

Cervical motion preservation using mesenchymal progenitor cells and pentosan polysulfate, a novel chondrogenic agent: preliminary study in an ovine model

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Object. There is an unmet need for a procedure that could generate a biological disc substitute while at the same time preserving the normal surgical practice of achieving anterior cervical decompression. The objective of the present study was to test the hypothesis that adult allogeneic mesenchymal progenitor cells (MPCs) formulated with a chondrogenic agent could synthesize a cartilaginous matrix when implanted into a biodegradable carrier and cage, and over time, might serve as a dynamic interbody spacer following anterior cervical discectomy (ACD).

Methods. Eighteen ewes were divided randomly into 3 groups of 6 animals. Each animal was subjected to C3–4 and C4–5 ACD followed by implantation of bioresorbable interbody cages and graft containment plates. The cage was packed with 1 of 3 implants. In Group A, the implant was Gelfoam sponge only. In Group B, the implant consisted of Gelfoam sponge with 1 million MPCs only. In Group C, the implant was Gelfoam sponge with 1 million MPCs formulated with the chondrogenic agent pentosan polysulfate (PPS). In each animal the cartilaginous endplates were retained intact at 1 level, and perforated in a standardized manner at the other level. Allogeneic ovine MPCs were derived from a single batch of immunoselected and culture-expanded MPCs isolated from bone marrow of outbred sheep (mixed stock). Radiological and histological measures were used to assess cartilage formation and the presence or absence of new bone formation.

Results. The MPCs with or without PPS were safe and well-tolerated in the ovine cervical spine. There was no significant difference between groups in the radiographic or histological outcome measures, regardless of whether endplates were perforated or retained intact. According to CT scans obtained at 3 months after the operation, new bone formation within the interbody space was observed in the Gelfoam only group (Group A) in 9 (75%) of 12 interbody spaces, and 11 (92%) of 12 animals in the MPC cohort (Group B) had new bone formation within the interbody space. Significantly, in the MPC & PPS group (Group C), there were only 1 (8%) of 12 levels with new bone formation ($p = 0.0009$ vs Group A; $p = 0.0001$ vs Group B). According to histological results, there was significantly more cartilaginous tissue within the interbody cages of Group C (MPC & PPS) compared with both the control group (Group A; $p = 0.003$) and the MPC Group ($p = 0.017$).

Conclusions. This study demonstrated the feasibility of using MPCs in combination with PPS to produce cartilaginous tissue to replace the intervertebral disc following ACD. This biological approach may offer a means preserving spinal motion and offers an alternative to fusion to artificial prostheses. (DOI: 10.3171/2010.3.FOCUS1050)

KEY WORDS • anterior cervical discectomy • pentosan polysulfate •
 mesenchymal stem cell • tissue engineering • sheep • Gelfoam

CERVICAL discectomy is a common procedure, which is usually performed for neural decompression in patients experiencing radiculopathic or myelopathic symptoms. Cloward, Smith, and Robinson^{7,8,30,34} introduced

Abbreviations used in this paper: ACD = anterior cervical discectomy; ACDF = ACD and fusion; ICRS = International Cartilage Repair Society; MPC = mesenchymal progenitor cell; PPS = pentosan polysulfate.

ACDF in the 1950s to address this problem and modifications of their techniques are still widely used today.

Regardless of the cervical fusion technique employed, a long-term sequela of adjacent segment disease can be recurrent pain from adjacent nerve root compression.¹⁴ The incidence of symptomatic adjacent segment disease in a group of 374 patients followed for a maximum of 21 years after anterior cervical fusion was found to be 2.9% per year and up to 25.6% at 10 years.¹³ Other



FIG. 1. Lateral radiograph showing modified Cornerstone HSR cervical cage and Mystique Hydrosorb cervical plate at C3–4 and C4–5. The white dots are the radiolucent markers on the proximal and distal screws and plates (top and bottom); thus there are 5 points at each attachment point.

studies also report increased intradiscal pressure at adjacent levels to fused vertebrae, which may account for the adjacent segment disease.^{11,28} Many patients requiring ACDF also exhibit signs of preexisting spondylosis at other levels and it has been shown that an even higher incidence of symptoms may develop in these patients following ACDF.²⁰

