1   **Original Article**

2

3   **Engraftment of autologous bone marrow cells into the injured cranial cruciate ligament**
4   **in dogs**

5

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13

14 **Abstract**

15   Current research indicates that exogenous stem cells may accelerate reparative

16 processes in joint disease. However, no previous studies have evaluated whether bone marrow

17 cells (BMCs) target the injured cranial cruciate ligament (CCL) in dogs. The objective of this

18 study was to investigate engraftment of BMCs following intra-articular injection in dogs with

19 spontaneous CCL injury. Autologous PKH26-labeled BMCs were injected into the stifle joint

20 of eight client-owned dogs with CCL rupture. The effects of PKH26 staining on cell viability

21 and PKH26 fluorescence intensity were analyzed in vitro using a MTT assay and flow

22 cytometry. Labelled BMCs in injured CCL tissue were identified using fluorescence

23 microscopy of biopsies harvested 3 and 13 days after intra-articular BMC injection.

24

25   The intensity of PKH26 fluorescence declines with cell division but was still

26 detectable after 16 days. Labelling with PKH26 had no detectable effect on cell viability or

27 proliferation. Only rare PKH26-positive cells were present in biopsies of the injured CCL in

28 3/7 dogs and in synovial fluid in 1/7 dogs. No differences in transforming growth factor-β1,

29 and interleukin-6 before and after BMC treatment were found and no clinical complications

30 were noted during a 1 year follow-up period. In conclusion, BMCs were shown to engraft to

31 the injured CCL in dogs when injected into the articular cavity. Intra-articular application of

32 PKH26-labeled cultured mesenchymal stem cells is likely to result in higher numbers of

33 engrafted cells that can be tracked using this method in a clinical setting.

34

35 *Keywords:* Bone marrow cells; Cranial cruciate ligament; Dog; Mesenchymal stem cells,

36 PKH26; Transplantation.

37

Introduction

The cranial cruciate ligament (CCL) is essential for stifle joint stability and its rupture leads to functional impairment, meniscal lesions and early onset of osteoarthritis (Arnoczky and Marshall, 1977; Korvick et al., 1994). In dogs, CCL injury is common and has been treated using a variety of different surgical techniques since 1952 (Paatsama, 1952), but no single treatment option has been shown to be clearly superior. The goals of most reported techniques are to alleviate pain, decrease instability and minimize osteoarthritis (OA) (Moore and Read, 1995), but residual lameness is frequent and OA is a common sequel (Elkins et al., 1991; Innes et al., 2004; Rayward et al., 2004). The lack of ideal outcome following surgery has prompted interest in exploring new adjunctive treatment options, such as regenerative stem cell therapy. Knowledge of the benefits of these treatment strategies would be useful both for the treatment of spontaneous canine CCL injury and for investigations into degenerative anterior cruciate ligament (ACL) disease in humans using the dog as a model.

Mesenchymal stem cell (MSC) therapy is a newly developing therapeutic approach in OA that has proven useful in cartilage repair in a variety of animal models (Black et al., 2007, 2008; Chong et al., 2007; Guercio et al., 2012; Jorgensen and Noel, 2012; Khan et al., 2010; Kirkby and Lewis, 2012; Koga et al., 2008; Mokbel et al., 2011a, b; Murphy et al., 2003). Bone marrow-derived MSCs hold particular promise for tissue repair because of their ability to engraft into tissues and differentiate into the target tissue cell type, including fibroblasts, osteocytes, adipocytes, chondrocytes and myocytes (Chamberlain et al., 2007; Pittenger et al., 1999). In recent studies, MSCs were found to accelerate healing of transected ligaments in animal models (Agung et al., 2006; Kanaya et al., 2007; Kim et al., 2011) and evidence suggests that fresh whole bone marrow cells (BMCs) may have superior effects compared to purified MSCs, presumably because of an additional benefit of hematopoietic stem cells (Oe

63   et al., 2011). Indeed, injured rat ACLs treated with BMCs had more mature fibroblasts and

64   tighter collagen bundles compared to those treated with MSCs, leading the authors to

65   conclude that bone marrow (BM) transplantation is an effective treatment for ACL injury (Oe

66   et al., 2011). To date, no studies have investigated the potential benefit of stem cell adjunctive

67   treatment in dogs with experimental or spontaneous CCL injury.

