



Manufacturing and use of human placenta-derived mesenchymal stromal cells for phase I clinical trials: Establishment and evaluation of a protocol

Proizvodnja i upotreba humanih mezenhimskih stromalnih ćelija izolovanih iz placente za klinička istraživanja prve faze: uspostavljanje i procena protokola

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Abstract

Background/Aim. Mesenchymal stromal cells (MSCs) have been utilised in many clinical trials as an experimental treatment in numerous clinical settings. Bone marrow remains the traditional source tissue for MSCs but is relatively hard to access in large volumes. Alternatively, MSCs may be derived from other tissues including the placenta and adipose tissue. In an initial study no obvious differences in parameters such as cell surface phenotype, chemokine receptor display, mesodermal differentiation capacity or immunosuppressive ability, were detected when we compared human marrow derived-MSCs to human placenta-derived MSCs. The aim of this study was to establish and evaluate a protocol and related processes for preparation placenta-derived MSCs for early phase clinical trials. **Methods.** A full-term placenta was taken after delivery of the baby as a source of MSCs. Isolation, seeding, incubation, cryopreservation of human placenta-derived MSCs and used production release criteria were in accordance with the complex regulatory requirements applicable to Code of Good Manufacturing Practice manufacturing

of *ex vivo* expanded cells. **Results.** We established and evaluated instructions for MSCs preparation protocol and gave an overview of the three clinical areas application. In the first trial, MSCs were co-transplanted *in vivo* to patient receiving an allogeneic cord blood transplant as therapy for treatment-refractory acute myeloid leukemia. In the second trial, MSCs were administered *in vivo* in the treatment of idiopathic pulmonary fibrosis and without serious adverse effects. In the third trial, MSCs were injected directly into the site of tendon damage using ultrasound guidance in the treatment of chronic refractory tendinopathy. **Conclusion.** Clinical trials using both allogeneic and autologous cells demonstrated MSCs to be safe. A described protocol for human placenta-derived MSCs is appropriate for use in a clinical setting, relatively inexpensive and can be relatively easily adjusted to a different set of regulatory requirements, as applicable to early phase clinical trials.

Key words:
stromal cells; therapeutics; clinical protocols; clinical medicine.

Apstrakt

Uvod/Cilj. Mezenhimske matične (stromalne) ćelije (MSCs) trenutno se koriste u velikom broju kliničkih istraživanja za različite kliničke indikacije. Iako je koštana srž uobičajeni izvor početnog materijala za kultivaciju ovih ćelija, količina ćelija koja se pri tome dobija i dalje predstavlja ograničavajući faktor. Alternativno, MSCs sve više se izoluju iz drugih tkiva kao što su placenta novorođenih beba ili masno tkivo. U inicijalnom istraživanju nije otkrivena nikakva razlika u osnovnim fenotipskim karakteristikama ćelijskih receptora, hemokinskih receptora ili sposobnosti ćelija za normalnu mezodermnu diferencijaciju između MSCs izolo-

vanih iz placente i koštane srži. Cilj ovog rada bio je uspostavljanje i procena protokola za kultivaciju i odabir MSCs izolovanih iz placente i adekvatno pripremljenih za upotrebu u kliničkim istraživanjima prve faze. **Metode.** U ovoj studiji korišćena je placenta beba rođenih carskim rezom nakon normalne trudnoće. Izolacija, zasejavanje, inkubacija, krioprezervacija i kriterijumi za proizvodnju humanih MSCs bili su u skladu sa složenim regulatornim principima koji se u ovom trenutku primenjuju u Australiji. **Rezultati.** Uspostavljen je i procenjen detaljan protokol za pripremu MSCs i dat je pregled njihove upotrebe u tri različite kliničke studije. U prvoj studiji MSCs su date *in vivo* putem, pre alogene transplantacije matičnih ćelija krvi, u lečenju akutne mijeloidne

leukemije refraktorne na terapiju. U drugoj studiji, MSCs su date *in vivo* u lečenju idiopatske fibroze pluća, bez ozbiljnih neželjenih efekata. U trećoj studiji, MSCs su injektovane direktno u mesto oštećenja tetive pod kontrolom ultrazvuka, u lečenju hronične refraktorne tendinopatije. **Zaključak.** Kliničke studije, bazirane na primeni ćelija alogenog i autolognog porekla, demonstrirale su bezbednu upotrebu MSCs.

