A Comparison of Mesenchymal Precursor Cells and Amnion Epithelial Cells for Enhancing Cervical Interbody Fusion in an Ovine Model

**BACKGROUND:** Rapid, reliable fusion is the goal in anterior cervical diskectomy and fusion. Iliac crest autograft has a high rate of donor-site morbidity. Alternatives such as bone graft substitutes lack osteoinductivity, and recombinant bone morphogenetic proteins risk life-threatening complications. Both allogeneic mesenchymal precursor cells (MPCs) and amnion derived epithelial cells (AECs) have osteogenic potential.

**OBJECTIVE:** To compare for the first time the capacity of MPCs and AECs to promote osteogenesis in an ovine model.

**METHODS:** Five groups of 2-year-old ewes were subjected to C3-4 anterior cervical diskectomy and fusion with a Fidji interbody cage packed with iliac crest autograft alone (group A; n = 6), hydroxyapatite-tricalcium phosphate Mastergraft granules (HA/TCP) alone (group B; n = 6), HA/TCP containing 5 million MPCs (group C; n = 6), or HA/TCP containing 5 million AECs (group D; n = 5); group E was made up of age-matched nonoperative controls (n = 6). At 3 months, animals were euthanized and quantitative multislice computed tomography, functional radiography, biomechanics, histology, and histomorphometry were performed.

**RESULTS:** No procedure- or cell-related adverse events were observed. There was significantly more fusion in the MPC group (C) than in group A, B, or D. Computed tomography scan at 3 months revealed that 5 of 6 MPC-treated animals (83%) had continuous bony bridging compared with 0 of 5 AEC-treated and only 1 of 6 autograft- and 2 of 6 HA/TCP-treated animals (P = .01).

**CONCLUSION:** Implantation of allogeneic MPCs in combination with HA/TCP within an interbody spacer facilitates interbody fusion after diskectomy. The earlier, more robust fusion observed with MPCs relative to autograft and HA/TCP bone substitute indicates that this approach may offer a therapeutic benefit.

**KEY WORDS:** Amnion epithelial cell, Anterior cervical diskectomy and fusion, Biologics, Mesenchymal stem cell, Sheep, Spine fusion, Stem cells

**ABBREVIATIONS:** AEC, amnion epithelial cell; HA/TCP, hydroxyapatite/tricalcium phosphate; IVA, intervertebral angle; LA, lordosis angle; MPC, mesenchymal precursor stem cell
precursor cells within autographs decline with age, an issue that may reduce bone fusion in the elderly. Other factors that may independently decrease fusion rates include multilevel surgery, rheumatoid arthritis, smoking, and the concomitant use of antiinflammatory medications. If fusion does not occur, a pseudarthrosis results, commonly causing recurrent radiculopathy and neck pain that may require surgical revision.

Recombinant human bone morphogenetic proteins have been tried as osteoinductive agents in the cervical interbody space and have demonstrated increased fusion rates. Significantly, this advantage comes with an increase in complication rates of up to 24%, including life-threatening complications such as airway compromise.

The interbody cage (7.7 × 12 × 15 mm) was made from polyphosphate (HA/TCP; Mastergraft granules, Medtronic, Minneapolis, Minnesota) alone (group B; n = 6); HA/TCP containing 5 million MPCs (group C; n = 6); or HA/TCP containing 5 million AECs (group D; n = 5). The fifth group was made up of age-matched nonoperated controls (n = 6).

Allogeneic mesenchymal precursor stem cells (MPCs) are derived from donor bone marrow and were recently shown to increase bone fusion rates in other indications. Allogeneic amnion epithelial cells (AECs) are readily available from discarded afterbirth tissue; therefore, an invasive procedure such as bone marrow aspiration is not required to harvest them. AECs are derived from epiblast cells before gastrulation that migrate along the walls of the amniotic cavity to form the amnion epithelium. It has been hypothesized, therefore, that they retain pluripotency and are able to be differentiated down all 3 germ cell lineages. In relation to the present study, AECs are reported to show significant osteogenic differentiation potential.

