1    **Title**: Repair of large osteochondritis dissecans lesions using a multi-layered collagen-

2    based osteochondral graft substitute in an equine athlete.

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32

33    **Place where this case was operated:**

34    University College Dublin Veterinary Hospital, Large Animal Surgery Service, School

35    of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

36

37    **Ethical approval and consent form:**

38    As this was a true clinical case and not an experimental case, no ethical approval was

39    required. Authorisation from the Irish Department of Agriculture, Food and the Marine

40    was granted to use the multi-layer collagen-based osteochondral graft substitute on this

41    specific case. The horse was permanently stamped "Out of the Food Chain". The owner

42    signed a consent form discharging the University College Dublin Veterinary Hospital

43    and the Royal College of Surgeons in Ireland from any legal responsibilities.

44

45    **Oral/poster presentation of this work:**

46    This work has not been presented at any national or international meeting.

47

48    **Conflict of Interest:**

56

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**Abstract:** (250 words)

Osteochondral lesions, resulting from *osteochondritis dissecans*, are problematic to treat and present a significant challenge for clinicians. The aims of this study were to investigate the use of a scaffold-assisted microfracture approach, employing a novel multi-layered collagen-based osteochondral graft substitute in the treatment of severe *osteochondritis dissecans* of both lateral femoral trochlear ridges in an equine athlete, and to assess the potential of this novel scaffold to enhance repair of the osteochondral unit. A 15-month-old female filly presented with large *osteochondritis dissecans* lesions involving both femoral lateral trochlear ridges. After routine arthroscopic debridement and microfracture of the subchondral bone, multi-layered osteochondral defect repair scaffolds were implanted into the fragmentation beds in both left and right femoropatellar joints via mini-arthrotomy. Exploratory arthroscopy 5 months post-implantation revealed smooth cartilaginous repair tissue, contiguous with adjacent cartilage, covering the defect. At 22-month follow up, the filly had no signs of lameness and was exercising at her intended level. Radiographically, although still slightly flattened, the femoral trochlear ridges were smooth, with no evidence of osteoarthritis. Ultrasonographically the defects were filled with bone and covered with an overlying cartilaginous layer, with the trochlear ridge contour almost entirely restored. This report demonstrates the effective clinical use of this novel multi-layered osteochondral defect repair scaffold in the treatment of *osteochondritis dissecans* of an equine athlete. The successful repair achieved here using this novel scaffold in an equine patient with large bilateral lesions shows the potential for clinical translation in the treatment of human patients presenting with osteochondral defects.

## 1. Introduction

Repair of articular cartilage defects, occurring as a result of osteoarthritis, fracture, fragmentation, or surgical debridement of *osteochondritis dissecans* (OCD) lesions or subchondral bone cysts, represents a significant challenge for orthopaedic surgeons. Articular cartilage regeneration following injury is impaired by inherently poor vascular supply and the limited cellular content of hyaline cartilage (Nixon *et al*., 2011). Greater challenges present with larger defects and with subchondral bone involvement and currently no 'gold standard' technique exists for repair of such defects.

*Osteochondritis dissecans (OCD)*, a disruption of endochondral ossification, is a common orthopaedic developmental disease in many species, including humans and horses, and results in separation and instability of the overlying articular cartilage. OCD affects 10 to 30% of the equine population, depending on breed and joint (Desjardin *et al*., 2014) and as such is a major concern in the horse industry (Jeffcott, 1996). In humans, OCD is less common, with prevalence estimated at 15 to 21 per 100,000 (Hughston *et al.,* 1984), and defects most commonly occurring on the femoral condyles (Pascual-Garrido *et al.,* 2009). Arthroscopic debridement of OCD lesions is the primary surgical strategy to facilitate healing in horses (Van Weeren and Jeffcott, 2013; McIlwraith, 2013; McIlwraith *et al*., 2015). Prognosis is inversely proportionate to the lesion's size; with lesions in excess of 40 mm long carrying a 54% chance of achieving expected athletic performance with arthroscopic debridement alone (Foland *et al*., 1992). In order to enhance repair of OCD lesions in veterinary patients, similar approaches to those used in human surgery have been applied. Autologous osteochondral grafting (mosaicoplasty) has been used successfully in the repair of deep osteochondral defects resulting from debridement of subchondral bone cysts (Bodó *et al*., 2004; Bodó *et al*., 2014). Autologous chondrocyte implantation has shown success

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in the healing of experimentally created full-thickness cartilage lesions (1.5cm

diameter) in the lateral trochlear ridge of horses with improved histologic scores at 8

weeks post-implantation (Nixon *et al*., 2011). However, debridement, with or without

microfracture, remains the most commonly used treatment for OCD defects in horses

for practical reasons (McIlwraith, 2013; McIlwraith *et al*., 2015; Frisbie *et al*., 1999).

Equine models are currently recommended for preclinical assessment of new

biomaterial-based strategies for cartilage repair as they provide the closest

approximation to humans in terms of cartilage thickness (Malda *et al*., 2012). A number

of experimental studies have thus been carried out using equine models to assess the

regenerative potential of new biomaterials (Frisbie *et al*., 2008; Frisbie *et al*., 2009;

Kon *et al*., 2010b; Nixon *et al.,* 2011; Seo *et al*., 2013). Few studies have been carried

out in horses to investigate the use of these biomaterial-based strategies for the repair

of osteochondral defects resulting from injury or disease, such as OCD. In one case,

Tsuzuki *et al*. (2013) showed fibrocartilaginous repair of an OCD lesion using a gelatin

β-tricalcium phosphate sponge, impregnated with platelet-rich plasma, bone

morphogenetic protein-2, mesenchymal stem cells. While the biomaterial-based

solutions investigated to date have shown some potential, generally fibrocartilaginous

tissue results and an urgent need remains for repair strategies that will facilitate long

lasting repair of the osteochondral unit.

