**Introduction**

Despite osteoarthritis (OA), particularly in the knee, being a major global health concern 1, there are few effective disease modifying treatments to change its natural history and progression. A biological approach using cell therapy offers the promise of an early and perhaps permanent solution for treating OA, particularly if it could be simplified and made even more cost effective than the current option of autologous chondrocyte implantation (ACI). With this in mind, various cell therapies are currently being trialled, including intra-articular injections using mesenchymal stromal cell (MSCs), with the most common sources for clinical use being bone marrow-derived (BM)- MSCs 2 and adipose-derived (AD)-MSCs 3–8. We and others have investigated MSCs derived from human umbilical cords (hUC-MSCs), which have a similar trilineage differentiation capability, immunomodulatory ability and CD-immunoprofiles compared to hBM-MSCs, whilst also having greater proliferative capacity 9,10. While the exact mode of action (MoA) of MSCs remains unknown, increasing evidence suggests that MSCs function through trophic effects on endogenous cell populations, with the production of several soluble factors, such as growth factors and cytokines, immunomodulatory and anti-inflammatory molecules 11, as well as the secretion of extracellular vesicles (EVs) which contain many of these factors 12. Furthermore, through the prevention of T-lymphocyte maturation and reduction in macrophage activation, MSCs appear capable of minimising local inflammatory responses in arthritic joints 13.

An allogeneic MSC product offers several advantages over an autologous one; for example, the patient requires only a single procedure, as no cell harvest is required. This prevents short-term donor site morbidity, saves costs and allows easier logistics in delivering the treatment. In addition, there are lower production and commercial manufacturing costs for allogeneic cell products than autologous ones as several treatment batches can be prepared in one manufacturing run rather than a single treatment as with an autologous product. Furthermore, autologous MSCs derived from a patient with OA are likely to have reduced *in vitro* proliferation and differentiation potential compared to cells derived from tissues earlier in development 14. Human UC-MSCs therefore appear to have exciting potential as a source of allogeneic cells in the prevention/treatment of OA.

Previously, we have studied the effect of intra-articular injected hUC-MSCs *in vivo* in two small pre-clinical models. One was in a murine model of joint surface injury (JSI) 15 which in some ways resembles the clinical situation (i.e. an isolated cartilage defect) that ACI was designed to treat, rather than OA *per se*. The other was a murine model of established severe OA, the partial medial meniscectomy (PMM) model 16. In the JSI model, mice treated with hUC-MSCs demonstrated significantly improved repair tissue formation in the site of injury, compared to that seen in the no-cell (control) group of mice 15. However, these differential findings were not present in mice with end-stage OA in the PMM model (16). It is of note that in both of these xenogeneic models, the implanted hUC-MSCs did not appear to elicit any inflammatory reaction.

Following these murine models, the next logical step was to apply hUC-MSCs to a large animal (ovine) model of early to moderate OA. Hence, this study was undertaken to apply an intra-articular injection of hUC-MSCs into ovine knees (stifle joints), which had undergone a medial meniscectomy and to monitor the progression of OA using several different methods.

**Materials and Methods**

Human Samples

All human umbilical cords were collected after maternal donors had provided written informed consent, with favourable ethical approval being given by the National Research Ethics Service (10/H1013/62). Umbilical cords (*n* =3) were obtained following natural births, from healthy mothers aged 23-35 years, and processed within 24 h of delivery as previously described 9. Human UC-MSCs were isolated from the tissue enzymatically and culture-expanded via a hybrid process 9,15. Initially, hUC-MSCs were cultured for a single passage using standard tissue culture techniques in complete culture medium (Dulbecco's Modified Eagle's Medium (DMEM/F12, Life Sciences, Paisley, UK) containing 1% (*v/v*) penicillin and streptomycin (P/S, Life Sciences, Paisley, UK) and 10% (*v/v*) foetal calf serum (FCS, Life Sciences, Paisley, UK)) 9,15 for 12-20 days. They were then harvested via trypsinisation at 70-80% confluency and 5 million cells seeded into the Quantum® bioreactor (Terumo BCT Inc, Lakewood, Colorado, USA) in complete culture medium for 6-11 days before being harvested and characterised or stored frozen in liquid nitrogen until application 9.