Both AECs and MPCs exhibit low immunogenicity and antiinflammatory properties, which diminish their potential to elicit an immune response when transplanted to an allogeneic host. Human AECs fail to induce a xenogeneic mixed lymphocyte reaction in animal models and exhibit minimal major histocompatibility complex I and II expression, supporting the use of AECs xenogeneically. In the present study, we compared the capacity of allogeneic ovine MPCs with human AECs to promote cervical interbody fusion in an ovine model of cervical disectomy.

METHODS

Study Design

Twenty-nine 2-year-old Boarder-Leicester/Merino ewes were divided randomly into 5 groups. Four groups were subjected to C3-4 anterior cervical disectomy and prepared for fusion with a Fidji interbody cage (Abbott Spine, Austin, Texas). The C3-4 segment was used because of its similarities to the human cervical spine. The interbody cage was packed with iliac crest autograft alone (group A; n = 6); hydroxyapatite/tricalcium phosphate containing 15% hydroxyapatite and 85% tricalcium phosphate (HA/TCP; Mastergraft granules, Medtronic, Minneapolis, Minnesota) alone (group B; n = 6); HA/TCP containing 5 million MPCs (group C; n = 6); or HA/TCP containing 5 million AECs (group D; n = 5). The fifth group was made up of age-matched nonoperated controls (n = 6).

Mesenchymal Precursor Cells

Allogeneic ovine MPCs (Mesoblast Ltd, Melbourne, Australia) were isolated from bone marrow of outbred sheep using immunoselection with monoclonal antibodies and STRO-3+ and manufactured by Lonza Inc (Walkersville, Maryland) under good manufacturing practice guidelines. The MPCs were derived from a single batch and culture expanded to passage 4. The MPC surface marker characteristics have previously been reported. The optimal dose and matrix combination were determined previously by our group.

The cells were frozen and maintained in the vapor phase of a liquid nitrogen tank until thawed and used within 30 minutes. Cellular viability was determined before implantation using Trypan Blue exclusion and was > 85%.

Amnion Epithelial Cells

After institutional ethics approval (Monash Medical Centre, Clayton, Australia) and patient informed consent were obtained, discarded term placenta were collected from elective caesarean section deliveries. The cells were harvested by the method previously described, with autologous blood and then frozen and maintained in the vapor phase of a liquid nitrogen tank until thawed and used within 30 minutes. Cellular viability was > 80% using Trypan Blue exclusion. The cells were characterized with monoclonal antibodies and flow cytometry as previously described (Figure 1).

Interbody Cage and Carrier

The interbody cage (7.7 × 12 × 15 mm) was made from polyetheretherketone and was packed with iliac crest autograft cancellous bone in the autograft control group. The HA/TCP carrier was mixed with autologous blood and then packed into the cage in the HA/TCP alone group. In the cell-treated groups, the MPCs or AECs were added to the carrier within the cage and allowed to soak into the granules, avoiding spillage (Figure 2).

Surgical Technique and Postoperative Care

The surgical procedure has been previously described and was carried out with institutional ethics approval (School of Biomedical Sciences, Monash University). Animals were fasted 12 to 24 hours before surgery and were allowed water ad libitum. Sheep were anesthetized by intravenous injection of thiopentone 20 mg/kg and anesthesia maintained by isoflurane (1%-3%) inhalation and were positioned supine on the operating table. Local anesthetic (0.5% Bupivicaine with 1:200 000 adrenaline) was injected before a right anterolateral approach through a longitudinal neck incision. The longus colli muscle was elevated bilaterally with diathermy, and the position of the C3-4 level was confirmed with fluoroscopy. Distraction was achieved with 16-mm Caspar pins followed by a total disectomy and removal of the cartilaginous end plates with a high-speed drill to reveal bleeding bone. The posterior longitudinal ligament was opened until the dura was visualized to directly simulate the clinical procedure and to investigate any effects of the cells around the neural elements. In sheep receiving autograft, the left iliac crest cortical bone was elevated, and cancellous bone was curetted and packed into the cage. All cages were inserted and countersunk by approximately 3 mm. The longus colli muscle was then approximated by suture, followed by layered closure and subcuticular suture to skin.