Prikazani protokol je pogodan za rane faze kliničkih istraživanja, relativno je pristupačan i može se lako prilagoditi različitim kliničkim uslovima i zakonskim regulativama.

Ključne reči:

ćelije, stromalne; lečenje; protokoli, klinički; medicina, klinička.

Introduction

Although stem cells and their differentiated progeny offer great promise for treatment of many congenital and acquired human diseases, the optimal type and source remain unclear^{1, 2}. Mesenchymal stromal cells (MSCs), derived from rare mesenchymal stem cells, are fibroblastoid cells that are present in the bone marrow and virtually all other tissues of the body and which can be readily isolated and expanded *ex vivo*³⁻¹⁰. MSCs are able to differentiate *in vitro* into cells of the mesodermal lineage such as osteoblasts, chondrocytes and adipocytes, but their ability to differentiate to cells outside the mesodermal lineage such as hepatocytes, endothelial cells and neuronal cells is controversial^{1, 2, 6}. MSCs are also able to modulate the activity of cells of the immune system. Preclinical studies to date have provided a significant amount of information on MSCs indicating that they can deliver therapeutic proteins in a paracrine fashion or participate in the repair of defects by mesodermal differentiation³⁻¹⁰. The same studies emphasize the potential of MSCs *in vivo* and their capacity to home to sites of tissue injury and inflammation. MSCs have the potential for cell banking^{5, 11}. With no need to match a donor and intended recipients for the antigens of the major histocompatibility complex (MHC), MSCs from a single manufacturing campaign can be utilised in numerous clinical trials and for a number of patients^{5, 11}. However, MSCs manufacture is a highly specialized, rather complex, time consuming and labour-intensive exercise¹¹⁻¹³. The placenta is a natural "waste product" at birth and can provide a practically unlimited supply of donor tissue^{6, 11, 12}. Human placenta-derived MSCs (also referred to as hpMSCs, in further text MSCs) need to be isolated, cultured and cryopreserved according to stringent regulatory requirements^{11, 12} including the current Code of Good Manufacturing Practice (cGMP) standard or its close equivalent^{11, 12}. The manufacturing process includes collection of source tissue (placenta), isolation of MSCs, and their subsequent processing, storage and transport^{2, 5, 11, 12, 14}. A set of policies and procedures is required to support the manufacturing process; this process needs to be based on the Quality Management System (QMS) principles, the International Organization for Standardization (ISO) standard and other complex regulatory requirements¹³⁻¹⁷. MSC from various sources have been used extensively in different disease models¹⁸⁻²⁰ and continue to be investigated for safety, efficacy and potency²¹⁻²⁴, their lifespan²⁵ as well as manufacturing protocol variations, improvements and adjustments²⁶⁻²⁸.

The primary aim of this paper was to establish a protocol for manufacturing/ preparation of clinical-grade human

placenta-derived MSCs that could be cell banked and used for various clinical applications on an "as needed" basis. Additionally, we aimed to evaluate the protocol and its feasibility in a clinical trial programme, in the areas of hematology (co-transplantation of human MSCs and allogeneic cord blood cells as therapy for treatment-refractory hematological malignancies), pulmonary medicine (intravenous administration of MSCs for idiopathic pulmonary fibrosis), and in a musculoskeletal application (local administration of MSCs for chronic refractory tendinopathies). The key parameters for evaluation of the protocol were: 1) manufacturing considerations of MSCs for use in clinical settings; 2) regulatory considerations related to MSCs manufacturing (*eg* could the protocol be relatively easily adjusted to comply with a different set of regulatory requirements as mandated for applications in early phase clinical trials in Australia or other comparable sets of regulations), and 3) safety, accessibility and usability of human placenta-derived MSC.

Methods

The material used for cell isolation, culture and cryopreservation is presented in Table 1. The placenta was collected from a healthy mother (the donor) during a routine term elective Caesarean section birth^{5, 6, 11}. A full informed consent was attained several weeks prior to the delivery^{5, 6, 11}. The donor selection guidelines of both the Australian Red Cross Blood Services and the Australian Cord Blood Registry (AusCord) were fulfilled^{11, 14}. A complex set of quality assurance system policies was applied to each step of the collection, processing, storage and transport of MSCs^{5, 6, 11}.