In an attempt to minimize the long-term problems associated with adjacent segment disease, a variety of motion preservation techniques and devices have been evaluated in select patient groups. For example, cervical disc arthroplasty has been evaluated in comparison with conventional interbody fusion in prospective trials, but the results have been mixed.^{2,9,10,22,26,27,29,31} Recently, one investigative group performed allograft disc transplants in humans in an attempt to provide a biological alternative to artificial disc prosthesis.³² Cervical discs, together with endplates and uncovertebral joints, were removed from donors and stored in 10% dimethyl sulfoxide with 10% calf serum and frozen in liquid nitrogen. In a series of 5 patients with a 5-year follow-up period, 1 patient displayed

radiological evidence of fusion across the transplanted disc space, whereas sagittal motion was maintained in 4 of 5 patients. As assessed by MR imaging, preservation of disc hydration was present in 2 of 5 patients, and although the authors were unable to provide evidence that the annulus and nucleus cells of the transplanted discs survived, they suggest that their approach led to no adjacent segment disease at 5 years.

There are clearly many practical difficulties associated with human disc transplantation and it is unlikely to become part of routine clinical practice in the immediate term. Nevertheless, there is an unmet need for a procedure that could generate a biological disc substitute, while at the same time preserving the normal surgical practice of achieving cervical spine decompression. The objective of the present study was to test the hypothesis that adult allogeneic MPCs formulated with a chondrogenic agent could synthesize a cartilaginous matrix when implanted into a biodegradable carrier and cage, which over time might serve as a flexible interbody spacer. It should be emphasized that in this preliminary experimental study, the objective was not to produce a new intervertebral disc, but rather a cartilaginous tissue within the body of the resorbable cage.

Methods

Study Design

Eighteen Border-Leicester/Merino ewes (2 years old) were divided randomly into 3 groups of 6 animals. Each animal was subjected to C3–4 and C4–5 ACD following the standardized surgical procedure.¹⁹ A custom-modified noncrystalline polylactide copolymer with a 70:30 ratio of poly(L-lactide) to poly(D,L-lactide) (Hydrosorb Cornerstone HSR, Medtronic), was packed with 1 of 3 implants then inserted into each disc space. In Group A, the implant consisted of Gelfoam only (Pharmacia & Upjohn Co.). In Group B, the implant was composed of Gelfoam containing 100 μ l of 1 million immunoselected MPCs (Mesoblast Ltd.) suspended in 1.0 ml Profreeze (Lonza Ltd.).²¹ The implant in Group C was Gelfoam containing 100 μ l of 1 million MPCs/ml Profreeze formulated prior to cryopreservation with 100 μ g/ml of PPS (Proteobio-actives Pty Ltd.). Group C was designated the “MPC & PPS” group.

Anterior plating to each level was then performed using a Mystique Hydrosorb cervical plate (Medtronic) and screws (Fig. 1). In total, 36 cervical spinal levels were included in the study. Animals were killed at 3 months. The study was approved by the Animal Research Ethics Committee of the School of Biomedical Sciences, Monash University, Victoria, Australia.

Endplate Treatment

The retention of the cartilaginous endplates, while potentially inhibiting fusion and promoting chondrogenesis within the interbody space, could also inhibit nutrition to the implanted cells. In the light of this possibility, 1 random level in each animal was selected and the endplates were perforated in a standardized manner using a



Fig. 2. Photographs of custom-made instrument used for standardized perforation of endplates.

custom-made tool (Fig. 2). This procedure ensured that 16 holes (0.5-mm wide × 0.5-mm deep) were made through the endplates into the vertebral bone at the designated levels. Each animal had the identical implant placed at both C3–4 and C4–5 levels, but 1 level was randomly assigned to have the endplates perforated and the other endplates were retained intact (Table 1).

Cervical Plate, Interbody Cage, and Carrier

The cages, plates, and screws were made from polylactic acid and were bioresorbable. The Mystique Cornerstone interbody cage (6-mm high × 11-mm long × 14-mm wide) was specifically modified to increase the internal volume to 472 mm³. This cage was packed with the same volume of Gelfoam carrier in every case. In the cell-treated groups, the MPCs were added to the carrier within the cage (Fig. 3 left) and allowed to soak into the sponge, avoiding spillage (Fig. 3 right). The Mystique Hydrosorb cervical plate (25 mm) was secured to each operated level using 4 Mystique (3 × 13 mm) screws (Fig. 4).