A fentanyl patch was administered postoperatively. After 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 regular observations were made. The sheep were allowed to graze ad libitum and supplemented with Lucerne chaff. Clinical pathology was performed on days 0 and 1 and 3 months after surgery for...
FIGURE 1. Characterization of human amnion epithelial cells by flow cytometry.
Radiographic Analysis

Fusion was assessed by plain and functional radiography, multislice thin-cut (0.6-mm) computed tomography (CT), and quantitative CT. Fusion was defined by continuous bridging of trabecular bone and the absence of radiolucent lines at 3 months on CT. Continuous bridging of trabecular bone was further subdivided into < 30% or > 30% of the interbody cage area as assessed by a blinded consultant radiologist using a semiquantitative score (see the Table and Figure 3). All animals had plain lateral and selected anteroposterior digital radiographs (Radlink, Atomscope HF 200A, Redondo Beach, California) of the cervical spine preoperatively, within 24 hours after surgery, and at 1, 2, and 3 months postoperatively. This was performed under sedation using medetomidine (0.025 mg/kg IV) and reversal with atipamezole (0.125 mg/kg IV).

Functional radiography was conducted by the method of Kandziora et al. After the sheep were euthanized, the superficial musculature of the neck and thorax was resected to expose the anterior and posterior cervical spinal segments for each degree of movement. Total displacement of the C3-4 joint was calculated from these curves.

Postmortem Analysis

Clinical veterinarians performed comprehensive autopsies in a blinded fashion. Samples from all organs and tissues from the perisurgical site were reviewed by a blinded, board-certified veterinary pathologist.

Histomorphological, Histomorphometric, and Fluorochrome Analyses

The fluorochromes calcein green 10 mg/kg, oxytetracycline 50 mg/kg, and alizarin complexone 30 mg/kg were administered intravenously at 3, 6, and 9 weeks, respectively. After the sheep were euthanized, the C3-4 segment was excised and fixed in 10% normal buffered formalin.

Undecalcified bone histology was performed as previously described. Blocks were dehydrated in ascending concentrations of ethanol under agitation and then cleared in butanol before embedding in glycolmethacylate (Technovit 7100; Kulzer, Wehrheim, Germany) following a slow embedding and hardening protocol.

For fluorochrome analysis, the midsagittal section was ground to approximately 40-μm thickness with a diamond blade Macrotome (MR Ltd, Cambridge, United Kingdom). For light microscopy, the 10-μm sections were cut with a sledge microtome (Leitz, Wetzlar, Germany). Histological sections were stained with hematoxylin and eosin, Safranin-O/light green, von Kossa, Alcian Blue, and Masson-Goldner, the last stain being used for histomorphometric analysis.

Slides were scanned with the Olympus dot slide System (with a BX51 microscope) at ×2 magnification for each fluorescent label using
U-MNIBA3, U-WIG3, and U-MWU2 filters and a Peltier-cooled high-sensitivity camera at consistent exposure. The images were then uploaded into the Metamorph quantitative analysis software program (version 7.6; Molecular Devices, MDS Inc, Sunnyvale, California), and the area within the cage was identified and demarcated with the Metamorph proprietary software package. The intensity of each fluorescent label within the cage was measured and expressed as intensity per unit area (square microns). This gave a quantitative assessment of the amount of bone deposition at each time point.

A certified veterinary pathologist performed a semiquantitative analysis of the histological sections in a blinded manner. A score, using the criteria of Zdeblick et al. to assess fusion was assigned separately to the cage-vertebra interface and the tissue inside the cage using the following system: empty (score 0), fibrous tissue (score 1), and bone (score 2). Four points indicate a successful fusion, and 3 points represent a developing fusion.