*Human UC-MSC Characterisation*

Human UC-MSC populations were assessed for the presence of the International Society for Cellular Therapy (ISCT) MSC markers 9: CD19, CD34, CD45, Human Leukocyte Antigen (HLA)-DR (-ve), CD73, CD90, CD105 (+ve), as well as some chondrogenic/MSC markers: CD271, Receptor Tyrosine Kinase-like Orphan Receptor 2 (ROR2), Fibroblast Growth Factor Receptor 3 (FGFR3), CD151, CD39, CD44, CD49, CD163, CD166 and immunomodulatory markers: CD106 and CD317, by flow cytometry with appropriate isotype-matched IgG negative controls as previously described 9,15.

*Animals*

All animal experimental protocols were carried out in accordance with the Animal Scientific Procedures Act (1986) and are reported in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. This study was approved by the UK Home Office and the Animal Welfare and Ethical Review Board at Cambridge University. Female, skeletally mature Welsh Mountain sheep (aged 3-4 years), underwent a medial meniscus transection surgery in the left stifle joint, a model that induces OA over 12 weeks 17, as previously described 18.

*Animal Anesthesia, Preparation, and Surgical Techniques*

Sheep (n=14) were anaesthetised with an intravenous injection of 0.25mg/mg alfaxalone and anaesthesia maintained via inhalation of isofluorane. The surgical procedure was performed identically for all subjects and performed under strict sterile conditions by a single surgeon (FH). Prior to surgery each stifle joint was physically examined to ensure there were no abnormalities; if any gross pathology or instability was present, the animal was excluded from participating in the study.

If suitable, the sheep was placed in dorsal recumbency and the (left) medial femorotibial joint opened from the medial aspect, cranial to the collateral ligament, leaving the medial collateral ligament intact. The medial meniscus was identified and the superior surface transected, using a No. 11 scalpel blade as previously described 17,18. The tibial plateau was protected via a rounded soft polypropylene spacer inserted via an incision at the inferior surface junction to allow access. The incision was then closed using vertical mattress sutures (0 Vicryl, Ethicon) through the joint capsule and Vicryl absorbable sutures (3–0, Ethicon) to close the skin. For the first 72 hours following surgery, post-operative analgesia (intramuscular 4mg/kg carprofen) was provided once daily to provide pain relief. No animals received any immobilization techniques, splints or casts. All animals were allowed to fully weight bear following surgery, initially being kept in a small pen for 48 hours for monitoring and to reduce ambulation, before being maintained for a further 5 days in a large indoor pen, after which they went outdoors to pasture in a field. Sheep were monitored for 4 weeks post-surgery for behavioural changes or poor wound healing.

*Cell application*

All animals in this study were randomly allocated to their treatment groups – either cells + delivery vehicle (hUC-MSCs in DMEM/F12) or delivery vehicle alone (DMEM/F12). On the day prior to surgery a member of the technical staff (not part of the research team) randomly chose which animals were to be used the following day and in what order animals would be operated on and subsequently treated. Treatments were given by intra-articular injection into the medial femoro-tibial joint of the operated joint under aseptic conditions 4 weeks post-surgery as described below.

Prior to treatment, the Quantum® expanded hUC-MSCs from three individual human donors were thawed, washed in DMEM/F12 and pooled to ensure a consistent cell product was injected into all 7 animals. All hUC-MSCs were found to be negative (< 2%) for CD19, CD34, CD45, Human Leukocyte Antigen (HLA)-DR and positive (> 95%) for CD73, CD90, CD105.Ten million cells in 50μl of DMEM/F12 were injected intra-articularly in the treatment group (n=7; Figure 1). No cells were administered to those in the control group (n=7), but 50μl of DMEM/F12 was injected representing the vehicle control. Following surgery, all animals were housed again as a single flock and all further analyses performed in a random order e.g. gait analysis.

Two sheep in the treatment group demonstrated lameness 1 day post injection, so received anti-inflammatory medication (Carprofen) for 48 hours, as per the Home Office guidelines, after which the lameness had resolved. Sheep were sacrificed, using an overdose of injectable anaesthetic, 8 weeks post-injection of cells or vehicle alone. Joints were retrieved, assessed for their macroscopic appearance, imaged with X-rays and magnetic resonance imaging (MRI) and then fixed in formalin (4% formaldehyde v/v) for histological analysis.