To meet this need a biocompatible, biomimetic and highly porous (> 95%) multi-

layered collagen-based scaffold has been developed within the Tissue Engineering

Research Group in the Royal College of Surgeons in Ireland (Gleeson *et al.*, 2010;

Levingstone *et al*., 2014). This novel construct mimics the inherent graduated structure

of healthy osteochondral tissue: a bone layer composed of type I collagen and

140 hydroxyapatite (HA), with demonstrated osteogenic properties (David *et al*., 2015;

141 Gleeson *et al*., 2010; Murphy *et al.,* 2014), an intermediate layer composed of type I

142 collagen and hyaluronic acid (HyA) and a cartilaginous region composed of type I

143 collagen, type II collagen and HyA. The properties of the material are designed to

144 provide the biological and biomechanical cues required to encourage infiltration of host

145 cells from the bone marrow and to promote differentiation of these cells towards the

146 required lineage in each region. The regenerative potential of this scaffold has been

147 demonstrated *in vitro* and *in vivo* in both rabbits and goats (Levingstone *et al*., 2014;

148 Levingstone *et al*., Under review a; Levingstone *et al*., Under review b).

149 This study describes the use of this novel, multi-layered osteochondral scaffold in the

150 treatment of bilateral OCD of the lateral trochlear ridges in the femoropatellar joints of

151 an Irish Sport Horse. The extent of the osteochondral lesions in this horse was such that

152 the prognosis for future athleticism was poor. Loss of articular cartilage and destruction

153 of subchondral bone architecture, resulting from the required curettage of the lesions,

154 was expected to disrupt the contour of the trochlear ridges. The degree of disruption

155 would likely have led to instability in the joint, resulting in osteoarthritis (McIlwraith,

156 2013). The aims of this study were to assess the potential of a scaffold-assisted

157 microfracture approach, combining microfracture with implantation of a novel multi-

158 layered collagen-based scaffold to enhance the repair of the osteochondral unit of both

159 lateral femoral trochlear ridges in a horse affected by OCD.

160

161 **2.     Materials and methods**

162 **2.1     Case description**

163 A 15-month-old female Irish Sport Horse presented to the University College Dublin

164 Veterinary Hospital (UCDVH) with severe effusion of both femoropatellar (FP) joints.

Radiographic examination of the FP joints demonstrated marked intra-articular soft tissue swelling and the contour of the middle and proximal aspects of the lateral trochlear ridge (LTR) of the distal femur of both the left and right hind limbs was markedly irregular, with large areas of resorption deep in the subchondral bone. Non-detached osteochondral fragments were visible in these lesions. There was a small, mineralized fragment detached from the parent bone in the cranial aspect of the right FP joint (Figure 1). The proximodistal length and craniocaudal depth of the osteochondral lesions on the left side were 46 mm and 22 mm, respectively, and on the right side were 49 mm and 23 mm, respectively. Ultrasonographic (US) examination confirmed the presence of marked synovial effusion of the FP joints. The surface of the osteochondral unit was undulating, irregular in shape and thickness, and was deeply fissured. Non-detached osteochondral fragments were noted (Figure 2). The mediolateral measurement of the lesion was 20 mm for the left trochlea and 19 mm for the right trochlea. A free ossified fragment was found in the right FP joint. These changes were consistent with a diagnosis of OCD and were classified according to the International Cartilage Repair Society (ICRS) OCD lesion classification scale as grade IV (i.e. lesions with a complete discontinuity, with a dislocated fragment or a loose fragment within the bed; grade IV is the most severe grade) (Brittberg and Winalski, 2003).

## 2.2 Scaffold fabrication

Multi-layered scaffold sheets, 60 mm x 60 mm x 20 mm, were fabricated using a unique iterative layering fabrication method (Levingstone *et al*., 2014). The multi-layered scaffold consisted of a bone layer containing type I collagen (Col1) [Southern Lights Biomaterials, Napier, New Zealand] and hydroxyapatite (HA) [Plasma Biotal, UK], an

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190 intermediate layer, consisting of Col1 and hyaluronic acid sodium salt derived from

191 *Streptococcus equi* (HyA) [Contipro, Dolní Dobrouč, Czech Republic] and a cartilage

192 layer, consisting of Col1, type II collagen (Col2) [porcine type II collagen, Symatese,

193 Chaponost, France] and HyA. Scaffolds were freeze-dried as previously described in

194 order to produce a multi-layered scaffold with seamless layer integration (Levingstone

195 *et al.*, 2014; Gleeson *et al.*, 2009). Following freeze-drying the scaffold was cross-

196 linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)/N-

197 hydroxysuccinimide (NHS) (Sigma–Aldrich, Arklow, Ireland) at a concentration of 6

198 mM EDAC $g^{-1}$ of collagen, and a 5:2 M ratio of EDAC:NHS for 2 hours at room

199 temperature (Haugh et al. 2011). Scaffolds were then dehydrothermally (DHT) cross-

200 linked in a vacuum oven (VacuCell, MMM, Germany) at 105°C and a pressure of 50

201 mTorr for 24 hours to generate cross-links through a condensation reaction and also to

202 sterilise the scaffolds.

203

204 **2.3    Surgical procedure**

205 The horse was anaesthetized and placed in dorsal recumbency. A routine arthroscopic

206 approach to the left FP joint was made between the middle and lateral patellotibial

207 ligaments (McIlwraith et al., 2015). The instrument portal was made through stab

208 incision lateral to the lateral patellotibial ligament. The cartilage covering the middle

209 and proximal parts of the LTR was undulating, irregular and deeply fissured with non-

210 detached but unstable fragments (Figure 3). The cartilage lesions were debrided and the

211 subchondral bone was curetted until solid, bleeding bone was encountered.

212 Microfracture of the subchondral bone was carried out with a 30° microfracture pick

213 (Sontec Instruments, Colorado, USA) and a mallet, as previously described (Frisbie *et*

214 *al.*, 1999). Debris and small fragments were then lavaged from the FP joint with the aid

of a third portal created in the suprapatellar pouch with an 11 mm laparoscopic cannula (Vinardell *et al.*, 2008; McNally *et al.*, 2011). A second surgical team performed an identical procedure concurrently on the right FP joint. As the defects were extensive and the prognosis for athleticism was poor, a tissue engineering approach was applied in this horse. To enable scaffold implantation a small arthrotomy (50 mm) was created directly over the LTR of each stifle (lateral to the lateral patellotibial ligament). Self-retaining retractors were placed to expose the fragmentation bed (Figure 4). A sterile foil template was press-fitted into the fragmentation bed and the sterilized multi-layered scaffold sheets were cut to match the size and shape of the templates. The scaffolds were then soaked in sterile saline and pressed gently into the defect. The left scaffold was secured with a combination of fibrin glue (Tisseel, Baxter, Dublin, Ireland) and cyanoacrylate glue (Histoacryl, B Braun Medical, Dublin, Ireland) (Figure 4). The scaffold in the right defect was retained using a press-fit approach. Closure of the arthrotomy was performed in five layers and the arthroscopy portals were closed in one layer. After closure a combination of morphine [Morphine Sulphate, Mercury Pharma] 10 mg, bupivacaine [Marcain, AstraZeneca] 200 mg and gentamicin [Gentaject, Franklin] 500 mg was injected intra-articularly. Sterile adhesive dressings were placed prior to recovery from anesthesia.