68

69   The purpose of the present study was to assess the engraftment potential of autologous

70   BMCs injected into the articular cavity in dogs with partial or complete CCL rupture and to

71   determine whether PKH26 red fluorescent labelling is a safe and effective way to track canine

72   BMCs.

73

74   **Material and methods**

75   *Animals*

76   Client-owned dogs presented for surgical treatment of spontaneous partial or complete

77   CCL rupture to the Division of Small Animal Surgery and Orthopaedics of the University of

78   Berne were considered for inclusion in the study (see Appendix: Supplementary data for

79   details). Informed client consent was obtained for each dog. Study inclusion criteria were

80   diagnosis of partial or complete CCL rupture confirmed by arthroscopy and unremarkable

81   results of routine haematological and serum biochemical analyses. Dogs were excluded if

82   there was a recent history of illness other than pelvic limb lameness or if they had undergone

83   previous intra-articular application of any substance or previous surgery on the affected limb.

84   Dogs were screened using an orthopaedic examination with various parameters: lameness,

85   functional disability, range of motion and pain on manipulation. For each parameter a score

86   was recorded at first time of presentation and 3 month after surgery. The scores assigned were

87   based on a 4-point scale, 0 (no/best) to 4 (worst) (For details see appendix: Supplementary

88   material).

89

90   All animal experiments were reviewed and approved by the Commission of Animal

91   Experimentation of the Canton of Berne, Switzerland (BE42/12; date of approval, 30/4/2012).

92

93   *Isolation of bone marrow cells*

94   Bone marrow was harvested from the proximal humerus in each dog using a 13-G BM

95   biopsy needle connected to a 10-mL syringe containing 2 mL of heparin solution (3,000

96   U/mL). A total of 15 mL BM was aspirated and immediately injected into a transfer bag

97   containing 7 mL citrate phosphate dextrose adenine solution. The BM aspirate was passed

98   through a blood transfer filter set into a 20-mL syringe, and cells were separated by density

99   gradient centrifugation at 445 *g* for 35 min. The interface with the nucleated cell fraction was

100   transferred and washed twice in phosphate buffered saline (PBS) before counting and

101   partitioning for PKH26 labelling, intra-articular injection, and cultivation (See Appendix:

102   Supplementary material for details).

103

104   *Flow cytometric characterization of cells*

105   Freshly isolated cells and cultured cells were evaluated by flow cytometry for the

106   specific MSC markers, CD90 (YKIX337.217, eBioscience) and CD44 (FAB5449A, R&D),

107   and for the hematopoietic stem cell marker, CD45 (YKIX716.13, eBioscience). Data were

108   analyzed using a flow cytometer (LSR II, BD Bioscience) and commercial software

109   (FACSDiva, BD Bioscience).

110

111   *PKH26 labelling*

112   Labelling of cell membranes was performed using the PKH26 Red Fluorescence Kit

113   (Sigma-Aldrich) according to the manufacturer's instructions. After staining, a portion of

114   PKH26-BMCs was resuspended in PBS at a concentration of 1×10^7 cells/mL for intra-

115   articular injection. In addition, stained cells were suspended in complete medium for

116   evaluation of dye cytotoxicity, growth characteristics and fluorescence intensity.

117

118   *Evaluation of PKH26 cytotoxicity*

119   The effect of PKH26 labelling on cell viability was tested using a colorimetric MTT

120   assay. For this, MSCs from the second passage were stained with PKH26 dye at $2 \times 10^-$

121   ^{6}M/10^6 cells and 4×10^{-6}M/10^6 cells. The assay was conducted in replicate with MSCs from

122   four dogs as described elsewhere (Waldherr et al., 2012). Cell viability in each well, measured

123   as the optical density (OD), was calculated as follows: $100 \times$ (OD of sample well – OD of

124   blank well)/(OD of control well – OD of blank well). Mean values of repeated measurements

125   were used for analysis.