Figure 1.

*Activity Monitoring*

Dynamic incapacitance (weight bearing was measured using an “Accugait” force plate (AMTI, Massachusetts, USA) prior to surgical treatment and ~12 weeks post-surgical treatment, prior to euthanasia 19. Dynamic weight bearing was calculated as a percentage of the weight bearing for each sheep post-treatment compared to that prior to the injury (KN, FH).

*Postmortem Joint Evaluation*

At post-mortem, the gross morphology of the operated knee joints was scored by two blinded observers (KN, FH) as previously described 20. In brief, the stifle joints were divided into four compartments: the medial femoral condyle (MFC), lateral femoral condyle (LFC), medial tibial plateau (MTP) and lateral tibial plateau (LTP). At each anatomical site, macroscopic scoring of gross articular damage (0-16) and osteophyte development (0-12) was performed. Macroscopic synovial changes were also assessed (0-5) 20. Hence total scores could range from 0-33, where a high score indicated greatest degeneration to the joint.

*Radiographic Evaluation*

Following euthanasia, cranio-caudal radiographs were obtained of the operated limbs using a Cloud DR scanner (BCF Technologies). Two blinded observers (JP, PC) scored the scans with the Kellgren-Lawrence (KL) scoring system 21, ranging from 0-4, where a high KL score indicates increased osteoarthritic damage to the joint, whilst a low score indicates a normal healthy joint.

*Magnetic Resonance Imaging*

Joints were also imaged ex vivo, with a 0.25-T MRI scanner (Esaote). The imaging protocol used the following sequence: T1 echo train = 1, TR = 0.0 ms, TE = 26.0 ms, slice thickness = 2.5 mm, dimension size = 2.5 × 2.5 mm2, matrix size = 256 × 256; T2 echo train = 8, TR = 0.0 ms, TE = 120.0 ms, slice thickness = 4.0 mm, dimension size = 4.4 × 4.4 mm2, matrix size = 512 × 512 and 3D T2-weighted hybrid contrast-enhanced (Hyce) echo train = 1, TR = 0.0 ms, TE = 21.1 ms, slice thickness = 2.5mm, dimension size = 2.5 × 2.5 mm2, matrix size 512 × 512.

The sheep Magnetic resonance OsteoArthritis Knee Score (sMOAKS) 22 was used to evaluate joint degeneration in the ovine knee joints and the images were scored by two blinded observers (JP and PC) from 0-234 22. The joint was divided into the three main compartments: the femoro-tibial medial joint (FTMJ) and femoro-tibial lateral joint (FTLJ) and the patellofemoral joint (PFJ). In all compartments, articular cartilage loss (0-72), bone marrow lesions (BMLs) and cysts (0-108) and osteophyte formation (0-48) were measured, alongside Hoffa's synovitis (0-3) and effusion (0-3). In all cases a high score indicates increased osteoarthritic damage to the joint, whilst a low score indicates a normal healthy joint.

The thickness and total volume of the femoral cartilage were also measured on the MR scans of the operated ovine knee joints using pyKNEER, as previously described 23. High-resolution 3-Tesla MR sheep scans from a normal ovine knee joint were used as the reference template. In brief, the automatic image analysis workflow preprocesses, segments and analyses femoral knee cartilage from MR images. T1 and T2 weighted images were used for the cartilage segmentation and manually checked by two authors (JP, PC).

*Histological Evaluation*

Following imaging, the four main joint quadrants (MFC, LFC, MTP, LTP) and synovium from the operated knee joints were isolated for histological processing. After fixation in 10% neutral buffered formalin, samples were decalcified in 4% EDTA for 2-4 weeks before transferring to 70% ethanol. Samples were then paraffin wax embedded and sectioned at 5µm thickness. Sections were stained with haematoxylin and eosin (H&E; Sigma-Aldrich, Dorset, UK) or toluidine blue (Sigma-Aldrich). Semi-quantitative scoring systems were used to assess cartilage (JP & HM) and synovial changes (JP & TH) in each joint by two blinded observers as previously described 20,24. To assess synovial changes, the intimal hyperplasia, inflammatory infiltrate, subintimal fibrosis and vascularity were all scored from 0-3, to give an aggregate score of 0-12 24. Cartilage changes were assessed using a modified Mankin score (0-100, summed from 0-25 per joint quadrant) 20. The following parameters were scored: cartilage structure (0-10), chondrocyte density (0-4), cell cloning (0-4), metachromasia (interterritorial toluidine blue staining; 0-4) and tidemark/ calcified cartilage/ subchondral bone (0-3). For both cartilage and synovial changes, a higher score indicates a higher degree of tissue abnormality.