**2.4    Post-operative evolution**

The Anderson Sling system [Charles D. Anderson, Care for Disabled Animals, Potter Valley, CA] was used in recovery to support the horse until fully conscious and weight bearing on all limbs, reducing stress on the scaffolds (Taylor *et al*., 2005). Perioperative antibiotics (Procaine penicillin [Depocillin, Interchem] 22,000 IU/kg IM q 12 hours and gentamicin sulphate [Gentaject, Franklin] 6.6 mg/kg IV q 24 hours) were continued

for five days. Non-steroidal anti-inflammatory (phenylbutazone [Phenylarthrite, Vetoquinol] 2.2 mg/kg IV q 12 hours) was continued for five days on a tapering dose. The horse was discharged from the hospital on day eight post-operatively, with recommendations to remove the skin sutures on day 12-14 post-surgery and to restrict exercise to a large box stall for the first 6 weeks, then to walk the horse in hand for 6 weeks and finally to turn her out to a paddock for the following 3 months.

Follow up US was performed at 8 days, 5 months and 22 months postoperatively. Follow up radiographic examination was performed at 5 and 22 months postoperatively. A radiographic scoring system, devised by Sparks *et al*., (2011) was used to evaluate radiographs at 5 and 22 months. This system assessed the postoperative radiographic appearance of the subchondral filling (scale: 0 = No evidence of prior OCD to 3 = Worsening/progression of the subchondral lucencies) and subchondral bone contour (scale: 0 = smooth, contiguous to 4 = free floating mineralized bodies).


## 3.    Results

### 3.1    Post-operative outcome

The horse recovered uneventfully from anesthesia using the Anderson Sling system. Moderate effusion of the FP joints was observed at suture removal 12 days post-operatively. The incisions were intact at this time. Eight days later, both arthrotomy sites began discharging clear serous fluid and dehisced fully over the next 2 days. The horse was treated with trimethoprim-sulphadiazine [Noroprim granules, Norbrook] (30 mg/kg q 12 hours *per os*) for 2 weeks from the onset of dehiscence. The wounds were cleaned daily with dilute chlorohexidine solution and had fully healed 7 weeks later. No fever or signs of lameness developed during this period. Exercise was restricted to box rest during the entire management of this complication (total of 10 weeks). Once

265   wound healing was achieved, a walking programme was introduced consisting of five

266   minutes walking in hand daily for 1 week with the duration of daily walks increased by

267   five minutes per week. This was continued for 6 weeks. The horse was then turned out

268   in a small paddock until undergoing a second bilateral FP arthroscopy. Twenty-two

269   months after scaffold implantation the horse was sound and in full training: ridden

270   exercise under saddle at walk, trot, canter and jumping. There was evidence of mild

271   joint effusion and small scars were present at the arthrotomy sites.

272

273   **3.2    Diagnostic imaging**

274   Ultrasonographic examination performed eight days postoperatively showed the

275   subchondral bone to have a concave contour, representing the area of the LTR that had

276   been debrided (Figure 2). These defects were filled with homogeneous echogenic

277   material, which was determined, based on previous experience, to be due to the

278   presence of the scaffolds. The scaffold was well fixated and in line with joint surface

279   on the left side, and in line with the joint surface, but was partially dislodged

280   distolaterally on the right side. On ultrasonographic examination, 5 months

281   postoperatively the defects in the LTRs resulting from the curettage were still evident;

282   however, the defects were noticeably shallower. The defects were smooth and filled

283   with mildly echogenic heterogeneous material. Ultrasonography, 22 months

284   postoperatively, showed mild effusion of the left FP joint with some synovial

285   proliferation. The subchondral defects, created by the surgical debridement, into which

286   the scaffolds had been positioned, were no longer evident. These areas were instead,

287   filled with bone as evidenced by a mildly undulating hyperechogenic line, contiguous

288   with adjacent normal subchondral bone. Repair of the overlying cartilaginous layer was

289   also evident. While this cartilage layer was thinner than the surrounding cartilage tissue,

290     the echogenicity was largely similar to that of the adjacent normal hyaline cartilage.

291     Some areas of increased echogenicity were observed within the repair tissue indicating

292     that some tissue remodeling may still be ongoing.

293     Radiographic examination performed at 5 months demonstrated modest reduction in

294     intra-articular soft tissue swelling. The subchondral lucencies within the LTRs were

295     reduced in size but still apparent. Lucent areas were less well defined by a rim of

296     sclerosis. The proximal and distal limits of the defects were smooth and round.

297     Evaluation of the radiographs of both left and right sides showed Grade 2 subchondral

298     filling (i.e. <50% resolution of lucencies) and Grade 2 subchondral contour both (i.e.

299     mild/moderate irregularities). The length (proximodistal) and depth (craniocaudal) of

300     the osteochondral lesions at 5 months in the left were 46 mm and 17 mm, and for the

301     right were 40 mm and 18 mm, respectively. Radiographic examination performed 22

302     months postoperatively showed no evidence of osteoarthritis and no free bodies. The

303     LTRs were smooth, although with a flatter appearance than normal. Repair of the

304     subchondral bone was evident; however, a small area of incomplete healing was seen

305     to persist on the left side. Assessment of the radiographs at 22 months postoperatively

306     (Figure 1) showed Grade 1 subchondral filling (i.e. >50% resolution of subchondral

307     lucencies) and Grade 1 subchondral contour (i.e. flattened). The length (proximodistal)

308     and depth (craniocaudal) of the osteochondral lesions at 22 months in the left were 33

309     mm and 17 mm, and for the right were 35 mm and 17 mm, respectively.

310

311     **3.3     Exploratory arthroscopy**

312     The horse was represented to UCDVH 5 months postoperatively due to mild persistent

313     effusion of the femoropatellar joints. The horse did not exhibit lameness at the walk or

314     trot. Small scars (20 mm x 10 mm) were present at site of the previous arthrotomies.