The placenta (Figure 1) was collected and double-bagged aseptically in the operating suite and transferred in a cool box to the processing laboratory^{5, 6, 11}. The external membranes and the umbilical cord were removed from the placenta in a biosafety flow-cabinet using an aseptic technique. The placental tissue was divided into smaller pieces (up to 10 g each)^{5, 6, 11}. The small pieces of placental tissue were washed with 500 mL/100 g of Hanks Balanced Saline Solution (HBSS, Invitrogen)⁶ and this process was repeated with the same volumes of HBSS for several times.

Additional details on MSCs isolation, seeding, incubation and cryopreservation are described in Table 2. Each passage consisting of 90 tissue culture flasks (T175) yielded between 4×10^8 and 1×10^9 cells. At the end of each passage (P2 to P5), cells were reseeded and also cryopreserved for clinical trial use and further testing⁶. The MSCs intended for cryopreservation were washed with 50 mL HBSS twice, centrifuged and the pellet was resuspended in 65% Plasma-

Table 1
Materials used for human placenta-derived mesenchymal stromal cells isolation, culture and cryopreservation^{5, 6, 11}.

Equipment and solutions	Media and reagents (1)	Media and reagents (2)
<ul style="list-style-type: none"> - On-demand label printer (Birch) - Weighing balance (Sartorius) - Heat Sealer CRG (Ljungberg and Kogel AB) - Bench Centrifuge (Sigma) - Cryocyte Freezing bags, 50 mL (Baxter) - Controlled-rate freezer (Planar) - Sterile surgical gowns, face masks, over-shoes and hair nets (Kimberley-Clark) - 0.2 µm sterile filter (Becton Dickinson) - Incubated Orbital Shaker (Bioline) - 70 µm cell strainer (BD Pharmingen) - Centrifuge tubes, 50 mL, conical bottom (Nunc) - FACS tubes (BD Pharmingen) - Syringes (60 mL) (Terumo) - Mixing canula (Unomedical) - Seriological pipettes; 5, 10 and 25 mL (Sarstedt) and motorized pipetter. - Tissue culture flasks (175 cm² and 25 cm²) (Nunc) - Sterile 70% ethanol (Pharmacia) - Sterile water (Baxter) 	<ul style="list-style-type: none"> - Hanks Balanced Salt Solution (HBSS) (Invitrogen) - TrypLE Select (recombinant trypsin-like substitute) (Invitrogen). - Dulbecco's Modified Eagle Medium, low glucose 1g/100 mL (DMEM-LG, Invitro gen) - Trypan Blue (Invitro gen) - Gentamicin, 40 mg/mL (Pharmacia) - Ficoll-Paque Premium (GE Healthcare) - Fetal calf serum, Australian sourced (Invitrogen) - Digest Media: 50 mL Collagenase I stock (Worthington (stock is 0.2 µm filter sterilised 2000 U/mL colla genases in sterile RO-grade water) - Pulmozyme (recombinant cGMP grade DNase I) (Genentech) - Albumex 40 (40% human serum al bum, Australian Red Cross Blood Service) - Tissue culture (TC) medium (TCM): DMEM-LG supplemented with 20 % fetal bovine serum (Invitrogen) and 50 µg/mL gentamicin (40 mg/mL stock) 	<ul style="list-style-type: none"> - Bovine serum albumin (BSA)(Sigma) - Plasma-Lyte 148 Replacement In travenous Infusion (Baxter) - CryoSure-Dimethyl-sulphoxide (DMSO) (Wak Chemie) - Cryopreservation media: 65 mL Plasmalyte, 25 mL Albumex-20 and 10 mL DMSO (Note: to avoid pro tein precipitation always add Albumex-20 to the Plasmalyte and mix before adding DMSO) - 7-Amino-actinomycin D (7AAD) (BD Pharmingen) - mAbs: CD45APC-Cy7, CD73PE, CD105FITC CD45APC-Cy7, CD73PE, CD105FITC (BD Pharm ingen) - Calibrite fluorescent beads (BD Pharmingen) - Sterile phosphate buffered saline (PBS) (Invitrogen)

Table 2
Methods used for human placenta-derived mesenchymal stromal cells isolation, cell seeding, incubation and cryopreservation^{5, 6, 11}