Immunofluorescence (IF) was used to determine if hUC-MSCs could be identified in the ovine knee joints, using a mouse monoclonal antibody, MANEM4, clone 6C4, against the epitope peculiar to human emerin which is not present in ovine species (kindly provided by Dr Heidi Fuller (Oswestry)). In brief, all steps were performed at room temperature and sections were washed three times in 0.2% Triton X-100 in PBS between steps unless otherwise stated 15. Following deparaffinisation and rehydration, antigen retrieval was performed by heating in sodium citrate buffer, pH 6 for 20 min at 96°C. Sections were then blocked for 30 min in IF blocking buffer (1% bovine serum albumin/10% horse serum/10% FCS in PBS; Paisley, Life Technologies, UK). Sections were blotted and incubated with the primary antibody (MANEM4, diluted 1:10 in IF blocking buffer) for 2 hours. Adjacent sections were stained with an isotype-matched IgG1 (Dako, Glostrup, Denmark) as a negative control. Sections were then incubated with the secondary antibody, goat anti-mouse IgG Alexa Fluor 488 (diluted 1:400 in IF blocking buffer) for 1 h, before washing and mounting the slides in hard-set medium with DAPI (VECTASHIELD®, Vector Laboratories, Burlingame, CA, USA). Images were obtained using a Leica SP5 confocal microscope.

*Statistics*

Data were tested for normality using the Shapiro-Wilk test, where appropriate. Non-parametric data were analysed for statistical significance using the Mann-Whitney *U* test and expressed as the median ± IQR. Parametric data were assessed using either the unpaired t-test with Welch's correction or the paired t-test and expressed as the mean ± 95% CI. All data were analysed using GraphPad Prism 8 (version 8.1.2; San Diego, USA) and deemed statistically significant when *P* <0.05.

**Results**

*Activity Monitoring*

The change in mean dynamic weight bearing from before treatment and ~12 weeks after treatment was very similar (p = 0.980) between sheep that had received hUC-MSCs compared to the no-cell control group (Figure 2).

Figure 2.

*Post-Mortem Macroscopic Score*

There was an improved (lower) total macroscopic OA joint score in sheep that had received hUC-MSCs (11±4) compared to the no cell control group (14±6), but this did not quite reach significance (p=0.054; Figure 3A; Table 1). Assessment of each individual scoring parameter also demonstrated that both parameters, gross articular cartilage damage and osteophyte development, were lower in the hUC-MSCs treatment group compared to the no cell control group, but not significantly (p=0.089 and p=0.096, respectively; Figure 3B-C). There was no significant difference for the macroscopic scoring of the synovium between the two treatment groups (p=0.735; Figure 3D).

Figure 3.

*Kellgren–Lawrence Score*

The Kellgren-Lawrence (KL) score, ranging from 0-4, showed that joints treated with hUC-MSCs had significantly better (lower) K-L scores (2.0±0) compared to the no cell control group (3.0±0; p=0.028; Figure 4). Typical X-rays from each treatment group are shown in Figure 4B.

Figure 4.

*Magnetic Resonance Imaging*

No difference was observed in the sMOAKS score between the hUC-MSC treated group and the no-cell controls (hUC-MSCs 18±10 vs. no cells 22±9; p=0.784; Figure 5A). Likewise, no significant difference in the thickness of the cartilage on the femoral condyles was observed between the treatment group and the no cell control group (hUC-MSCs 0.82mm, 95% CI 0.585, 1.046; vs. no cells 0.75mm, 95% CI 0.522, 0.982; p=0.632; Figure 5B). In terms of cartilage volume again, no significant differences were observed between the treatment group and the no cell control group (hUC-MSCs 988.7mm3, 95% CI 128.0, 1849; vs. no cells 924.8mm3, 95% CI 207.6, 1642; p=0.890; data not shown).

