermal matrix group. Fiber arrangement was

262   significantly more abnormal in the dermal matrix group than in the DBM group (p =

263   0.039). There were no other significant inter-group differences.

264

265   Tenocyte Nuclei

266

267   Rounding of nuclei (indicating persistent degeneration) was identified in all groups

268   following tendon reattachment (Figure 7). The median score was 1.75 (95% CI 1.12

269   to 2.55) in the controls, 1.50 (95% CI 1.03 to 1.97) in the DBM group, and 2 (95% CI

270   1.56 to 2.10) in the dermal matrix group. There were no significant inter-group

271   differences.

272

273   Cellularity

274

275   Specimens were evaluated for an increase in cellularity, indicating persistent

276   degeneration. The median score was 1.50 (95% CI 0.97 to 2.20) in the controls, 1.25

277   (95% CI 0.70 to 1.97) in the DBM group, and 2 (95% CI 1.69 to 2.48) in the

278   accellular human dermal matrix group. Cellularity was significantly less in the DBM

279   group than in the dermal matrix group (p = 0.037), but there were no other significant

280   inter-group differences.

281

282

283

284

285 Vascularity

286

287 Specimens were evaluated for an increase in vascularity, indicating persistent

288 degeneration. [11] The median score was 1.50 (95% CI 0.54 to 2.12) in the controls,

289 0.25 (95% CI -0.35 to 2.01) in the DBM group, and 1 (95% CI 0.30 to 1.53) in the

290 accellular human dermal matrix group. There were no significant inter-group

291 differences.

292

293 Hyalinisation

294

295 Hyalinisation was not observed in any of the specimens.

296

297 pQCT

298

299 For comparison with the operated side, control specimens were harvested from the

300 contralateral non-operated shoulder of the animals not used in this study. In this group

301 (n = 6), the median total bone mineral density at the supraspinatus tendon-bone

302 insertion was 793.25 mg/ccm (95% CI 754.24 to 844.70) (Figure 8). This

303 significantly decreased at six weeks following augmented tendon repair with DBM

304 and accellular human dermal matrix to a median of 721.20 mg/ccm (95% CI 537.52

305 to 771.68) (p = 0.004) and 620.55 mg/ccm (95% CI 551.01 to 733.80) (p = 0.006)

306 respectively. Following attempted direct repair of the supraspinatus tendon to bone

307 without the addition of an augmentation strategy, median bone mineral density at the

308   enthesis was 756.30 mg/ccm (95% CI 648.01 to 818.46). This was not significantly

309   different to controls (p = 0.078).

310

311

312   DISCUSSION

313

314   In this study, we hypothesised that: 1. DBM would regenerate a morphologically

315   superior enthesis characterized by greater fibrocartilage formation and improved

316   collagen fiber organisation compared to a commercially available accellular human

317   dermal matrix scaffold (GraftJacket); 2. DBM would result in a higher bone mineral

318   density at the footprint insertion site. However, our results do not support either

319   hypothesis. Using a previously validated rat model of chronic rotator cuff

320   degeneration, [2] the supraspinatus tendon was reattached to its bony insertion with

321   interposed DBM, dermal collagen, or no augmentation (controls) and analysed after

322   six weeks. All groups demonstrated closure of the tendon-bone gap with a

323   fibrocartilagenous interface, but the degenerative process at this time point could not

324   be reversed (there was a persistently high Modified Movin score in all groups).

325   Although there was no significant difference in enthesis maturation scores at the

326   conclusion of the study, the dermal collagen specimens exhibited a disorganized

327   structure with significantly more abnormal collagen fiber arrangement and cellularity

328   than the DBM repairs (indicating more severe degeneration).

329

330   Bone mineral density at the enthesis was the second parameter examined because it

331   has been shown to determine the quality and degree of tendon-bone healing, and the

332   pullout strength of suture anchors in the clinical setting. [3; 12] Following tendon

333   reattachment with DBM and dermal collagen, this did not recover to the baseline

334   levels (bone mineral density at the non-operated tendon insertion site). In contrast, the

335   non-augmented repairs exhibited a significantly higher bone mineral density than

336 DBM and dermal collagen specimens, and were not significantly different to control

337 specimens.