Histomorphometric analysis was performed with quantitative image analysis for percentage of osteoid formation relative to mineralized bone within the cage area. This was conducted on the midsagittal slice stained with Masson-Goldner in a blinded fashion. Consistent inclusive threshold mapping was then used to measure the threshold percentage within the total cage area, within the section, containing red staining regions representing osteoid volume and green staining regions representing mineralized bone volume, respectively (Figure 6).

Statistical Analysis
Comparison of nonparametric data was evaluated by the Kruskal-Wallis test on the median values followed by the Dunn multiple-comparison test when significant differences were observed. Parametric data were analyzed with 1-way analysis of variance followed by the Dunnett multiple-comparison test when significant differences were observed. The 2-tailed Student t test was used for comparison of parametric data, and the Fisher exact test was used for contingency data. Prism 5.0 (Graph Pad Software) was used for the statistical analysis. Values are expressed as means and ranges unless otherwise stated. All data figures show mean ± SD unless otherwise specified. A value of $P < .05$ was considered statistically significant.
RESULTS

Adverse Events

No cell-related adverse events were observed during the study or at postmortem. One ewe in the MPC group developed pneumonia and hypoproteinemia that resolved under close veterinary treatment. Two ewes, both from the autograft group, lost significant weight during the study period. One ewe in the AEC group had anterior protrusion of the cage by approximately 5 mm. Veterinary assessment, clinical pathology, and gross pathological and histopathological analyses showed no differences between groups.

Radiographic Results

By CT scan at 3 months, 5 of 6 MPC-treated animals (83%) had continuous bony bridging compared with no bony bridging in any of the AEC-treated animals \( (P = .01) \). In the control animals, there was continuous bony bridging in 1 of 6 autograft-treated animals and 2 of 6 animals treated with HA/TCP alone (Figure 7). This finding was confirmed by objective quantitative
CT, which revealed that MPC-treated animals had significantly higher bone callus density compared with AEC-treated animals ($P = .01$; Figure 9).

Functional radiography scores (Figure 8) showed a reduction in the IVA in all operated groups compared with nonoperated controls ($P = .0003$). MPC-treated animals had a significant reduction in IVA compared with HA/TCP ($P = .01$) and autograft ($P = .03$). The IVA was also significantly reduced ($P = .005$) in MPC-treated animals compared with AEC-treated animals, and there was no reduction in the IVA of AEC-treated animals compared with either operated control.

The LA was significantly reduced in cell-treated animals compared with nonoperated controls ($P = .02$). MPC-treated animals had a significantly reduced LA compared with HA/TCP ($P = .02$) but not compared with autograft- or AEC-treated animals; in addition, LA was not reduced in AEC-treated animals compared with operated controls.

There was no radiological evidence of ectopic bone formation posteriorly around the spinal canal or at the neural exit foramina in any animals. Bone callus formation was noted anterior to the interbody space extending immediately above and below the anterior aspects of the vertebral bodies. This was found at a mean maximal distance of 6.58 mm anterior to the cage in the AEC group compared with 3.84 mm in the MPC-treated group ($P = .03$). Similar reactive bone formation was seen in the autograft and HA/TCP controls with a mean of 6.10 and 6.68 mm, respectively. There was no evidence of calcification within the muscles or ligaments anterior to the spine.

### Biomechanical Results

Significantly more stiffness at the C3-4 level was observed in the operated groups compared with nonoperated controls in flexion ($P < .015$); however, this did not achieve significance in extension or lateral bending. No significant difference between operated groups was observed in any of the 4 degrees of motion.

### Histological Results

There was no evidence of inflammatory or neoplastic changes in any specimen within the fusion area as reviewed by the veterinary pathologist. The semiquantitative histological scoring system revealed that 5 of 6 of the MPC-treated animals (83%) had either a developing or complete fusion compared with 2 of 5 AEC-treated animals (40%). In the controls, 3 of 6 (50%) treated with HA/TCP alone and 1 of 6 (17%) in the autograft group had a developing or complete fusion. This finding was supported by the histomorphometric analysis (Figure 10), which demonstrated significantly more mineralized bone in the MPC-treated group compared with the AEC-treated group ($P = .04$). There was a significant difference between the MPC-treated group and autograft-treated group ($P < .0001$) and between the MPC group and the HA/TCP group ($P = .06$). There was, however, no difference between the AEC-treated group and either control.