Surgical Technique and Postoperative Care

The surgical procedure has been previously described.¹⁹ Animals were fasted 12–24 hours before surgery and were allowed water ad libitum. Sheep were positioned supine on the operating table and anesthetized by intravenous injection of thiopentone (20 mg/kg); anesthesia was maintained using isoflurane (1–3%) inhalation. Local anesthetic (0.5% bupivacaine with 1:200,000 adrenaline) was injected prior to a right anterolateral ap-

proach through a longitudinal neck incision. The longus colli muscle was elevated bilaterally using diathermy and the position of the C3–4 and C4–5 levels were confirmed with fluoroscopy. Distraction was achieved with 16-mm Caspar pins followed by a total discectomy, but the cartilaginous endplates were carefully preserved. No attempt was made to open the posterior longitudinal ligament. All cages were inserted and countersunk by approximately 3 mm. As per the manufacturer’s instructions, the Mystique plates were then molded using hot saline to reconstruct the anterior column and to act as a graft containment device. The longus colli muscle was then approximated by suture, followed by layered closure and subcuticular suture to skin.

A fentanyl patch was administered postoperatively. Following extubation, the sheep were transferred to a metabolic cage for observation. After 3 days, the sheep were transferred to open pastures for the duration of the study and observed regularly. The sheep were allowed to graze ad libitum and supplemented with Lucerne chaff.

Postmortem Analysis

All sheep were killed at 3 months by overdose using 150 mg/kg of pentobarbitone sodium. Clinical veterinarians performed autopsy examinations on the sheep in a blinded fashion.

Radiographic Analysis

Plain lateral and selected anteroposterior digital radiographs were obtained in all animals (Radlink Atomscope HF 200A) of the cervical spine preoperatively, within 24 hours following surgery, and at 1-, 2-, and 3-months postoperatively. The radiography was performed while the sheep were under sedation using metamidone (0.025 mg/kg intravenously) and reversed using atipamezole (0.125 mg/kg intravenously). After the sheep were killed at 3 months, multislice CT (Siemens Sensation 64) was performed on the isolated spine, and multiplanar images were acquired with 0.6-mm collimation on a 64-slice-scanner and reconstructed in the sagittal, axial, and coronal planes. Fusion, or lack thereof, was assessed by plain radiography and fusion was defined by continuous bridging of the trabecular bone and the absence of ra-

TABLE 1: Summary of study design and number of operated levels

| Treated Levels | No. of Operated Levels | | |
|-----------------|------------------------|---------------------|-------------------|
| | MPC Group (B) | MPC & PPS Group (C) | Control Group (A) |
| C3–4 perforated | 3 | 3 | 3 |
| C4–5 intact | 3 | 3 | 3 |
| C3–4 intact | 3 | 3 | 3 |
| C4–5 perforated | 3 | 3 | 3 |

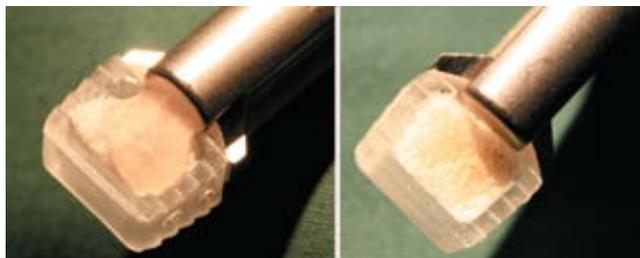


Fig. 3. Modified Cornerstone HSR cervical cage packed with Gel-foam with MSCs added (**left**) and after MSCs have soaked in (**right**).

diolucent lines at 3 months on CT.^{3,5} The interbody space was assessed, in a blinded manner, for the presence or absence of new bone formation (Fig. 5).

Histological Analysis

Following extraction, the spine was placed in 10% normal buffered formalin. The C3–4 and C4–5 motion segments were then cut from the remainder of the spine using a band saw. These 2 segments were cut in the mid-sagittal plane into 2 blocks, each containing the cage, with 3 mm of the superior and inferior vertebral bodies on either side. Decalcification was performed using 10% formic acid for 2 weeks. The blocks were then dehydrated in ascending concentrations of ethanol before being placed in neat chloroform overnight to dissolve the polylactic acid cage and plates. Dehydration was again performed in ascending concentrations of ethanol under agitation before clearing in xylene prior to embedding in paraffin. Six-micron sections were then cut in the sagittal plane and stained with H & E, Alcian blue, toluidine blue, or Masson trichrome. A modified scoring system based on the ICRS scoring system (Table 2) was used for scoring the sections (Fig. 6).²⁵ A board-certified veterinary pathologist blinded to treatment condition scored each section.