Cell isolation	Cell seeding	Cell incubation and cryopreservation
<ul style="list-style-type: none"> - The pieces of tissue were finely diced and transferred to 50 mL centrifuge tubes (approximately 10 g per tube). The 16 x 50 mL tubes were used. Dulbecco's modified Eagle's medium-low Glucose (DMEM-LG; SACS Biosciences) with 100 U/mL collagenase, type I (Worthington Biochemical Corporation) and 5 µg/mL DNase I (Pulmozyme) was added to each tube up to a total volume of 25 mL. - Tubes were placed in an incubated shaker (37°C, 2 hr), then pulse spun at (540 g, 5 sec, 4°C) to remove large particulate matter and the cell suspensions were collected and filtered into 16 x 50 mL centrifuge tubes using 70 µm filters (BD Falcon). - 15 mL HBSS was added to the original tubes containing large particulate placental matter and the tubes were inverted several times to elute remaining loose cells into suspension⁶. Tubes were pulse centrifuged as before to pellet large particulate matter (540 g, 5 sec, 4°C), and cell suspensions were transferred to fresh tubes through 70 µm filters. 	<ul style="list-style-type: none"> - The cells were pelleted by centrifugation (540 g, 5 min, 4°C), the supernatant discarded and the cell pellets resuspended in 30 mL HBSS. 12 mL Ficoll-Paque™ Premium (1.073 g/mL) was then under-layered. Samples were centrifuged (540 g, 20°C, 20 min, no brake). - The cells at the interface of were transferred to 12 x 50 mL centrifuge tubes, HBSS as added to 50 mL and the cells were pelleted by centrifugation (540 g, 20°C, 10 min). The cells were resuspended in 10 mL DMEM media: (DMEM (1g/l glucose), 20 % FCS (Invitrogen, cat # 10099-141, Australian sourced), 50 µg/mL gentamicin (Pharmacia). - Cells were initially seeded into 8 T175 cm² tissue culture flasks (T175). The cells were cultured in 30 mL DMEM media (37°C, 5% CO₂, humidified incubator). The relatively high FCS concentration was used to maximise cell expansion rate. When the cells were about 90–99% confluent, the DMEM media was removed and flasks washed with 20 mL HBSS. 5 mL TrypLE select (a GMP grade trypsin-like substitute) (Invitrogen), was added to each flask and incubated (15 min, 37°C). 	<ul style="list-style-type: none"> - The cells were removed by pipetting HBSS across the monolayer. The cells were pelleted by centrifugation (350 g, 5 min, 4°C) and cell pellets resuspended in DMEM media. The cells were equally divided between 90 T175 flasks, each in a final volume of 60 mL DMEM media and further incubated (as above). This equated to an approximate cell concentration of 0.7–1.4 x 10⁴/mL (in our first two production runs). - The relatively large volume of 60 mL was used because the media was left on the cells for 6–7 days without being changed. When these were confluent after approximately 6–7 days, the cells were detached from the flasks. - The majority of cells were then cryopreserved, whilst a fraction of the cells were used to seed a further 90 T175 flasks. This procedure was repeated a further three times.

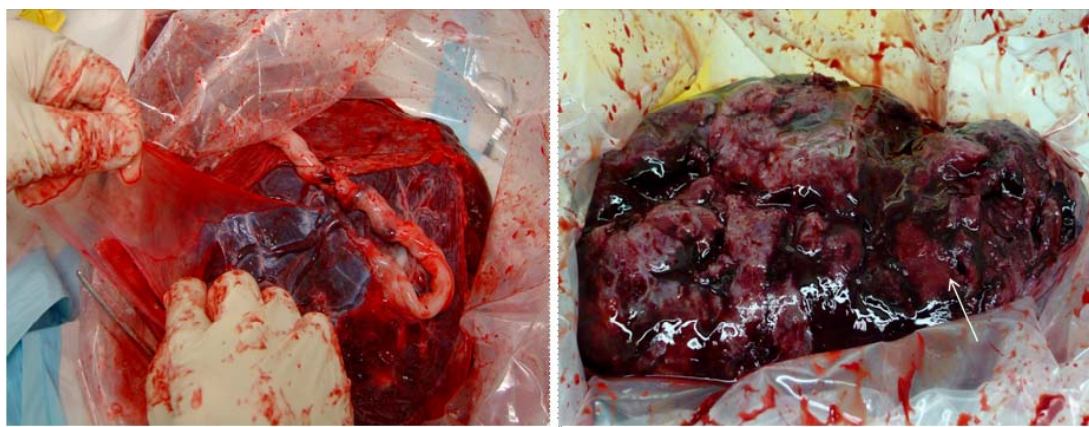


Fig. 1 – The human term placenta. (A) The fetal portion of the human placenta with the umbilical cord still attached. Mechanical separation of the fetal membranes, the amniotic membrane and the chorionic membrane is shown; (B) The portion of the placenta that embeds into the mother's uterus, the decidua. /Reproduced with permission from Heazlewood et al. ¹⁴.