The FP joints were explored arthroscopically under general anaesthesia as described earlier. Repair tissue within the graft implantation sites was visualized arthroscopically (Figure 3) and scored using the International Cartilage Repair Society (ICRS) cartilage repair assessment tool as shown in Table 1 (Brittberg and Peterson, 1998; Peterson *et al*., 2000). The sites of the scaffold implantation were covered by immature cartilage that was contiguous with the adjacent normal cartilage along the entire perimeter (Figure 3). The cartilage was smooth although the repair tissue maintained a lower profile than the trochlear ridge proximally and distally. An area of proliferative synovitis/granuloma covered the joint capsule at the site of the arthrotomies. This was more marked in the left femoropatellar joint. Macroscopic assessment of repair tissue using the ICRS scoring system resulted in a score of 10/12 for each defect site, placing them in the Grade II (nearly normal) category. Exploration of the entirety of the joint demonstrated an absence of debris or fragments. The arthroscopy portals were closed and sterile adhesive dressings were placed prior to recovery. The filly recovered uneventfully from anesthesia using the Wilderjans rope recovery system (Niimura del Barrio *et al.*, Under review).

## 3.4    Synovial fluid analysis

Synovial fluid analysis 5 months postoperatively showed that synovial fluid from both joints was subjectively less viscous than normal synovial fluid with nucleated cell count from the left and right femoropatellar joints found to be normal ($0.3 \times 10^9$ cells/L) and mildly elevated ($3.6 \times 10^9$ cells/L), respectively. The percentage neutrophils from the left and right femoropatellar joints were 7% and 12%, respectively. The total protein from the left and right FP joints was 8 g/L and 12 g/L, respectively and synovial fluid serum amyloid A (SAA) levels was less than 5 μg/mL for both joints. Percentage neutrophils,

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340    total protein and synovial fluid SAA levels were normal in both joints (Stack *et al.*,

341    Under review).

342

343    **4.    Discussion**

344    This study demonstrates the successful use of a multi-layered collagen-based scaffold

345    in the treatment of large OCD lesions in both femoral trochlear ridges of an equine

346    athlete. The results show the potential of this scaffold to promote repair of bone and

347    cartilage within the defect sites, leading to restoration of the joint surface. Exploratory

348    second-look arthroscopy, 5 months post scaffold placement, revealed that the defects

349    were covered with immature cartilage, contiguous with the surrounding cartilage,

350    although the profile of repair tissue was slightly below the normal joint surface. At 22

351    months post-implantation, the horse was undergoing a full athletic regimen.

352    Radiographic and US examinations 22 months postoperatively confirmed filling of the

353    subchondral bone defects with new bone and restoration of the integrity of the joint

354    surface.

355    *Osteochondritis dissecans*, a manifestation of osteochondrosis (OC), is the most

356    common cause of lameness and the most common indication for surgery in the equine

357    stifle joint (van Weeren, 2012). It occurs due to derangement of the normal

358    endochondral ossification process and results in irregular, thickened cartilage, necrosis

359    of the underlying subchondral bone, and often, fissuring and fragmentation of the

360    affected area of cartilage (Laverty and Girard, 2013; Sparks *et al*., 2011). The origin is

361    still unclear, however it is commonly accepted that there are a number of contributing

362    factors including dietary imbalance, biomechanical factors, genetic susceptibility,

363    declining metabolic rate in the young adult and physiological factors; such as growth,

364    conformation and hormonal imbalance (Desjardin *et al*., 2014; Erickson *et al.*, 2013;

365 Laverty and Girard, 2013; Van Weeren, 2012). Clinical signs develop when the joint

366 surface is breached by the dissecting lesion or when a fragment completely detaches,

367 leading to synovitis, varying degrees of lameness and development of osteoarthritis

368 (Desjardin *et al*., 2014). The horse presented in this study had no history of trauma and

369 was free from OCD in the other predilection sites. The specific aetiology in this horse

370 is undefined, as is often the case.

371 Currently, surgical removal of OCD fragments, curettage of necrotic bone and cartilage

372 alone, or in conjunction with microfracture of the subchondral bone, remains the

373 recommended clinical approach for treatment of OCD defects in horses (Foland *et al*.,

374 1992; Frisbie *et al*., 1999; McIlwraith, 2013; McIlwraith *et al*., 2015). Microfracture

375 alone has shown clinical success; however, often repair tissue is composed of

376 fibrocartilage rather than hyaline cartilage and can break down over time (Miller *et al*.,

377 2004; Steadman *et al*., 2003). In the case presented here, the OCD lesions were large

378 with extensive lytic lesions in the subchondral bone and thus, the prognosis for

379 athleticism using standard approaches was poor (Foland *et al*., 1992; Sparks *et al*.,

380 2011). Debridement, with or without microfracture, would likely have resulted in joint

381 instability and osteoarthritis as a sequel (McIlwraith and Nixon, 1996). The large size

382 of the lesion precluded use of osteochondral grafting procedures, such as mosaicoplasty

383 (Bodó *et al*, 2014), as the osteochondral tissue required to fill the defects could not have

384 been harvested without substantial donor site morbidity. Autologous cartilage

385 implantation (ACI) (Frisbie *et al*, 2008; Nixon *et al,* 2011) was deemed inappropriate

386 due to the extent of diseased subchondral bone requiring debridement, and the

387 requirement for an additional surgery to harvest cartilage for expansion. Thus in this

388 study, a scaffold-assisted microfracture approach was utilised. The technique involved

389 curettage of diseased cartilage and bone, combined with microfracture of the

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390  subchondral bone, followed by implantation of a novel multi-layer osteochondral defect

391  repair scaffold. Similar enhanced microfracture procedures have shown promise in the

392  repair of chondral and osteochondral lesions in humans (Anders *et al*., 2013;

393  Dhollander *et al*., 2012; Gille *et al*., 2010; Kon *et* al., 2014; Kusano *et al*., 2012). Thus,

394  we hypothesised that the use of this approach would lead to enhanced regenerative

395  responses within the defect sites. To the authors' knowledge, this is the first time this

396  scaffold-assisted microfracture approach has been used to treat OCD lesions of the

397  distal femoral trochlear ridges in horses. Additionally, this is the first use of this multi-

398  layered osteochondral defect repair scaffold in the treatment of disease in a veterinary

399  patient.

400  The multi-layered scaffold employed in this case has been designed for osteochondral

401  defect repair, and specifically for use as an off-the-shelf, cell-free biomaterial for press-

402  fit implantation into an osteochondral defect site. The use of microfracture here serves

403  to enable cells, most notably stem cells, from the bone marrow to populate the defect.