Statistical Analysis

Data were analyzed using 1-way ANOVA followed by the Dunnett multiple comparison test where significant differences were observed. The 2-tailed Student t-test was used for comparison of parametric data and the



Fig. 4. **Left:** Intraoperative photograph showing interbody cage and carrier in disc space following discectomy. **Right:** Photograph at 3 months postmortem showing plate, screws, and cage with its contents.

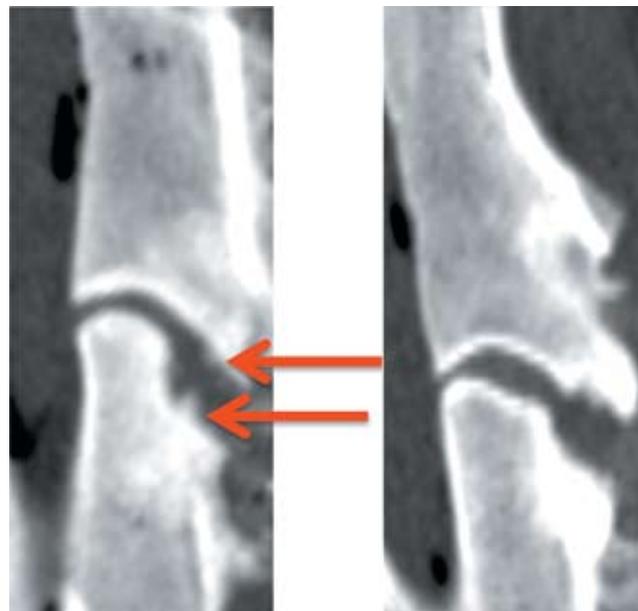


Fig. 5. Sagittal CT scans showing an example of new bone formation (**left**) within the interbody space (*arrow*) and no new bone formation within the interbody space (**right**). Note anterior subperiosteal bone formation in both images.

Fisher exact test was used for contingency data. Prism 5.0 (Graph Pad Software) was used for the statistical analysis. Values were expressed as means and SDs unless otherwise stated. A probability value < 0.05 was considered statistically significant.

Results

Adverse Events

No surgical- or cell-related adverse events were observed during the study or at postmortem. All animals were ambulant within 2 hours of surgery. A degree of graft extrusion was noted in all animals by 3 months. This extrusion was generally due to anterior vertebral subperiosteal bone formation above and below the disc space, which elevated the plate and caused migration of the cage (Fig. 7). The cages migrated anteriorly a mean distance of 5 mm (range 3–10 mm) but there were no differences in extent of cage or plate movement between the experimental groups.

Endplate Treatment

There was no significant difference between groups

TABLE 2: Modified ICRS histological scoring system used for this study

| Matrix | Score* |
|-------------------------------|--------|
| bone | 0 |
| fibrous tissue | 1 |
| fibrocartilage | 2 |
| mixed fibro/hyaline cartilage | 3 |
| hyaline cartilage | 4 |

* Evidence of ossification within cartilage, subtract 1.