Figure 5.

*Histological and Immunohistochemical Analyses*

Histological scoring demonstrated improved (lower) microscopic cartilage scores in sheep receiving hUC-MSCs (37±6) compared to the no cell control (41±13), but again, this finding did not quite reach significance (p=0.064; Figure 6A). When assessing each of the joint quadrants individually, again there was no significant difference between the two treatment groups (MFC, p=0.142; LFC, p=0.453; MTP, p=0.357 and LTP, p=0.548; Figure 6B-E), although it should be noted that in all cases the median score was lower in the treated group compared to the no cell control and scores were generally more severe on the medial (operated) side of the joint.

Figure 6.

Furthermore, there was no significant differences in synovitis scores between the two treatment groups (hUC-MSCs: 3.0±0.5 vs. no cells 3.0±2.5; p=0.900; Figure 7).

Figure 7.

No human cells were detected by immunostaining with the antibody to human emerin in paraffin wax-embedded sections of the joints from sheep receiving human UC-MSCs 8 weeks post-implantation (Figure 8). They were, however, clearly visible in sections of human umbilical cord, used as a positive control tissue.

Figure 8.

**Discussion**

Extensive work has been undertaken to evaluate the characteristics of hUC-MSCs and also their effect in murine preclinical models of OA 15,16. This study of hUC-MSCs in a model of early to moderate OA in a large animal is the next logical progression in the translational pipeline for developing hUC-MSCs as a treatment for human patients with OA.

The hUC-MSCs were obtained via a ‘hybrid’ process of growing hUC-MSCs, with culture expansion, initially in monolayer, followed by use of the Quantum® bioreactor system. Such up-scale expansion can provide a cost-effective method of producing large numbers of cell doses whilst complying with GMP- regulations 25. Previous work in our group has shown little difference with regards to MSC characterisation, tri-lineage differentiation or telomere length, between hUC-MSCs grown using this bioreactor compared to traditional tissue culture plastic protocols 9. In addition, there were very high levels of gene expression for the immunomodulatory molecule, indoleamine 2, 3-dioxygenase (IDO), by Quantum® generated hUC-MSCs when stimulated with interferon gamma, thus warranting further in vivo investigation of Quantum® expanded hUC-MSCs potency.

In order for cell therapy to be considered a useful and cost-effective treatment for OA, it should alter the disease progression and pathogenesis, so by necessity be applied earlier in the OA spectrum than the end-stage when joint prostheses are used, perhaps even being applied at the pre-OA stage 26. Sheep are considered a suitable large animal model of musculoskeletal issues, since the biomechanics of the ovine stifle joint is similar to the human knee 20,27 and it is highly translatable to the human clinical setting 28. Meniscal release procedures, such as has been used in this study, result in a mild OA with moderate changes within 12 weeks 17,18, thus allowing the application of a treatment modality part way through the pathogenesis of OA.

As hypothesized, the hUC-MSCs appeared to delay the progression of OA, with significantly lower KL scores in the treatment arm compared to the no-cell control group. Likewise, the macroscopic and histological scoring showed better scores following treatment with hUC-MSCs, although this did not quite reach significance. This suggests that perhaps the study was underpowered and indeed the numbers in each group were fairly small. However, this number had been calculated to be adequate from an earlier study based on histology as an outcome measure to achieve a 0.8 level of power (personal information; F Henson, Cambridge). Furthermore, when looking at the synovitis scores, a non-significant difference between the two treatment groups suggests that the hUC-MSCs did not evoke an inflammatory response in any of the treated animals, at least at the time-point measured.

The chronology of OA development may also in part be responsible for a lack of significance in some parameters, for example, in gait analysis. Cake et al 17 found vertical ground reaction forces varied considerably with time post meniscal injury compared to baseline, with maximal reduction of peak forces at 2.5 and 8 weeks but a tendency to return towards normal by 12 weeks 17. Similarly, the dynamic weight bearing in sheep which had undergone the same meniscal destabilization as in the current study, showed an improvement after 3 weeks when treated with 120µg of a GDF-5 analogue compared to untreated controls, but this difference between treated and controls decreased with time to 11 weeks and, as in the current study, did not reach significance 19. Indeed Newell et al showed there to be a correlation between activity and the severity of pathology 18. With several outcome measures showing a trend to improvement in pathological OA features but not reaching significance, perhaps larger sample numbers and extending the timescale to 24 or 26 weeks may have rendered a higher level of significance. Furthermore, the continual monitoring of the sheep using smart technology, such as commercially available telemetric systems e.g. Fitbark, which measures movement behaviour, may have allowed more subtle changes to be monitored over time 18. In addition, in order to gain more information on the mode of action of the implanted cells, studying changes in the inflammatory status of different tissues such as the meniscus, particularly at different timepoints, may provide useful and critical information.