126

127   *Population doubling time*

128   Growth characteristics of MSCs at the first and second passages unstained and stained

129   with PKH26 (2×10^{-6} M and 4×10^{-6} M) from six dogs were investigated. The MSCs were

130   seeded into a 24-well plate at a density of 2.1×10^3 cells/cm^2. After a recovery time of 48 h,

131   three wells were detached daily for a period of 8 days and cell numbers were counted in a

132   hemocytometer. The population doubling time was computed using an online calculator[1].

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[1] See: http://www.doubling-time.com/compute.php (accessed 15 August 2014)

133

134

135   PKH26 *fluorescence intensity*

136   Fluorescence intensity was assessed in freshly isolated BMCs and MSCs during cell

137   proliferation over 16 days as described in detail in the Appendix: Supplementary material.

138

139   *In vivo experimental protocol*

140   The experimental schedule is summarized in Fig. 1. On day 0, dogs presenting with

141   signs of CCL injury were clinically examined. BM was harvested and pre-operative

142   radiographs were performed under general anaesthesia. BMCs were isolated and labelled with

143   PKH26 (final concentration: 2×10^{-6} M PKH26 and 1×10^7 cells/mL) within 3 h of

144   harvesting. Synovial fluid was first aspirated and a total of 1×10^7 PKH26-labeled BMCs

145   diluted in 1 mL PBS was injected immediately afterwards through the same needle under

146   aseptic conditions. An aliquot of remaining BMCs was used for microbiological quality

147   control. The dogs were then presented again for stifle arthroscopy and tibial plateau levelling

148   osteotomy (TPLO) either 13 days (Group 1) or 3 days (Group 2) following intra-articular

149   BMC injection. Prior to arthroscopy, synovial fluid was again collected.

150

151   *Tissue collection*

152   During arthroscopy immediately prior to surgical treatment by TPLO, the gross

153   appearance of the stifle joint was evaluated and biopsies of the damaged CCL and synovial

154   membrane were excised. Synovial membrane was harvested craniomedially and

155   craniolaterally to the optic port that was located lateral to the patellar ligament halfway

156   between patella and tibial tuberosity. Tissues were snap frozen on dry ice in O.C.T.

157   compound (Tissue-Tek). Each block was cut into 5 µm sections at 10 µm intervals and placed

158   on specimen slides. Sections were stored at -80 °C pending fluorescence microscopy.

159

160   *Fluorescence microscopy of harvested samples*

161   Slides were examined for PKH26 fluorescence using a confocal laser scanning

162   fluorescence microscope (FluoView FV1000, Olympus) after counterstaining with TOTO-3

163   iodide (Life Technologies). Sections were defined as positive if a clear cell structure with

164   spindle-shaped fibroblast-like morphology was detected showing at least partial red

165   fluorescence in the membrane and far red fluorescence of the nucleus. Synovial fluid samples

166   were examined for PKH26 fluorescence after centrifugation in a 96-well plate.

167

168   *Synovial fluid analyses*

169   Because of a possible effect of BMCs on cytokine production and immune cell

170   attraction, synovial fluid obtained before and after intra-articular BM injection was examined

171   cytologically, and transforming growth factor (TGF)-β1 and interleukin (IL)-6 were

172   quantified using a commercial ELISA (canine TGF-ß1, IL-6 Quantikine ELISA Kit, R&D)

173   according to the manufacturer´s protocol.

174

175   *Follow-up examinations*

176   Dogs were discharged from hospital 1 day after surgery with a soft-padded bandage on

177   the operated leg for 3 days and administered carprofen (Rimadyl, Pfizer, 4 mg/kg PO once

178   daily for 7 days). Owners were instructed to restrict activity initially to leash walks, followed

179   by a gradual increase in activity. Dogs were re-examined 14 days and 3 months after surgery.