404  The scaffold was seen to fill with blood on implantation demonstrating that the

405  hydrophilic nature and porous scaffold architecture enables infiltration of blood and

406  cells from the host bone marrow through the scaffold's seamlessly integrated multi-

407  layered structure (Figure 4). Clot formation within the defect also results in improved

408  retention of the scaffold within the defect site (Frisbie *et al*., 1999). The incorporated

409  extracellular matrix macromolecules, and scaffold biostructural and biomechanical

410  properties have been previously shown to direct the differentiation of mesenchymal

411  stem cells (MSC) to produce bone, calcified cartilage and cartilage within the requisite

412  regions of the defect site, with restoration of the anatomical tidemark and to result in

413  joint regeneration (Levingstone *et al.*, Under review a; Levingstone *et al.*, Under review

414  b). The scaffold demonstrated numerous advantages, including sufficient mechanical

17

415    strength, durability and flexibility to withstand surgical handling. It was easily cut to

416    shape and sculpted to conform well to the debridement bed, so much so that in the right

417    limb a press-fit implantation could be achieved.

418    Successful clinical results were demonstrated using both press-fit and gluing fixation

419    methods. Fixation poses a significant challenge for biomaterials in articular cartilage

420    repair. Suturing has proved successful but is limited by technical challenges and the

421    long surgery times required. Other potential fixation approaches include polydioxanone

422    (PDS) staples (Frisbie *et al*., 2009) and PDS pins (Nixon *et al*., 2004; Sparks *et al*.,

423    2011). The scaffold employed here, once hydrated, becomes compliant with reduced

424    mechanical strength, although still sufficient for surgical handling, this poses

425    challenges for retention of staples, pins or sutures. Press-fit implantation offers

426    advantages, such as reduced surgical time and avoidance of the need to include

427    additional materials, and has been used successfully in small focal lesions (Levingstone

428    et al., Under Review a; Levingstone et al., Under Review b); however larger lesions

429    present greater challenges. In this case, ultrasound confirmed persistence of the scaffold

430    within both defect sites eight days postoperatively. Incomplete fixation was observed

431    on the right side with some detachment evident. However, despite this, the bulk of the

432    scaffold remained *in situ*. Tissue glues, such as fibrin glue and cyanoacrylate glue, have

433    demonstrated some success in scaffold fixation (Ayan *et al* 2007; Patel *et al*., 2010).

434    The gluing approach employed here, using a combination of fibrin and cyanoacrylate

435    glue proved more successful, with scaffold fixation observed ultrasonographically eight

436    days postoperatively. This approach is thus recommended for fixation of this multi-

437    layered scaffold in large defects in the future. Recovery from anaesthesia was an

438    important consideration to ensure protection of the scaffold in the initial postoperative

439    phase. Use of the Anderson sling recovery system resulted in optimal recovery with

440 minimal flexion of the stifle joints. Furthermore, the use of the sling resulted in less

441 tension and trauma to the laterally placed arthrotomy suture lines.

442 On US examination at 5 months postoperatively, the defects were filled with slightly

443 echogenic material. During exploratory arthroscopic surgery performed the following

444 the day this was confirmed to be immature cartilage, with ICRS macroscopic scores

445 placing it in the near normal category. Synovial fluid analysed 5 months postoperatively

446 was normal in the left femoropatellar joint and showed signs of mild inflammation in

447 the right. The reason for mild synovitis in the right femoropatellar joint is unclear but

448 may be related to the arthrotomy site dehiscence and chronic synovial proliferation.

449 The mild increase in nucleated cell count may also reflect the normal inflammatory

450 process involved in healing of osteochondral defects. The lack of marked inflammatory

451 response at any time during the study demonstrates the biocompatibility and safety of

452 the scaffold. Modest radiographic healing was observed with the debridement bed

453 slowly filling with new bone over time. Due to the size of the lesions in this case, it was

454 not expected that the trochlear ridges would appear radiographically normal at any time

455 point (McIlwraith and Nixon, 1996), thus the aim here was to improve the trochlear

456 function and offer the horse an improved athletic prognosis. Healing was evidenced by

457 the loss of the sclerotic rim demarcating the abnormal from normal subchondral bone;

458 the new bone formation and the uniformity of the subchondral bone. In addition, there

459 was no evidence of osteoarthritis.

460 Ultrasound performed 22 months postoperatively revealed replacement of the deeper

461 layers of the scaffolds with bone, and cartilage superficially. Whilst the original profile

462 of the trochlear ridges had not been fully achieved, the articular surface of the LTRs

463 was resurfaced and functional. These results demonstrate the benefits of the

464 osteoinductive properties of the base layer of the scaffold provided by the

465 hydroxyapatite component and the chondrogenic properties of the top layer provided

466 by the type II collagen and hyaluronic acid. Notably, the cartilage and bone healing

467 observed here was achieved in the absence of additional chondrogenic or osteogenic

468 growth factors or cells. A more complete evaluation of the quality of the cartilage repair

469 and expected long term clinical function could have been made if the tissues were

470 biopsied during the second surgery (Krishnan *et al.*, 2008). However, this was not

471 possible due to ethical considerations. While the cartilage covering the healed

472 subchondral bone was functional at 22 months post-surgery, the long term durability of

473 the repaired cartilage remains unknown. At 22 months postoperatively, the horse was

474 performing athletically at its intended level, with no evidence of lameness. The reason

475 for the persistence of some effusion and synovial hypertrophy in the left femoropatellar

476 joint is not clear, but is likely to be related to the synovial proliferation and granuloma

477 formation at the arthrotomy site. The use of an arthrotomy was necessary in order to

478 provide sufficient accessibility to place the scaffolds, however, this resulted in some

479 side issues as highlighted by the dehiscence of both arthrotomy sites; a recognised

480 complication of arthrotomy in the horse (Trotter *et al*., 1983; Pascoe *et al*., 1980).

481 Dehiscence was partial in this case with the multiple layer closure used enabling the

482 joint capsule to remain intact and preventing development of synovial communication

483 or herniation; however, permanent scarring of the area resulted. While arthroscopic

484 implantation of this scaffold is not currently possible, it is likely that use of arthroscopic

485 methods would have reduced the likelihood of these complications occurring.

486 Arthroscopy has been shown to minimise trauma to surrounding tissues, decrease

487 muscular deficits, decrease chances of wound infection and improve cosmetic

488 outcomes (Jameson *et al*., 2011; McIlwraith *et al*., 2015; Small, 1993).