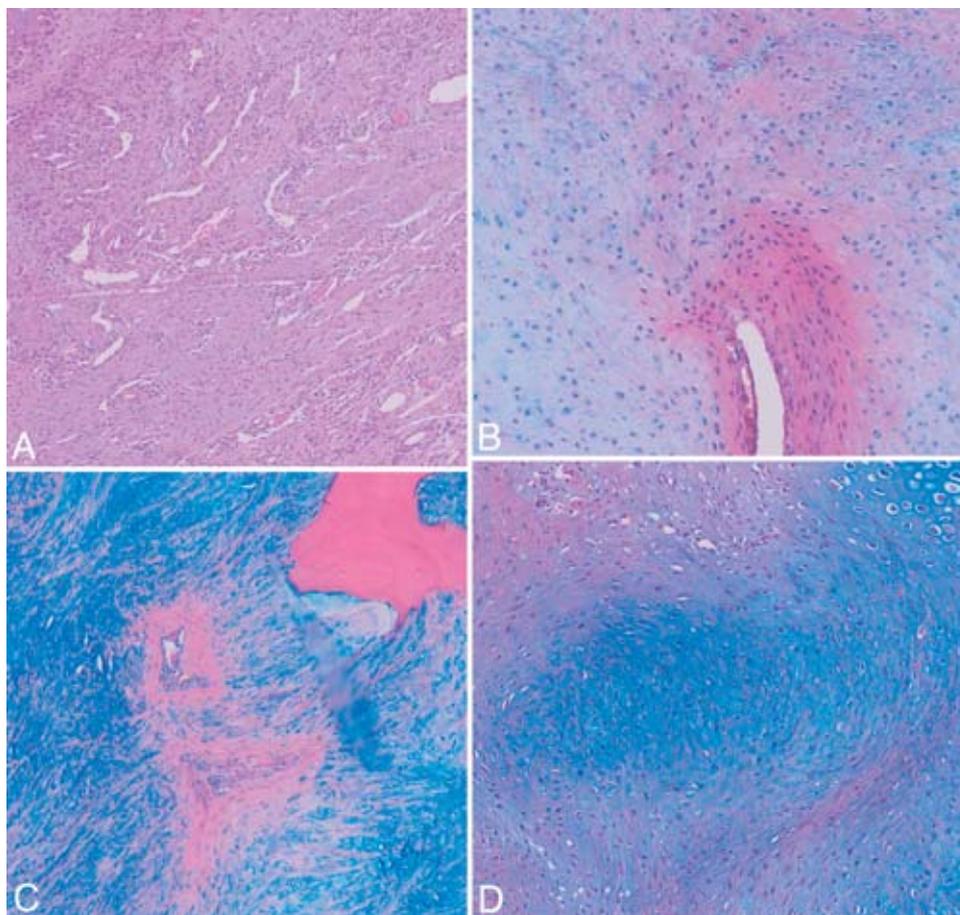


FIG. 6. Photomicrographs showing examples of the modified ICRS histological grading system: **A**, fibrous tissue (score 1); **B**, fibrocartilage (score 2); **C**, hyaline cartilage with ossification (score 3); **D**, hyaline cartilage (score 4). H & E and Alcian blue. Magnification $\times 4$.

in the radiographic or histological outcome measures, regardless of whether endplates were perforated or retained intact.

Radiographic Results

Continuous bridging of trabecular bone was not observed in any animal, at any time point, by plain radiography or by CT scan. According to CT scans obtained at 3 months, new bone formation within the interbody space was observed (Table 3, Fig. 8) in Group A (Gelfoam only) in 9 (75%) of 12 interbody spaces, with 4 of 6 levels having perforated endplates and 5 of 6 with intact endplates. Eleven (92%) of 12 animals in the MPC cohort (Group B) had new bone formation within the interbody space, with 5 of 6 levels having perforated endplates and 6 of 6 with intact endplates. Significantly, in the MPC & PPS group (Group C), there was only 1 (8%) of 12 levels with new bone formation and this occurred in a perforated endplate level ($p = 0.0009$ vs Group A and $p = 0.0001$ vs Group B).

Histological Results

No inflammatory, infective, or neoplastic changes were evident in any specimen recovered from any group. No difference was observed in the modified ICRS histology score between control Group A and MPC Group B;

mean scores were 0.92 ± 0.29 and 1.08 ± 0.79 , respectively, and were indicative of predominantly fibrous tissue within the interbody cage area. Significantly, in the MPC & PPS group (Group C), predominantly cartilaginous tissue within the interbody cages was found with a mean modified ICRS histology score of 2.28 ± 1.35 . This score was significantly higher than the control group ($p = 0.003$) and the MPC group ($p = 0.017$; Fig. 9).

Discussion

The results of the histological studies provide proof of concept for our working hypothesis that it is possible to perform a conventional ACD while at the same time generating, in situ, a cartilaginous tissue that has the potential to form an interbody mobile segment. Moreover, the MPC & PPS group, in contrast to the other experimental groups, failed to deposit bone to any large extent within the confines of the cage.

Although the present studies have shown that it is possible to grow cartilage from MPC & PPS seeded into a collagen scaffold within a Hydrosorb cage placed in a spinal location that normally supports fusion, the duration of the study was too short to determine if complete interbody chondrification occurred after the cage

was resorbed. Furthermore, based on the existing literature,^{11,13,14,19,27} we can only speculate as to whether the long-term clinical outcomes would be superior to the current practice of spinal fusion. In particular, the biomechanical characteristics of a cartilaginous tissue as a replacement for the tougher fibrocartilaginous annulus fibrosus must be questioned in relation to maintaining the functional stability of the cervical spine during articulation and axial loading. Additional longer-term multidisciplinary studies are clearly required to address these important issues.