Two of the treated sheep demonstrated a local reaction the day after injection. Initial inflammation as an adverse reaction is reported by one other group using an intra-articular injection of xenogeneic cell products. Punzon et al found an increase in pain and lameness in 4 of the 40 dogs which were treated with equine UC-MSCs in their dog OA study 29. As in our study, they treated it with anti-inflammatory drugs and suggest that it did not influence the long-term benefit of the cells.

The mode of action (MoA) of MSCs, including those derived from UCs, remains uncertain. Whilst the previously held belief was that MSCs would differentiate into the cell type of the tissue undergoing repair, depending on local cues (e.g. into chondrocytes for cartilage injuries), this is no longer thought to be the main MoA 30. Rather, MSCs do appear to be responsive to the host environment, but with a paracrine response such that if inflammation is present they may be stimulated to synthesise anti-inflammatory molecules and so diminish or dampen it down. For example, previous in vitro studies in our centre have shown that hUC-MSCs upregulate IDO gene expression after exposure to a pro-inflammatory stimulus (IFN-γ) 31. IDO, a potent immunomodulatory molecule, appears to deplete tryptophan via the kynurenine pathway causing the suppression of T-cells, minimising the local inflammatory response 32. In addition, they may also modulate the host’s cells to mount a therapeutic response 30. Long-term retention of viable MSCs may not be necessary for this, with many studies finding as we did, that implanted MSCs were not engrafted into the treated tissues, with few or no cells possible to identify soon after implantation 33. This finding is not universal, however, and some studies are able to identify implanted cells some time post-implantation. For example, labelled autologous MSCs which had been implanted into intervertebral discs were detected in 75% of those discs 8 months later 34. Similarly, in a study of Hartley-Dunkin guinea pigs with naturally occurring OA, MSCs which had been injected intra-articularly with hyaluronan, could be identified at 5 weeks post-implantation, the latest time point in that study 35.

Umbilical cord cells, prepared in different ways, are progressing along the translational pathway to the clinic as appropriate cells for allogeneic cell therapies in several fields. ORBCELL™(Orbsen Therapeutics, Ireland), which are CD362-enriched hUC-MSCs, are being used in the UK in a phase I/II trial of Acute Respiratory Distress Syndrome (REALIST; 36 and also in a phase II trial of autoimmune hepatitis (http://clinicaltrials.gov: NCT02585622). In Vietnam, Vinmec are running a phase I/II trial of UC-MSCs in chronic obstructive pulmonary diseases (COPD; <https://clinicaltrials.gov>: NCT04433104). In the veterinary world equine UC-MSCs already have approval for use in the treatment of OA in dogs 29 and horses 37. Their product, HorStem®, received a marketing authorisation by the European Medicines Authority (EMA) in 2019 37. The study leading to this approval describes how of 33 horses with mild to moderate OA, 16 received an injection of HorStem® into the affected joint and 17 horses received a placebo injection 37. They were examined for lameness (on a scale of 0 to 5, where 0 is normal) at days 14, 35 and 63. Treatment was considered successful if the lameness reduced to 0 or 1. There was a success rate of 75% in the cell treated group at day 63, compared to 25% in the placebo group 37.

The results of the present study provide further support to the premise that hUC-MSCs could provide a suitable allogeneic therapy for treating OA in humans in the early stages of the disease. Even without knowing the exact MoA of the cells, one cannot ignore the mounting evidence indicating the ability of MSCs to alter the joint environment and appear to stimulate regeneration in some tissues 38 and retard destruction of other tissues 39. In conclusion, we suggest that a phase I/II clinical trial of hUC-MSCs for treating early OA in humans is warranted, whilst also addressing the outstanding challenge of identifying clear potency markers 30.

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