At 3 weeks postoperatively, fluorochrome analysis showed more prominent deposition of mineralized bone in the MPC-treated group compared with the AEC-treated group ($P = .03$). Although there was no significant difference between the cell-treated groups at 6 weeks, by 9 weeks, significant differences were observed for the MPC-treated group compared with the AEC-treated group ($P = .05$)

### DISCUSSION

These results demonstrate the safety of allogeneic implantation of both human AECs and ovine MPCs when implanted anteriorly into the cervical interbody space. The low immunogenicity and antiinflammatory properties of these cells have previously been described$^{18,21}$; however, to the best of our knowledge, the present study is the first to test both cell types within the cervical interbody space adjacent to the spinal cord, trachea, esophagus, and other neck structures.

Despite the safety profile common to both cell types, efficacy in facilitating new bone formation was distinct. The multiple modalities of assessment used in this study revealed significantly improved fusion with MPCs compared with both autograft and
HA/TCP controls, which represent the current standard of care, and animals implanted with AECs. At the 3-month time point, no animals implanted with AECs had bridging of trabecular bone between the operated vertebral bodies, and the AECs appeared to have a negative effect on fusion. The fusion results with MPCs were similar to those reported for growth factors, including bone morphogenetic protein, when applied to an ovine cervical model at the 3-month end point. Other studies in lumbar posterolateral or interbody spinal fusion have shown superior effects with bone morphogenetic proteins.

Human amnion-derived cells have been evaluated in a variety of animal models of human disease without any evidence of xenogeneic reactions. Because of the difficulties inherent with undecalcified tissue, it was not possible to definitively identify human AECs in the histological sections in the present study; however, the identification of human AECs in tissues in other animal models and the lack of inflammation at the perioperative site in the present study suggest that the inability of AECs to contribute to interbody fusion is unlikely to have a xenogeneic basis.

The different techniques used for isolation of human AECs and MPCs could account for their difference in efficacy. The monoclonal antibody selection technique used for MPC isolation yields a homogeneous population of cells with batch-to-batch consistency. In contrast, enzymatic digestion, followed by density gradient and plastic adherence selection of the AECs, yields a heterogeneous population of cells, which introduces variability between batches. Although the cell marker characterization of the batch of cells used in the present study (Figure 1) closely resembles the characteristics of other batches of AECs and is similar to the cell surface marker profile defining mesenchymal stem cell populations, it is possible that different results may have been obtained with another batch of AECs. Studies by Bilic et al have demonstrated different outcomes in osteogenic differentiation of AECs between batches. Further work in identifying the characteristics of those AECs with highest osteogenic potential and then improving selection of this subpopulation should be undertaken before further testing this cell type for the present indication. It is also possible that a higher dose of AECs in vivo may have a superior effect on fusion. Alternatively, it is possible that AECs may be inhibitory to fusion altogether. Should this be the case, a fusion inhibition effect would be beneficial in other applications such as motion preservation. Further studies with AECs in these applications are underway.
The bone formation observed anterior to the cage was similar to that shown in previous preclinical studies. Zdeblick et al suggested that the countersinking of the cages, as was undertaken in the present study, results in exposed reamed bone lacking cartilaginous endplates that acts as a source for new bone formation. It is also possible, however, that the mechanism of this bone formation is related, in part, to the instability of the vertebral joints, as has been invoked for the development of osteophytes in cervical spondylolisthesis. In the present study, there was significantly more anterior bone formation in the AEC group compared with the MPC group. The former group failed to fuse, and functional assessment revealed increased mobility, which may account in whole or in part for this difference.