489 While equine models are recommended for preclinical assessment of new tissue

490 engineering strategies for cartilage repair, few studies have been carried out in horses

491 using tissue engineering strategies for the repair of cartilage defects resulting from

492 injury or disease. This study provided the opportunity to treat a veterinary patient, while

493 also providing important information for clinical translation. The promising results in

494 this study demonstrate that the use of this novel multi-layered osteochondral defect

495 repair scaffold in this scaffold-assisted microfracture procedure is an effective approach

496 to promote enhanced filling of osteochondral defects, following curettage, in the

497 treatment of OCD.

498

499 **5 Conclusions**

500 This case study investigated the use of a scaffold-assisted microfracture approach,

501 employing a novel multi-layered collagen-based scaffold, designed for osteochondral

502 defect repair in humans, in the treatment of large *osteochondritis dissecans* (OCD) of

503 both lateral femoral trochlear ridges in a horse. Clinical follow-up at 5 and 22 months

504 revealed almost complete filling of the subchondral bone defect, and restoration of a

505 smooth articular surface to the trochlear ridges. These promising results demonstrate

506 the effective clinical use of this novel multi-layered osteochondral defect repair scaffold

507 in the promotion of enhanced filling of osteochondral defects following debridement in

508 OCD. The successful functional repair of the complete osteochondral unit achieved

509 here in an equine athlete with large bilateral lesions shows potential for clinical

510 translation to human patients presenting with large osteochondral defects.

511

512 **Acknowledgements**

21

517
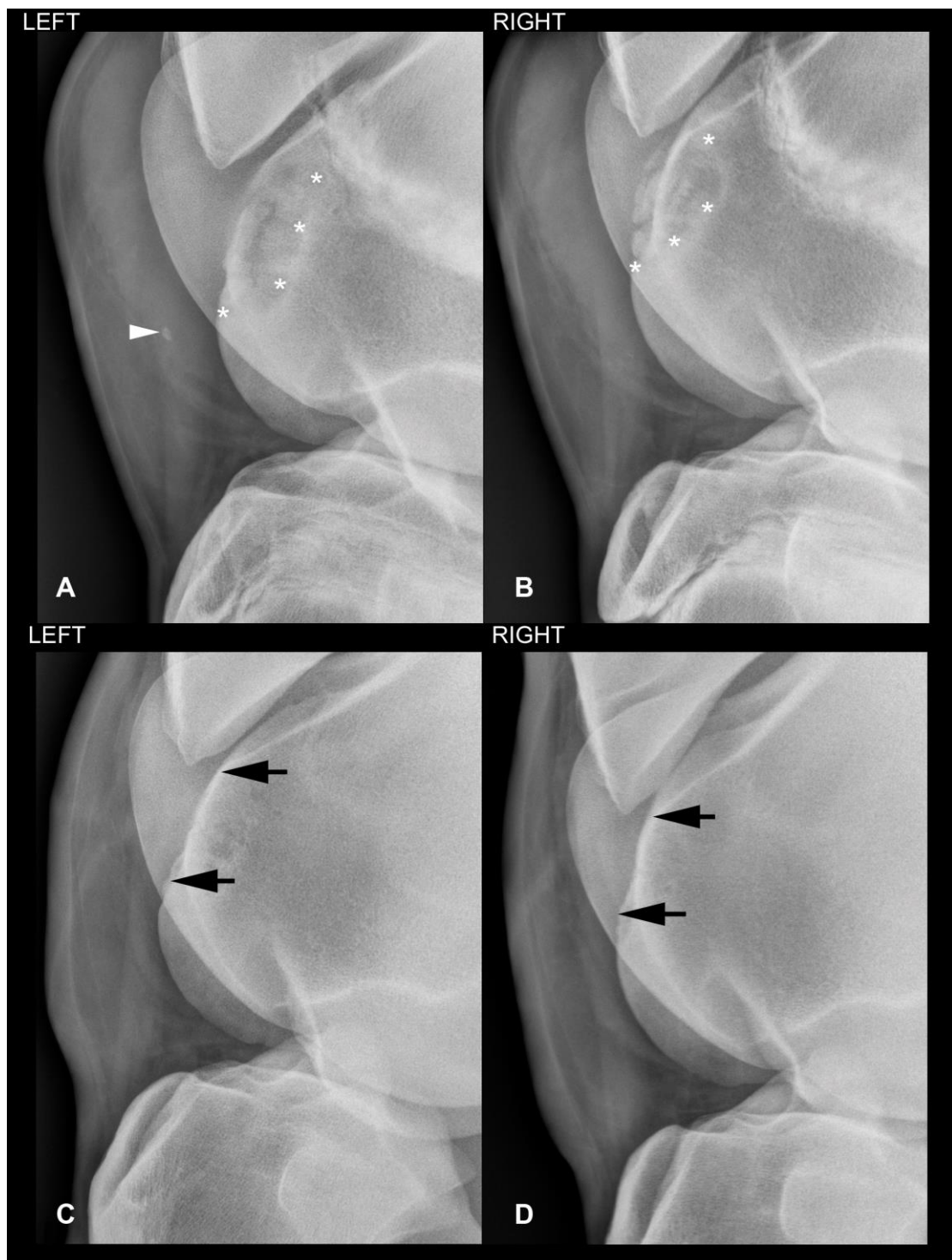
**Figures**

520  Figure 1. Lateromedial radiographs of left and right femoropatellar joints before (A, B)

521  and 22 months after arthroscopic debridement and scaffold placement (C, D). The

522  contour of the lateral trochlear ridges of the distal femur of both the left and right hind

523  limbs were markedly irregular, with non-detached osteochondral fragments overlying

524  large areas of cystic resorption deep in the subchondral bone (Lesion borders

525  highlighted with white *). There was a small, free mineralized fragment in the cranial

526  aspect of the right femoropatellar joint (white arrowhead). At 22 months

527  postoperatively there is Grade 1 subchondral filling of the subchondral lucencies (i.e.