180   The same clinician carried out both initial and follow-up orthopaedic and clinical

181   examinations. In addition, a final follow-up inquiry with the owners was performed by

182   telephone 12 months after surgery. Complications, including infection, pain or worsening of

183   articular function were recorded during follow-up examinations.

184

185   *Statistical analyses*

186   For each donor and experimental condition at least triplicate samples were used for

187   each assessment unless otherwise stated. Statistical analysis was performed with NCSS 2007

188   software. Differences in population doubling time, TGF-ß1 and IL-6 were evaluated using a

189   paired samples *t*-test after testing for normality. A *P*–value of <0.05 was considered as

190   significant.

191

192   **Results**

193   *Animals*

194   Eight cases were initially included, but one was subsequently excluded because it was

195   not presented for arthroscopy as scheduled. Four dogs underwent arthroscopy 13 days after

196   injection. Because of a low rate of detected PKH26 positive cells the interval between BMC

197   transplantation and tissue harvesting was shortened, therefore the second group of three dogs

198   went to surgery 3 days after injection (Fig. 1).

199

200   *Isolation, cultivation and flow cytometric characterization of cells*

201   The mean value of nucleated cell fraction recovered after BM aspiration (eight dogs)

202   and density gradient centrifugation was 23.0×10^7 cells (range, $0.35\text{-}33.2 \times 10^7$ cells). Freshly

203   isolated BMCs stained largely positive for CD45 with only a small proportion (<1%) of cells

204 negative for CD45 and double positive for CD44 and CD90. Cultured cells were adherent

205 within 2 to 3 days showing spindle-shaped fibroblast-like morphology generating

206 subsequently colony-forming units. After 8 to 15 days in culture, colonies became confluent

207 and were passaged for the first time. Primary cultured cells (second and fifth passages)

208 stained on average 92% ± 5% positive for CD44, 45% ± 3% double positive for CD44 and

209 CD90, and 100% negative for CD45 on flow cytometry, confirming phenotype consistent

210 with MSCs in most cells. Lack of expression of CD45 on cultured cells indicated that cells of

211 haematopoietic origin had been excluded during cell culture.

212

213 *Evaluation of PKH26 cytotoxicity*

214 The colorimetric MTT assay performed on MSCs from the 2nd passage in four dogs

215 revealed that the mean relative number of viable MSCs 24 h after PKH26 staining compared

216 to unstained MSCs was 93.4% ± 3.5% at 2 x 10^{-6} M and 98.6% ± 9.2% at 5 x 10^{-6} M PKH26.

217

218 *Population doubling time*

219 Mean population doubling time of unlabelled MSCs and PKH26-labeled MSCs from 6

220 dogs was 146.7 ± 63.5 h (range, 44.5-238.0 h) and 107.2 ± 37.5 h (range, 43.0-173.0 h),

221 respectively. No significant difference was found between these population doubling times by

222 using a paired samples *t*-test ($P = 0.43$).

223

224 *PKH26 fluorescence intensity*

225 Labelled BMCs plated in culture dishes attached efficiently and showed uniformly

226 distributed red fluorescence on microscopy. The labelling rate of BMCs assessed by flow

227 cytometry was 97.3 ± 3.3% and labelling intensity decreased to 67.5 ± 8.3% at the end of the

228   16-day observation period. The labelling rates of second passage MSCs assessed by flow

229   cytometry on days 0 and 16 were $94.0 \pm 2.1\%$ and $15.1 \pm 4.6\%$, respectively, with a mean

230   number of cell divisions after 16 days of 8 ± 3.