338

339 Several *in vivo* studies have demonstrated the ability of DBM to enhance tendon-bone

340 healing, but not in a degenerative rotator cuff model. [9; 12; 16; 17] In the first study to

341 examine the use of DBM at the healing enthesis, ▮▮▮▮▮ [16] created an acute tear

342 in an ovine patellar tendon model and repaired it with the scaffold. Compared to

343 controls at 12-weeks, the DBM group showed increased amounts of mineralised

344 fibrocartilage and improved functional weight-bearing. To determine the effect of

345 DBM on tendon healing within a bone tunnel, Kilicoglu et al [9] developed a rabbit

346 model and retrieved specimens three, six, and nine weeks after surgery. At three

347 weeks, a higher number of Sharpey's fibers, slightly increased fibrocartilage

348 formation, and new bone formation was observed in the DBM group, but this

349 difference was not significant at later time-points. In a further study examining the

350 healing potential of a DBM paste in a tendon-bone tunnel, Lovric et al [12] created a rat

351 model of anterior cruciate ligament (ACL) reconstruction. No reconstitution of the

352 fibrocartilage layer was observed in either the DBM or control groups. The main

353 finding was a significantly greater amount of new bone formation in DBM-augmented

354 animals associated with a significantly higher peak load to failure of the tendon-bone

355 interface at six weeks. Similar findings were noted in an ovine model of severe tendon

356 retraction, where DBM was used to enhance healing across a 1 cm defect. In this

357 study, there was an improvement in functional weight bearing at successive time

358 points and the development of a direct enthesis characterised by fibrocartilage. [17]

359

360 The current study could not reproduce the results of DBM-induced tendon-bone

361 healing observed in other animal models. Considering that the overall contact area

362 between tendon and bone is a major determinant of healing, the limited tendon-bone

363 surface area in a rat model does not present an environment that is as conducive to

364 healing as large animal models and those that utilise a tendon-bone tunnel. [5]

365 Furthermore, the high shear forces from contraction of supraspinatus are distributed

366 over a relatively small surface area and therefore may not have allowed adequate

367 tendon healing to take place. [6] Perhaps the most important finding of this study was

368 that the non-augmented control group demonstrated a similar histological outcome to

369 those tendons repaired with DBM and dermal collagen, and also resulted in a bone

370 mineral density at the tendon insertion comparable to non-operated controls. This

371 raises considerable doubt as to the suitability of a rat model to investigate rotator cuff

372 tendon-bone healing, because in humans spontaneous healing is thought not to occur.

373

374 There are several limitations to this study. Previous work has shown that most control

375 tendons in a rat model heal by eight weeks, making it challenging to detect

376 differences between control and experimental groups because it is difficult to improve

377 on a solid mass of healed scar tissue. [6] Earlier time points in the healing phase (two

378 and four weeks) may have highlighted differences that later became non-significant

379 between groups. Similarly, later time points (nine and 12 weeks) may have allowed

380 greater time for the scaffolds to remodel and exert their restorative effect on tendon-

381 bone healing. Finally, biomechanical evaluation of the repair construct would have

382 strengthened the results of this study, but due to the limited number of specimens this

383 could not be performed.

384

385   CONCLUSION

386

387   This study has highlighted the difficulty of developing a scaffold to solve the problem

388   of rotator cuff tendon-bone healing. Although the application of DBM to a chronic

389   rotator cuff tear does result in an enthesis comprised of fibrocartilage, this was not

390   significantly more mature than non-augmented controls or a commercially available

391   alternative accellular human dermal matrix scaffold.

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471

472   FIGURE AND TABLE LEGENDS

473

474   Figure 1: Supraspinatus tendon-bone fixation.

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476   Figure 2: Supraspinatus tendon-bone fixation, post-mortem, with cortical DBM (A),

477   GraftJacket (B), and direct repair of tendon to bone (Control) (C).

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479   Figure 3: Photomicrograph of the enthesis at six weeks. Specimens stained with H&E.

480   (a) Control: Direct tendon-bone repair characterized by a graded enthesis comprising

481   tendon (T), fibrocartilage (FC), mineralised fibrocartilage, and bone (B). (b) DBM:

482   DBM neo enthesis comprising a well organised, graded enthesis. (c) GraftJacket:

483   GraftJacket neo enthesis with a disorganized structure.

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485   Figure 4: Box and whiskers plot illustrating the enthesis maturation scores following

486   tendon reattachment using no augmentation strategy (controls), DBM, and

487   GraftJacket.

488

489   Figure 5: Box and whiskers plot illustrating the modified Movin scores following

490   tendon reattachment using no augmentation strategy (controls), DBM, and

491   GraftJacket.

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493   Figure 6: Photomicrograph (under polarized light) showing collagen fiber structure.

494   (a) Controls (direct tendon-bone repair). (b) DBM group. (c) GraftJacket group.

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496   Figure 7: Photomicrograph illustrating rounded nuclei in control (direct tendon-bone

497   repair) (a), DBM (b), and GraftJacket (c).

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499   Figure 8: Box and whiskers plot showing total bone mineral density at the

500   supraspinatus tendon-bone insertion 6 weeks following direct tendon to bone repair,

501   repair with cortical DBM, and repair with GraftJacket.

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