lyte (Baxter Healthcare), 25% human serum albumin (Australian Red Cross) and 10% DMSO (Wak-Chemie Medical GmbH). The cells were frozen either in 50 mL freezing bags (Baxter Healthcare) or in 1.8 mL cryovials (Nunc) ⁶. A set of release criteria ⁶ is shown in Table 3.

the adipogenic differentiation was measured as cells were stained with Oil Red O ⁶. Reagents used for differentiation techniques were mostly obtained from Sigma ⁶ while mAb were manufactured by BD Pharmingen and prepared in PBS with 2% Human Serum Albumin (1 : 5 dilution) ⁶.

The production release criteria used for manufacturing human placenta-derived mesenchymal stromal cells ^{5, 6, 11, 14} **Table 3**

Test	Pre-donation	Passage 0	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5	Day 180**
Gram Stain	N/A	N/A	N/A	✓	✓	✓	✓	N/A
14 day microbiological culture	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Mycoplasma detection	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Endotoxin detection	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Purity (by FACS phenotype)	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Karyotype (by cytogenetic analysis)	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Donor serology*	✓	N/A	N/A	N/A	N/A	N/A	N/A	✓
Donor health questionnaire	✓	N/A	N/A	N/A	N/A	N/A	N/A	✓

* For infectious disease markers; ** Donor/mother and the baby follow-up; ✓ – applicable; N/A – not applicable.

The osteogenic differentiation was obtained while confluent MSCs were cultured for three weeks (solution: DMEM-HG, 10% FCS, 0.1 μ M dexamethasone, 50 μ M L-ascorbic acid-2-phosphate, 10 mM β -glycerol phosphate disodium salt pentahydrate and 0.3 mM sodium phosphate) ^{2, 5, 6, 11}. It was assessed by staining cells in wells with Alizarin Red S ⁶. The chondrogenic differentiation was also obtained and the cells (5×10^5 MSCs) were cultured over a 3-week period (solution: DMEM-HG, 0.1 μ M dexamethasone, 1 mM sodium pyruvate, 50 μ M L-ascorbic acid-2-phosphate, 35 mM L-proline, 10 ng/mL TGF β ₁ (R&D Systems) and 50 mg/mL ITS Premix (insulin, human transferrin and selenious acid; BD Biosciences) ⁶. Chondrogenic differentiation was evaluated by staining the cell pellets with periodic acid Schiff (PAS) ⁶.

Adipogenic differentiation was initiated and confluent MSCs were cultured for three weeks (solution: DMEM-HG, 1 μ M dexamethasone, 5 μ g/mL insulin, 60 μ M indomethacin and 0.5 mM 3-Isobutyl-1-methylxanthine, IBMX) ⁶. Level of

MSCs were detached from flasks using TrypLE select (Life Sciences), washed and added to FACS tubes in order to prepare them for flow cytometry analysis ⁶. MSC were incubated as follows: 1×10^6 cells in 100 μ L of mAb mix (15 min at 4°C) with directly conjugated mouse anti-human CD45-FITC, CD14-APC-Cy7, CD73-PE and CD105-APC antibodies ⁶. An additional tube with MSC was prepared and it was stained with 5 μ g/mL isotype control mAb (IgG1 FITC, IgG1 PE, IgG1 APC and IgG1 APC-Cy7) ⁶. The excess antibody was removed and cells washed with the phosphate buffered saline (PBS). After the last wash, cells were resuspended in the solution consisting of 200 μ L PBS and 7-Amino-actinomycin D(7AAD) (BD Pharmingen) (1 : 40 dilution) in order to exclude the dead cells. An LSRII flow cytometer (Becton Dickinson) was used and the data were analysed by FCS Express Version 3 software (DeNovo) ⁶. A defined threshold for fulfilling our MSCs purity criterion was set ($> 85\%$ CD73⁺/CD105⁺ (double positive), $< 1\%$ CD45⁺) ^{5, 6, 11}.