231

232   *Fluorescent microscopy of harvested samples*

233   Fluorescence microscopy of control CCL tissue co-cultivated with PKH26-labelled

234   BMCs revealed adhesion and migration of BMCs based on numerous red fluorescent cells

235   located superficially and within the tissue (Fig. 2; positive control). Tissue of CCL and

236   synovial membrane were obtained from seven dogs after BMCs transplantation. A total of

237   280 sections (40 sections per dog) of CCL and synovial membrane were examined for PKH26

238   fluorescence. Positive cells were only detected in eight slides from three dogs of which one

239   were sampled 3 days after BMC injection and two were sampled 13 days after BMC injection

240   (Fig. 3). Positive cells were located within the organized CCL tissue and arranged

241   predominantly as single cells and occasionally in groups. However, the numbers of positive

242   cells was extremely small with no more than 10 per section. The intensity of PKH26-positive

243   cells was clearly less in these samples than in ex vivo CCL samples co-cultured with PKH26-

244   labeled BMCs. A single synovial sample (harvested on day 3) showed PKH26 positive cells.

245

246   *Synovial fluid analyses*

247   Cytological examination of synovial fluid taken both prior to BMC injection and prior

248   to arthroscopy exhibited less than 5% neutrophils. No significant difference was found in

249   TGF-ß1 ($P = 0.21$) and IL-6 ($P = 0.29$) concentrations between samples harvested prior to and

250   those harvested after BMCs treatment using a paired samples *t*-test (Fig. 4).

251

252   *Clinical assessment and follow-up of study dogs*

253   Pre-operative arthroscopy of BMC-treated dogs revealed no gross changes in the stifle

254   joint other than those generally observed in dogs assessed for partial or complete CCL

255   rupture. Bacteriological cultures of the remaining portion of injected BMC preparations

256   revealed no growth in all samples.

257

258   Follow-up examination performed 3 months after surgery revealed mild lameness and

259   swelling of the knee joint in one dog. The other dogs showed improvement of lameness,

260   function, and pain on manipulation (see Appendix: Supplementary material). Four dogs had

261   improved scores when testing the range of motion but withdrew the affected leg at full range

262   manipulation. A final inquiry was performed in all dogs 12 months after surgery. Normal

263   activity was reported by all owners during these inquires. The owners of three dogs indicated

264   slight stiffness in the morning. No complications associated with the intra-articular injection

265   of the PKH26-labeled BMCs were observed in any of the dogs.

266

267   **Discussion**

268   Several previous studies have investigated the effects of intra-articular stem cell

269   application on cartilage in dogs with OA (Black et al., 2007, 2008; Guercio et al., 2012;

270   Mokbel et al., 2011a). However, most previous reports have focused on clinical outcome.

271   This study evaluated engraftment of fluorescent-labelled BMCs into injured ligaments when

272   applied into the articular cavity in dogs with spontaneous CCL injury. The decision to use

273   BMCs instead of MSCs was based on ease of processing, making the procedure practical for

274   future use in a clinical setting, as well as the potential additional benefit of BMCs based on

275   studies in a rat model (Oe et al., 2011).

276

277   Several pre-conditions of harvested cells were tested in order to ensure that the

278   selected study design was applicable regarding dye and cells. Separated BMCs of the eight

279   dogs comprise haematopoetic stem cells showing CD45+ which were the main fraction with

280   around 99% and a very small part of mesenchymal stem cells which showed a phenotype of

281   CD45- CD44+ and CD90+. These findings are in accordance with the findings of Alvarez-

282   Viejo et al. (2013).

283

284   Following cultivation, cells were replaced by a homologous layer of adherent cells

285   expressing putative surface specific antigens, such as CD44+ and CD90+ as markers for

286   MSCs and lacking the haematopoetic stem cell marker CD45. They presented MSC

287   characteristics as reported in other studies (Csaki et al., 2007; Kisiel et al., 2012). Lack of

288   expression of CD45+ on cultured MSCs indicated that cells of haematopoietic origin had been

289   excluded during the cell culture process. Cell numbers recovered following gradient

290   centrifugation varied between samples, but were largely similar to those previously reported

291   in dogs (Nishida et al., 2012; Sato et al., 2011). Likewise, in vitro growth potential varied

292   between dogs, demonstrated by the wide range in population doubling time. These results are

293   consistent with previously published data for humans and may reflect patient variability

294   (Bertolo et al., 2013), age-related replicative senescence (Mareschi et al., 2006; Zhou et al.,

295   2008), as well as variation in individual MSC differentiation potency (Ding et al., 2013).