528  >50% resolution) and the subchondral contour is smooth although slightly flattened

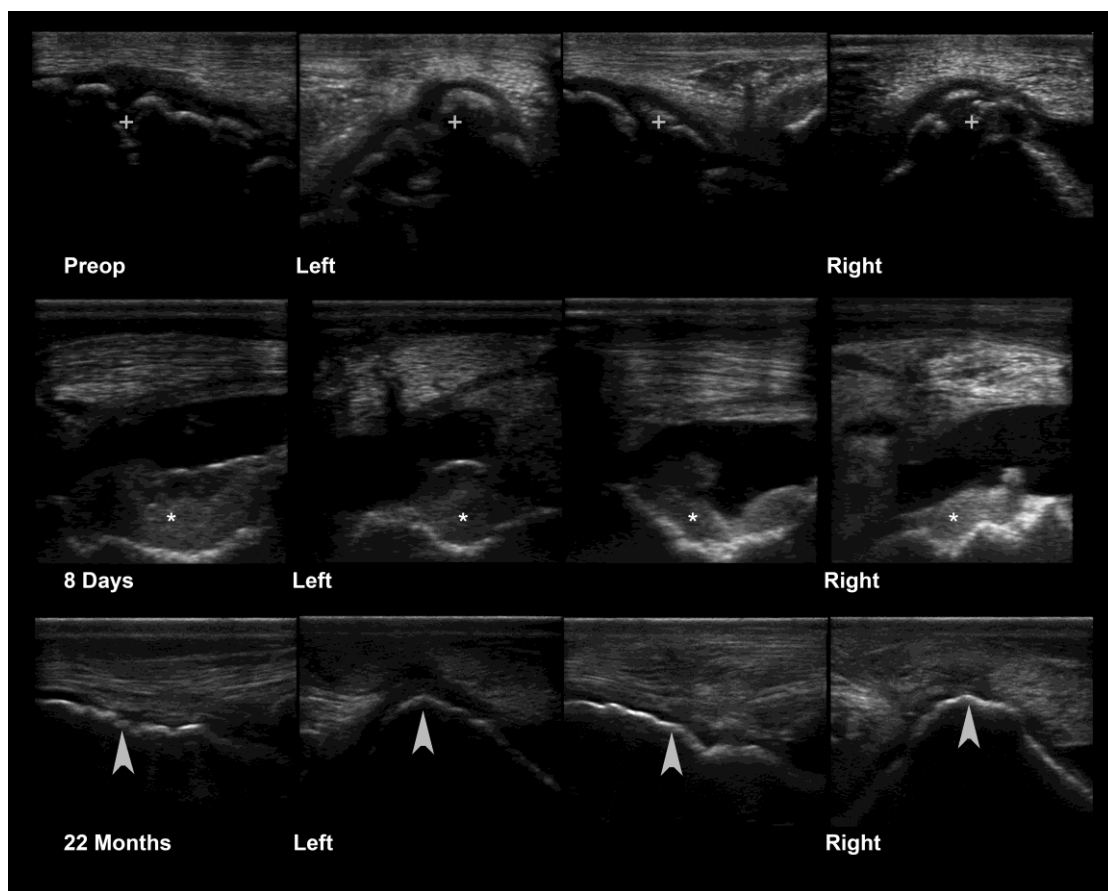529  (Grade 1) (black arrows).



530

531  Figure 2. Ultrasonographic images of the left and right lateral trochlear ridges in both

532  longitudinal (column 1 and 3) and transverse planes (column 2 and 4). Preoperatively

533  (top row), the surface of the osteochondral unit was undulating, irregular in shape and

534  thickness (white +), and was deeply fissured. At 8 days post implantation (middle row),

535  the scaffold (white *) is *in situ* in the left limb and largely *in situ* in the right limb with

24

536    a small area of detachment distolaterally. At 22 months (the bottom row) the

537    subchondral defects, created by the surgical debridement, into which the scaffolds had

538    been positioned, were no longer evident. These areas were instead, filled with bone as

539    evidenced by a mildly undulating hyperechogenic line (white arrows), contiguous with

540    adjacent normal subchondral bone. Proximal is to the right in longitudinal views.

541    Lateral is to the right in transverse views.
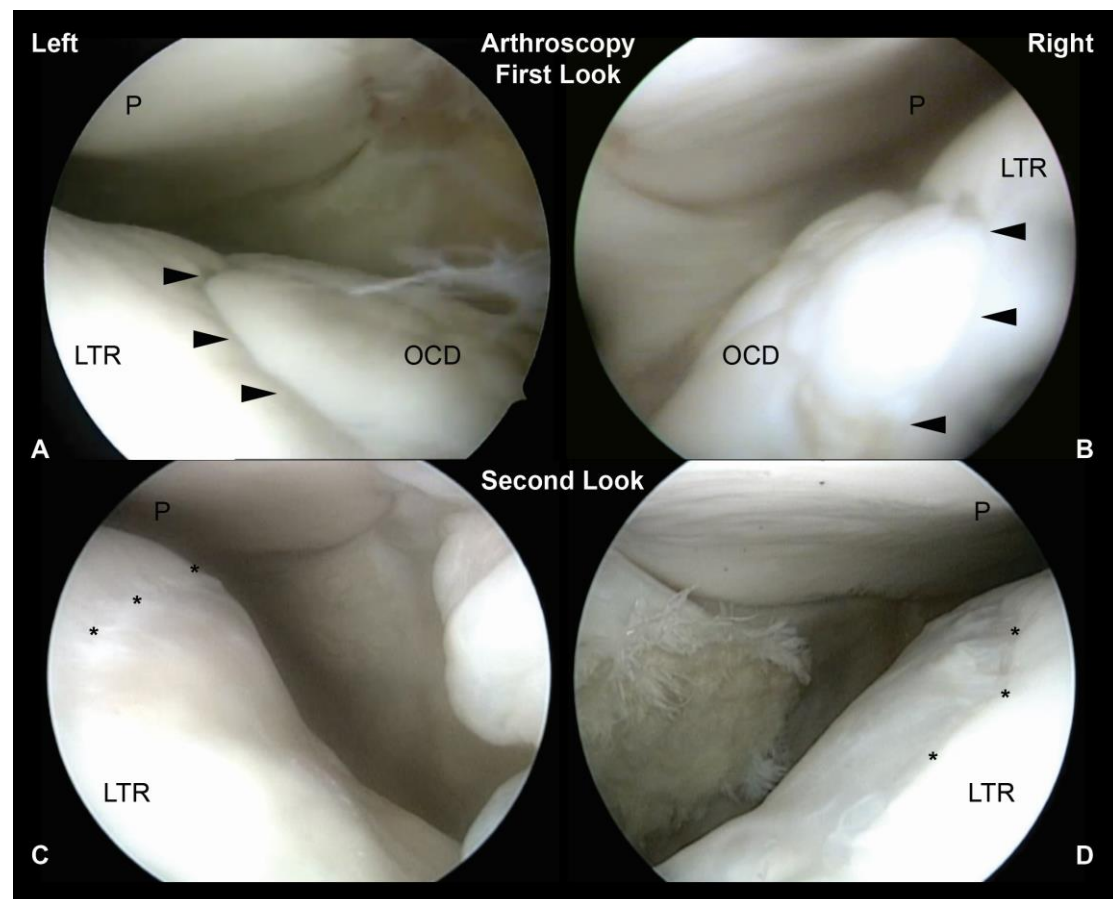
542



543

544    Figure 3. Arthroscopic images of the left (A, C) and right (B, D) femoropatellar joints

545    showing the lateral femoral trochlea (LTR) before debridement (top row) and during