Results

Establishing the protocol

Manufactured human placenta-derived MSCs were very similar to human bone marrow-derived MSCs (hbmMSCs)^{2, 5, 6}. We were unable to find major differences between human placenta-derived MSCs and bone marrow-derived MSCs in terms of morphology (Figure 2) and cell surface

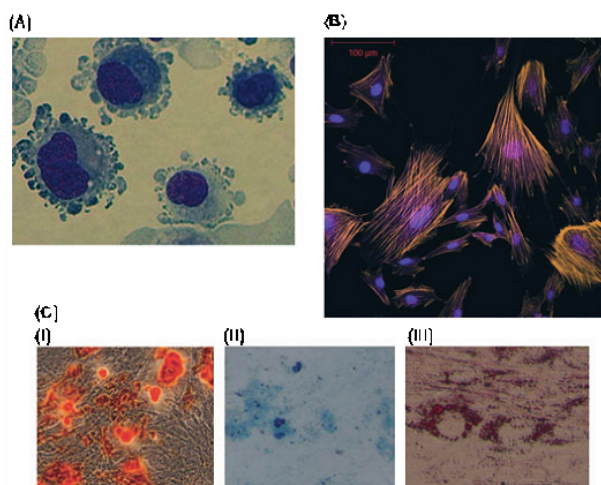


Fig. 2 – Photomicrograph showing morphology (Giemsa stain and Phalloidin stain) and mesodermal differentiation of human placenta-derived mesenchymal stromal cells (MSC). (A) Giemsa stain of human placenta-derived MSCs; (B) Morphology of human placenta-derived MSCs when stained with Phalloidin-AF546; (C) Osteogenic (i), chondrogenic (ii) and adipogenic (iii) differentiation and histological staining of human placenta-derived MSCs by alizarin red, Periodic acid schiff and Oil Red O, respectively.

Original magnification $\times 1000$ (A); $\times 400$ (B) and (Cii); $\times 100$ (Ci) and (Cii).

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phenotype^{2, 5, 6} (Figure 3), chemokine receptor display⁴, mesodermal differentiation², or immunosuppressive capacity on alloreactive T cells²⁹. Yen et al.¹⁷ established in their study that human placenta-derived MSCs expressed the early antigens SSEA4, Tra1-60 and Tra1-81, while the bone marrow derived MSCs lacked those antigens⁶. Conversely, we discovered a low level expression of SSEA4 and Tra1-60 on the human placenta-derived MSCs from our manufacturing process^{4, 6}.

All release from manufacturing criteria were fulfilled at the time of passage cryopreservation^{5, 6, 11} as presented in Table 3, including pre-donation serology screening, donor serology for infectious disease markers screening on day 180, pre-donation health questionnaire for the mother, and the day 180 health questionnaire for the mother and the baby^{5, 6, 11, 14}.

Evaluation of the protocol

Human placenta-derived MSCs manufactured at the Mater Medical Research Institute were used for three phases I (safety) clinical trials. These are briefly presented below.

First trial was a phase I multicentre open label dose-escalation study of unrelated, MHCs unmatched placenta-

derived MSCs in recipients of unrelated umbilical cord blood hematopoietic stem cell transplants.