296

297   The ability to track cells is undoubtedly necessary to evaluate the potential of cell

298   migration and new tissue transformation after in vivo transplantation. Differentiation between

299   graft and host cells after transplantation requires a method that labels cells of interest and

300   identifies them after harvesting at a later time. Previous studies used BMCs or MSCs

301   expressing green fluorescent protein (GFP) injected into the injured stifle joints of dogs

302   (Mokbel et al., 2011a), donkeys (Mokbel et al., 2011b) and goats (Murphy et al., 2003), as

303   well as GFP transgenic animals (Oe et al., 2011). An advantage of using GFP is its potential

304   use in long-term studies as daughter cells adopt GFP gene expression and, with it,

305   fluorescence is multiplied after several cell divisions. However, use of GFP requires a gene

306   transfer agent, such as a virus that is non-integrating or, preferably from a biosafety point of

307   view, a non-viral vector. These, however, are limited by the requirement of large cell

308   numbers, high levels of cell death and low transfection efficiency (Bakhshandeh et al., 2012).

309

310   The transformation procedures for GFP labelling are time consuming, making its use

311   impractical for implantation of autologous freshly isolated cells. Given this and some ethical

312   considerations as to potential adverse effects of GFP-modified cells, its use was considered

313   inappropriate for cell tracking in client-owned dogs. Instead, we used PKH26 red fluorescent

314   dye, a lipophilic cell membrane stain that has previously been used for tracking of a variety of

315   different cell types (Wisenberg et al., 2009). Data in the present study showed that cell

316   viability and population doubling time of MSCs in culture was not significantly affected by

317   PKH26 staining, corroborating findings of a previously reported study in which no effect on

318   cell growth or proliferation was observed (Shao-Fang et al., 2011). Furthermore, we

319   demonstrated that PKH26 labelling was highly effective and fluorescence was strong in

320   BMCs co-cultured with CCL tissue for 16 days. However, progressive loss of fluorescence

321   was observed in cultured MSCs over the same time frame. These findings suggest that BMCs

322   attached to tissue ex vivo do not proliferate as quickly as in monolayers due to their incipient

323   phenotype differentiation prior to replication.

324

325　　　　The findings of the present study, using the dog as a model of spontaneous CCL

326　　rupture, confirm previous observations in rats with transected ACL, showing that BMCs

327　　injected into the articular cavity engraft to the injured site of the ACL (Oe et al., 2011). In

328　　rats, GFP-transduced cells were found to be present in high numbers in the transected

329　　ligaments and seemed to be involved in appreciable neoligamental tissue transformation after

330　　4 weeks. However, we were only able to detect a very small number of PKH26 positive cells

331　　in a few CCL biopsies in 3/7 dogs. This may be due to the very low numbers of MSCs,

332　　estimated as 1 MSC per 10^4 mononuclear cells, which can differentiate into tissue in the

333　　transplanted BM as well as a low survival rate after in vivo transplantation (Pittenger et al.,

334　　1999; Wexler and Donaldson, 2003).

335

336　　　　Currently, no data are available with regard to the numbers of cells that can be safely

337　　transplanted into articular cavities in the dog. In previous studies, numbers of injected cells

338　　varied between 1.4 and 5×10^6 MSCs in different articular cavities in dogs (Black et al., 2007,

339　　2008; Guercio et al., 2012; Mokbel et al., 2011a) and 1×10^7 MSCs were injected into goat

340　　articular cavity without adverse effects (Murphy et al., 2003). Based on this empirical data,

341　　we injected 1×10^7 BMCs in the hope of achieving sufficient numbers of MSCs without

342　　untoward effects. Larger numbers of BMCs may result in higher numbers of engrafted MSCs,

343　　but further studies are needed to assess the safety and efficiency of larger transplants in dogs.