The beneficial effects on fusion resulting from the MPCs combined with HA/TCP observed in this study are possibly due to 2 separate mechanisms of action: direct osteogenic differentiation of the MPCs at the fusion site, or a paracrine effect on endogenous osteogenic precursors because the MPCs secrete many growth factors, including bone morphogenetic proteins. In combination with the HA/TCP, MPCs are therefore likely osteogenic, osteoconductive, and osteoinductive. Although autograft also possesses these 3 attributes, it is only weakly osteogenic and osteoinductive. The low number of osteogenic precursor cells or stem cells contained within fresh autogenous grafts is likely to account in whole or in part for this difference.

In addition to these fusion-enhancing properties, the immunological properties of the MPCs may have additional benefits. MPCs lack the ability to induce an allogeneic mixed lymphocyte reaction and secrete multiple antiinflammatory and immunosuppressive cytokines such as interleukin-10. These antiinflammatory properties could conceivably reduce radiculopathy or myelopathy, which are the common underlying problems necessitating cervical fusion. The potential beneficial properties of MPCs reported in this animal study support further evaluation in a human clinical trial.

CONCLUSION

MPCs facilitated a more robust fusion relative to controls representing the current standard of care in this study. In contradistinction, AECs reduced fusion. The osteogenic, osteoconductive, and osteoinductive potentials of MPCs obviate the donor-site morbidity associated with autograft harvest and the potential life-threatening complications associated with the use of bone morphogenetic proteins. The potential mechanisms by which MPC-mediated spinal fusion could include promotion of osteoblast differentiation and their secretion of trophic factors such as multiple bone morphogenetic proteins and other growth factors, which are known to upregulate osteogenesis.

Disclosure

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COMMENTS

Multiple materials have been studied to replace the use of autograft implanted by itself or in a vehicle in the interbody space after cervical disectomy. As the authors mention, autograft has many advantages, but obtaining the graft is associated with its own disadvantages. Consequently, the authors studied mesenchymal precursor cells (MPCs) and amnion epithelial cells (AECs) placed in interbody cages. They studied 5 groups of 2-year-old sheep undergoing the following treatments: (1) iliac autograft placed alone in an interbody cage (n = 6), (2) hydroxyapatite/tricalcium phosphate (HA/TCP) (n = 6), (3) HA containing MPC (n = 6), (4) HA and TCP containing AECs (n = 5), and (5) HA nonoperative control (n = 6).

At 3 months, the animals were euthanized, and the spines were studied with computed tomography, functional radiography, spinal mechanics, histology, and histomorphometry. Implantation of the allogeneic MPCs in combination with the HA/TCP in the interbody spacer facilitated interbody fusion. Interestingly, no fusion occurred in the AEC group. Consequently, the authors suggest that MPCs in an interbody cage might be used as an alternative for autograft. The authors found no undue reactions to either the MPCs or AECs. These observations are quite interesting. It is to be hoped that further studies by these authors or others will clarify the benefit of MPCs in promoting fusion.

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Goldschlager et al used an ovine model of anterior cervical disectomy and fusion at C3-4 to evaluate the capacity of allogeneic mesenchymal stem cell MPCs and human allogeneic amnion epithelial cells AECs in promoting cervical fusion compared with autograft or Mastergraft alone. Polyetherurethane cages of the same size were used in all experimental groups. The authors monitored all animals for adverse effects such as heterotopic bone formation, neoplastic transformation of transplanted cells, immunogenic reaction of host animals, and surgical complications and used radiographic, biomechanical, and histomorphological assessment of bone fusions. The results demonstrate an excellent fusion-promoting capacity of the MPC-Mastergraft combination in contrast to lack of fusion in the...
AEC-treated animals. There were corresponding biomechanical changes in all fusion animals, especially in the MPC-treated animals, which had a significant reduction in intervertebral and lordotic angles, although change in stiffness was not significant. Histological assessment of fusion also confirmed the superiority of MPC treatment with regard to evidence of bone fusion.

This well-done study looks at the important question of whether allogeneic stem cells are viable alternatives to human bone morphogenetic proteins in promoting anterior cervical fusion without the complications encountered with the use human bone morphogenetic proteins. Further studies are required to understand the molecular mechanisms of the fusion-promoting properties of the MPCs compared with AECs.

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