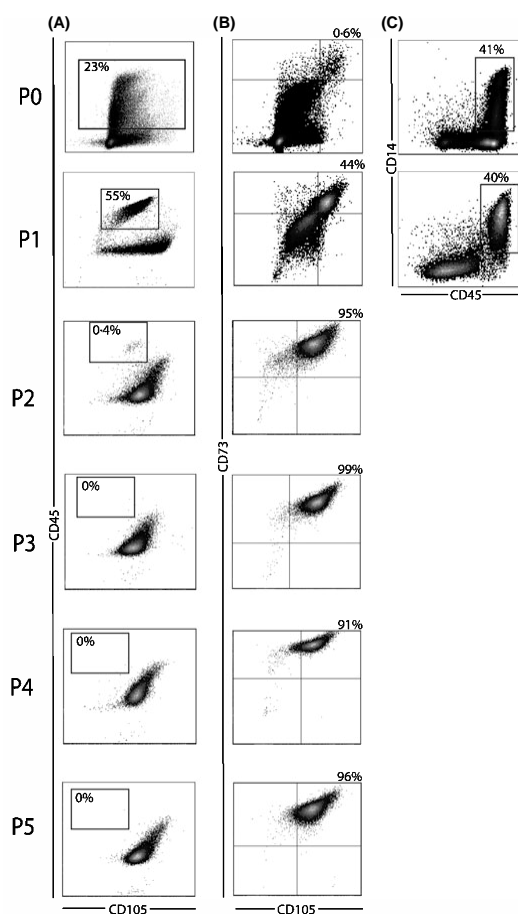


Fig. 3 – The flow cytometric phenotype of human-placenta derived mesenchymal stromal cells (MSCs) during isolation and passaging. (A) CD45⁺ frequency at each passage; (B) CD73⁺/CD105⁺ frequency at each passage; (C) CD14⁺/CD45⁺ frequency at early passage.

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In this phase I trial of safety and feasibility initiated in 2007 we co-transplanted human MSCs in a subject receiving an allogeneic cord blood transplant as therapy for treatment-refractory hematological malignancy. The trial was performed in collaboration with Professors Ken Bradstock and David Gottlieb of Westmead Hospital, Sydney. MSCs at a dose of 1×10^6 MSCs/kg were infused into the cord blood recipient 5 hours before infusion of cord blood cells⁶, in order to obtain a 5-hour window for observation of early adverse reactions due to MSCs (for example, infusional toxicity) unclouded by any possible side-effects from subsequent infusion of cord blood cells. The time from submission of the application until approval was received from the two institutional Human Research Ethics Committee (HRECs) involved was one year in total⁶. Two-way clinical trial agreements were put in place with an appropriate indemnification for each of the participating sites and for any adverse events attributed to MSCs. During this time, a request was made by one of the institutional HRECs for an external audit to be

conducted in the manufacturing processes outlined in the study protocol. This was performed by the staff of the Australian Red Cross Blood Service. We have conducted the two were external audits since then.

Clinical course ⁶: 24 hours after the pre-transplant myeloablative conditioning regimen completion (cyclophosphamide plus a total body irradiation), 100×10^6 human placental MSCs ($1 \times 10^6/\text{kg}$) were given intravenously to a 20-year-old Caucasian male with acute myeloid leukaemia in second remission ⁶. The cells were suspended in 30 mL and were infused over 7 min using a 200 μm in-line filter. No adverse events were noted. According to the study protocol, the patient received two allogeneic cord blood units five hours later. The total nucleated cell dose from two cord blood units was, post-thaw, $3.6 \times 10^7/\text{kg}$ while the total CD34⁺ cell dose was $1.2 \times 10^5/\text{kg}$ ⁶. The placenta-derived MSCs given to the patient were MHC-unmatched with both the recipient and the two cord units ⁶. MSCs donors and cord blood donors were unrelated to each other ⁶. At the day 70 posttransplant the patient developed cytomegalovirus (CMV) infection and he subsequently died from multiorgan failure ⁶. Of note, MSCs had been procured from a CMV-seronegative maternal donor. There were no reported serious adverse events related to the MSCs, including infusion toxicity or ectopic tissue formation. This trial was ceased when Westmead Hospital decided to no longer perform cord blood transplants in adult subjects. This was the first time, to our knowledge, that placenta-derived MSCs have been trialled in humans ^{6,11}.

Second trial was a phase I study to evaluate the potential role of MSCs in treatment of idiopathic pulmonary fibrosis.

In 2010 we initiated a phase I trial of MSCs in collaboration with Drs Daniel Chambers and Peter Hopkins of the Prince Charles Hospital in Brisbane. Idiopathic pulmonary fibrosis is a chronic, fibrosing lung disease of unknown cause predominantly occurring in middle-aged people that is characterized by severe, refractory and progressive breathlessness. There is no effective treatment for this disease except for lung transplantation, and this approach is limited by a shortage of lung organ donors. The rationale for this trial was based on literature reports of a beneficial effect noted in mice given murine MSCs after experimental pulmonary fibrosis was induced by the cytotoxic drug bleomycin.