Our previous studies with an ovine model of ACDF using the same allogeneic ovine MPC & Mastergraft hydroxyapatite/tricalcium phosphate granules in a Fidji interbody cage, when evaluated after 12 weeks showed 75% continuous bone bridge formation compared with 33% in the group that received granules alone.¹⁷ On the basis of these results, and notwithstanding the absence of the osteoconductive hydroxyapatite/tricalcium phosphate granules, it might be predicted that a greater amount of new bone formation was deposited within the cage after 12 weeks than was observed by CT in the present study. While it is possible that with time these small bone spurs may advance to frank bone bridging, the radiographic and histological studies clearly showed that after 12 weeks there was an increased frequency of bone spur deposition in cages containing MPC alone compared with the formulation containing MPC & PPS.

The inhibition of new bone formation by MPCs coupled with enhanced cartilage production arises from the ability of PPS to selectively direct the differentiation of MPCs and other mesenchymal stem cells down the chondrogenic lineage. In vitro studies have confirmed the concentration-dependent upregulation of cartilage associated genes SOX-9, aggrecan, and Type II collagen, and downregulation of bone-associated genes, Type I collagen, and RUNX2 gene expression by MPCs cultured in micromass, pellets,¹⁶ or Gelfoam sponges in the presence of PPS (Goldschlager T, Ghosh P, Wu AW, Shimmon S, Abdelkadar A, Jenkin G, et al., unpublished abstract [“Regeneration of the cervical intervertebral disc part 1: enhanced proliferation and chondrogenic differentiation of mesenchymal precursor stem cells (MPC) cultured in collagen sponges in the presence of pentosan polysulfate (PPS)”] presented at the Cervical Spine Research Society Annual Meeting, 2009). These findings were supplemented by biochemical assays that confirmed that PPS also directed chondrogenic differentiation of MPCs at the protein level¹⁶ (Goldschlager T, Ghosh P, Wu AW, Shimmon S, Abdelkadar A, Jenkin G, et al., unpublished abstract [“Regeneration of the cervical intervertebral disc part 1: enhanced proliferation and chondrogenic differentiation



FIG. 7. Sagittal section of the cervical spine after sacrifice at 3 months showing anterior migration of the cage and plate.

of mesenchymal precursor stem cells (MPC) cultured in collagen sponges in the presence of pentosan polysulfate (PPS)”] presented at the Cervical Spine Research Society Annual Meeting, 2009).

In contrast to the majority of previous investigations that have used transforming growth factor-β, bone morphogenetic proteins, or other proteinaceous growth factors to induce chondrogenic differentiation of mesenchymal stem cells,^{6,23} PPS is a semisynthetic sulfated polysaccharide that has been used for more than 40 years for

TABLE 3: Levels with new bone formation within the interbody space

| Group | C3-4 | | C4-5 | |
|---------------|--------|------------|--------|------------|
| | Intact | Perforated | Intact | Perforated |
| Control (A) | 2 | 3 | 3 | 1 |
| MPC (B) | 3 | 2 | 3 | 3 |
| MPC & PPS (C) | 0 | 1 | 0 | 0 |

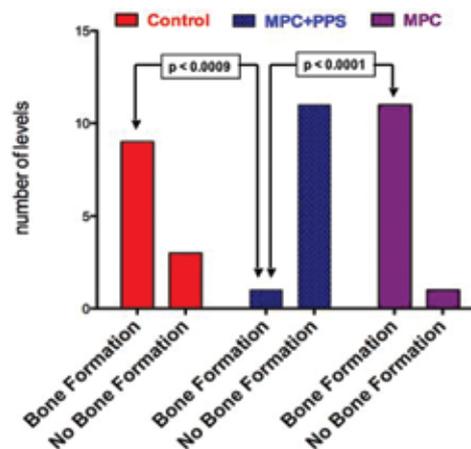


FIG. 8. Graph of new bone formation determined by CT at 3 months in each experimental group. p = 0.002 for ANOVA.