344

345　　　　Furthermore, a limitation in the evaluation of tissue samples in our study was the

346　　inability to obtain full-thickness biopsies in clinical patients. The small size of biopsies may

347　　therefore have led to some false negative results. Finally, in contrast to GFP expression, the

348　　fluorescence of PKH26 labelling decreases with cell division. The numbers of cells and time

349　　point at which labelled cells are assessed is therefore crucial for cell tracking. However, we

350   did not find a significantly greater number of PKH26 positive biopsies harvested 3 days

351   compared to those harvested 13 days after intra-articular BM injection.

352

353   Previous findings of enhanced ACL healing were associated with increased TGF-ß1

354   concentrations in synovial fluid and ACL material in rats treated with intra-articular BMCs

355   (Oe et al., 2011). Secretion of TGF-ß1 is directly influenced by the transplanted cells (Kuroda

356   et al., 2000). TGFb1 plays an anabolic role in the healing of ligaments by accelerating

357   proteoglycan synthesis and cell proliferation. However, the concentrations of TGF-ß1

358   measured prior to and after intra-articular BMC treatment in dogs in the present study were

359   not significantly different. Moreover, a possible inflammatory response after cell

360   transplantation was tested by analysing IL-6. IL-6 produced by several cell types functions to

361   increase the number of inflammatory cells in synovial tissue and its production is stimulated

362   by IL-1 and/or TNF (Venn et al., 1993). Our results revealed no inflammatory response after

363   BMC injection. IL-6 levels were no different prior to and after transplantation. For the

364   interpretation of the data, however, it should be noted that our results are based on a small

365   sample size.

366

367   **Conclusions**

368   Fresh BMCs injected into the articular cavity in dogs with spontaneous CCL injury

369   can engraft in the injured CCL, but were only rarely detected using this procedure. The low

370   recovery of transplanted cells implies that application of MSCs may be more useful for cell

371   tracking after PKH26 labelling in a clinical setting. Overall, BMC transplantation into the

372   stifle joint was well tolerated and showed no undesirable clinical effects on dogs followed for

373   up to 1 year. The clinical procedure was found to be practical and safe, but a decrease in

374   fluorescence with cell division renders the method inadequate for cellular tracking.

375

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

379

383

Appendix A: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:…

386
387 **References**

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559 **Figure legends**

560 Fig. 1. Timetable of the study design. Group 1 underwent the long procedure, group 2 the

561 shortened procedure. TPLO, Tibia plateau levelling osteotomy.

562

563 Fig. 2. Fluorescence photomicrographs of control tissues showing PKH26 positive (red) and

564 TOTO-3 positive (grey) cells (B, D); A and C are corresponding photomicrographs merged

565 with tissue. Control samples were made from tissues of CCL obtained from dogs undergoing

566 surgical treatment for CCL disease without intra-articular BM injection. Positive control

567 tissues were made by co-cultivation of tissue with PKH26-labeled BMCs at a density of $1 \times$

568 10^6cells/cm^2 in 12-well plates containing complete medium, harvested after 8 (C, D) and 16

569 (A, B) days and processed in an identical manner as study samples. Labelled cells were

570 associated with the surface (C, D) and were also integrated within the CCL explant (A, B).

571 Magnification: $\times$ 100.

572

573 Fig. 3. Fluorescence photomicrographs showing PKH26 positive (red) and TOTO-3 positive

574 (grey) cells (B, D); merged with tissue (A, C). In vivo specimens of torn CCLs from two dogs

575 at day 13 after transplantation of PKH26-labelled autologous BMCs. Labelled cells were

576 detected within the CCLs. Magnification: $\times$ 100.

577

578 Fig. 4. Scatter blot of concentrations of TGF-ß1 and IL-6 in synovial fluid sampled prior to

579 and post BMC transplantation in seven dogs. Samples of synovial fluid were collected from

580 all dogs on day 0 before BMC transplantation, additionally from dogs of group 1 (plotting

581 symbol: circle, black) on day 13 and of group 2 (plotting symbol: triangle, grey) at day 3 after

582 BMC transplantation. A median smooth line of the seven dogs is presented.