546    second-look arthroscopy five months later (bottom row). In A and B the cartilage

547    covering the middle and proximal parts of the LTR was undulating, irregular and deeply

fissured (black arrowheads) with evidence of non-detached osteochondral fragments (OCD). In C and D repair tissue within the graft implantation sites scored 10/12 (nearly normal) according to the International Cartilage Repair Society (ICRS) cartilage repair assessment tool. The patella (P) is visible above the LTR. Black * mark the junction between normal and repair cartilage.
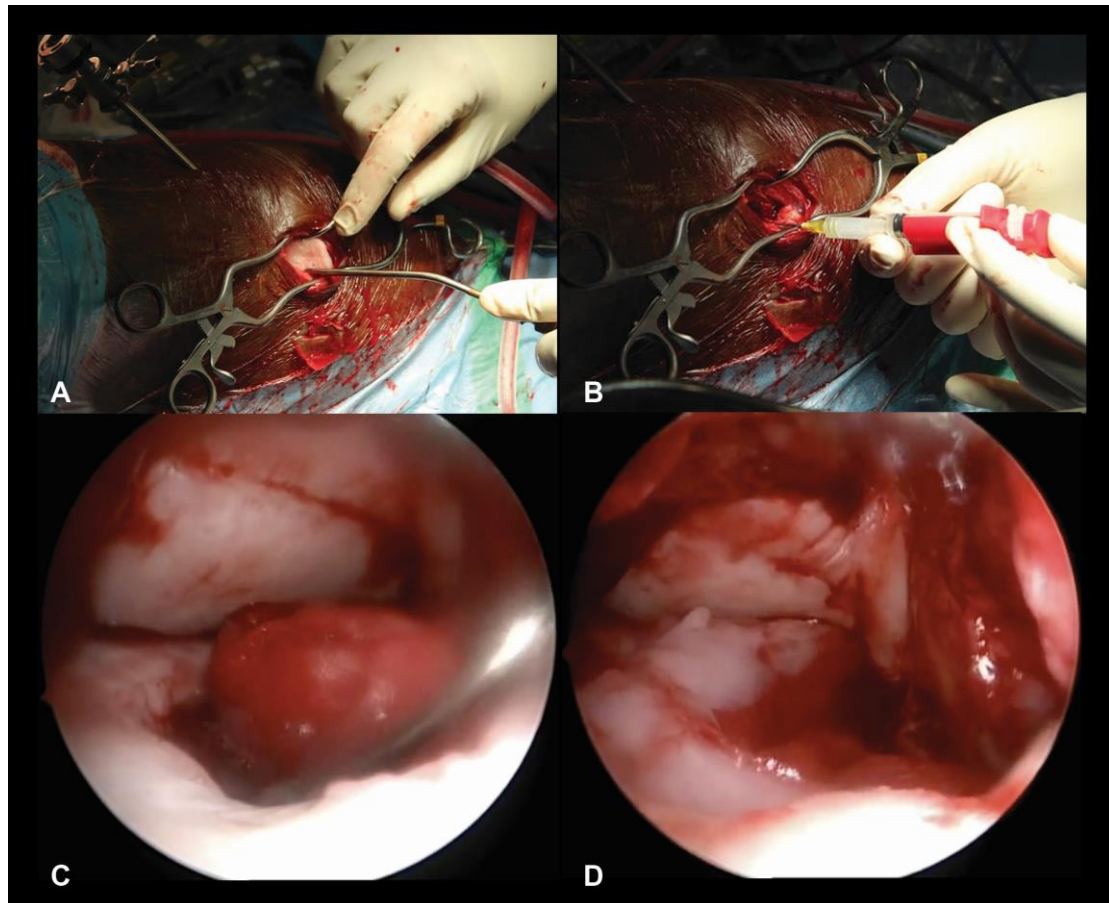


Figure 4. The scaffolds were soaked in sterile saline and pressed gently into the defect via arthrotomy (A). The left scaffold was secured with a combination of fibrin glue (Tisseel, Baxter, Dublin, Ireland) and cyanoacrylate glue (Histoacryl, B Braun Medical, Dublin, Ireland) (B, C, D). Note the scaffolds becoming blood soaked on implantation (C and D).

Table 1: International Cartilage Repair Society (ICRS) cartilage repair assessment tool
(Brittberg and Peterson, 1998; Peterson *et al*., 2000). This tool is used by surgeons to
evaluate the macroscopic appearance of cartilage repair tissue following interventions
such as ACI, subchondral drilling and microfracture and rates cartilage repair tissue as
Grade IV (severely abnormal), Grade III (abnormal), Grade II (nearly normal) or Grade
I (normal) based, on the degree of defect repair, degree of integration and macroscopic
appearance.

| | Criteria | Points |
|---|---|---|
| **Degree of Defect Repair** | Level with surrounding cartilage | 4 |
| | 75% repair of defect depth | 3 |
| | 50% repair of defect depth | 2 |
| | 25% repair of defect depth | 1 |
| | 0% repair of defect depth | 0 |
| **Integration to Border Zone** | Complete integration with surrounding cartilage | 4 |
| | Demarcating border < 1mm | 3 |
| | 3/4 of graft integrated, 1/4 with a notable border >1mm width | 2 |
| | 1/2 of graft integrated with surrounding cartilage,1/2 with a notable border > 1mm | 1 |
| | From no contact to 1/4 of graft integrated with surrounding cartilage | 0 |
| **Macroscopic Appearance** | Intact smooth surface | 4 |
| | Fibrillated surface | 3 |
| | Small, scattered fissures or cracks | 2 |
| | Several, small or few but large fissures | 1 |
| | Total degeneration of grafted area | 0 |

| Overall Score | | | |
|---|---|---|---|
| | Grade I | normal | 12 |
| | Grade II | nearly normal | 11-8 |
| | Grade III | abnormal | 7-4 |
| | Grade IV | severely abnormal | 3-1 |

566

567

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