Cervical motion preservation using mesenchymal progenitor cells

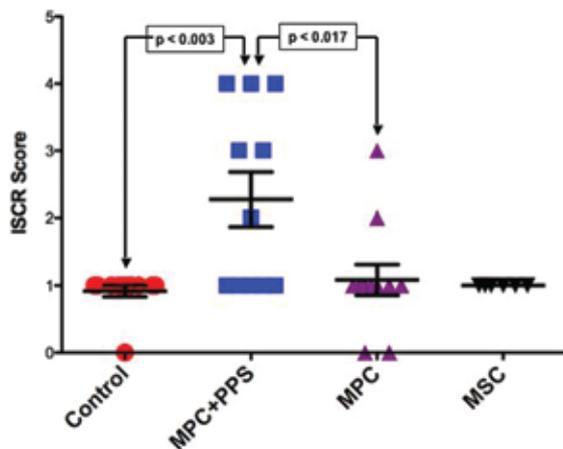


Fig. 9. Graph showing histological assessment of cartilage deposition at 3 months in each experimental group.

a number of clinical applications including osteoarthritis.^{12,18} Extensive *in vitro* and animal model studies have shown that PPS is antiinflammatory, promotes fibrinolysis, and stimulates cartilage matrix synthesis by chondrocytes.^{12,13,15} Pentosan polysulfate is also a potent inhibitor of serine proteinases, and it downregulates collagenase production by chondrocytes at the gene promoter level.^{1,12} More recently, PPS has been shown to inhibit the cartilage aggrecanases ADAMTS4 and ADAMTS5^{35,36} and their binding to the endogenous inhibitor TIMP-3. These data clearly demonstrate the ability of PPS to stimulate the biosynthesis of components of the extracellular matrix while concomitantly limiting their degradation by its direct and indirect anticatabolic effects. These beneficial pharmacological activities of PPS have resulted in its widespread use for the treatment of osteoarthritis in both veterinary^{4,12,24} and human practice.^{12,14} However, the present study is the first to demonstrate the ability of this drug to induce chondrogenic differentiation of MPC *in vivo*, thereby highlighting the potential of the MPC & PPS combination in neurological and orthopedic surgery and regenerative medicine.

A major problem encountered in this animal study was the migration or extrusion of the cervical cages and plates. We believe that the retention of the endplates was a likely cause because they reduce friction between the grooves of the cage and the cartilaginous endplate surface. This is less of a problem in the fusion setting where the endplates are removed and the interface between bone and the cage produces a greater degree of friction. However, there are reports of migration of bioresorbable implants in fusion surgery (Lebl DR, Metkar U, Grottkau B, Wood KB, unpublished abstract [“Early failure of bioabsorbable anterior cervical plates”] presented at the Cervical Spine Research Society Annual Meeting, 2009). In the studies described here, the cervical plate was used to contain the cage, as previous testing with a standalone cage in this model resulted in complete extrusion. Periosteal bone formation in the ovine model is well described³³ and caused elevation of the plate, thereby allowing migration of the cage. It is likely that the cage migration negated any potential difference arising from per-

forating or retaining the endplates. Further studies with custom-made implants for this purpose are in progress. In addition, biomechanical testing will be undertaken 18 months after implantation to ensure complete resorption of the polylactic acid implants occurred, to confirm motion preservation in this model.

Conclusions

This study has demonstrated the feasibility of MPCs in combination with PPS to produce cartilaginous tissue to replace the intervertebral disc following ACD. This biological approach may offer a means of preserving spinal motion and offer an alternative to fusion to artificial prostheses.

Disclosure

The research was partially funded through a sponsored research agreement from Mesoblast Ltd. awarded to Monash University. Peter Ghosh, Stan Gronthos, and Andrew Zannettino are consultants of Mesoblast Ltd. and own stock in this company. Silviu Itescu is an employee of Mesoblast Ltd. and owns stock in the company. Tony Goldschlager has received partial travel support from Mesoblast Ltd. to attend scientific conferences.

Author contributions to the study and manuscript preparation include the following. Conception and design: P Ghosh, T Goldschlager, S Itescu. Acquisition of data: T Goldschlager, G Jenkin. Analysis and interpretation of data: P Ghosh, T Goldschlager, A Zannettino, S Gronthos, G Jenkin. Drafting the article: P Ghosh, T Goldschlager, G Jenkin. Critically revising the article: P Ghosh, T Goldschlager, A Zannettino, S Gronthos, JV Rosenfeld. Reviewed final version of the manuscript and approved it for submission: P Ghosh, T Goldschlager, JV Rosenfeld. Statistical analysis: P Ghosh, T Goldschlager, S Itescu, G Jenkin. Administrative/technical/material support: A Zannettino, S Gronthos, S Itescu, G Jenkin. Study supervision: JV Rosenfeld, G Jenkin.

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