Clinical course: MSCs were administered *iv* as follows: 1×10^6 MSCs/kg (first cohort of 4 subjects) and 2×10^6 MSCs/kg (second cohort of 4 subjects). A validated dry shipper was used to transport MSCs to the collaborating hospital. The cells were kept cryopreserved until required for administration to subjects in the trial. Subsequently, the cells were thawed and infused intravenously. There were no reported serious adverse events related to MSCs, including infusion toxicity or ectopic tissue formation. The results of this trial were of interest since it involved subjects with impaired pulmonary function. MSCs injected intravenously immediately home to the lungs where they remained for approximately three days and could theoretically cause further pulmonary function compromise. This did not happen.

Third trial was a phase I study to evaluate the potential role of MSCs in treatment of chronic refractory tendinopathy.

In 2011 the phase I was initiated in subjects with Achilles tendinopathy refractory to conventional treatments. In contrast to the first two trials MSCs were injected directly into the site of tendon damage using ultrasound guidance. The Principal Investigator on this trial was Dr Mark Young, Visiting Medical Officer in Sports Medicine at Mater Private Hospital, Brisbane. Tendinopathy is a common condition associated with pain and diminished function in tendons. It occurs in active young people, and the incidence increases with age. The incidence of tendinopathy increases along with the life expectancy, which in turn places larger costs on the health system. The initial management of all tendinopathies is conservative, including activity modification, medications, corticosteroid injections and/or exercises. Surgical treatments are considered if a prolonged conservative management fails, but these interventions are costly and involve periods of immobilisation.

Clinical course: All the patients received their *iv* MSCs injections under the ultrasound guidance. The subjects were monitored for a period of at least 4 weeks before the next patient is treated so that any early adverse effects from the previous MSCs application could be assessed – both clinically and by ultrasound examination. The 3 patients of the first cohort received a single dose of 1.0×10^6 placenta-derived MSCs (1 mL of solution with a total of 1.0×10^6 cells per mL) each. The next cohort of 3 patients received a total of 4.0×10^6 MSC (1.0 mL of a solution with a total of 4.0×10^6 cells per mL) each.

Each of the above trials was subjected to oversight by a Data Safety Monitoring Committee. The Data Safety Monitoring Committee carried out an interim safety analysis after each cohort of patients had received their injections and no adverse events attributed to MSCs administration were noted. The overall safety outcomes were encouraging since these phase I trials were not designed to assess efficacy of the biologic drug but its safety. Clinical trial design and clinical outcomes (other than safety), patient inclusion and exclusion criteria, patient follow-up, relevant controls and clinical parameters are beyond the scope of this paper.

Discussion

In this study we presented our experience in establishing a protocol for manufacturing/ preparation of clinical-grade human placenta-derived MSCs that can be cell banked and used for various clinical applications on an “as needed” basis. We also evaluated the protocol based on manufacturing considerations of MSCs for use in clinical settings, regulatory considerations related to MSCs manufacturing (*eg* could the protocol be relatively easily adjusted to comply with a different set of regulatory requirements as mandated for applications in early phase clinical trials in Australia or other comparable sets of regulations), and safety, accessibility and usability of human placenta-derived MSCs. There are several issues to be discussed.

Firstly, there are manufacturing protocol considerations. These include, but are not limited to, the amount and type of tissue used as a source of cells, choice of manufacturing reagents (eg clinical grade reagents) and variations of the laboratory protocol, as well as the conditions of the *ex vivo* cell expansion procedure (eg “open” vs “closed” system). In our first manufacturing campaign, we used a part (300–500 g) of one placenta (placenta 1). This represented over a half of the total placenta mass and was used to seed 6×10^5 T175 flasks (P0)⁶. It yielded approximately 40×10^6 cells at first passage (P1)⁶ which were then split between 90 flasks at 4.38×10^5 cells/flask and plated at the initial density of 2500 cells/cm². The average yield for each subsequent passage was 742×10^6 cells (with a standard deviation of 82.6×10^6)⁶. At each passage, 40×10^6 cells were held back for the next passage and seeded to a new set of flasks at 4.38×10^5 cells/T175, and the remaining cells were cryopreserved⁶. It was noted that cell recovery at P4 and P5 of the first processed placenta was only 60% although cell recoveries were generally excellent⁶. The noted variability may have been due to the use of small aliquots of cells stored in cryovials for counting purposes and testing only^{5, 6}. The yield from cryovials, in our experience, tends to be slightly lower than that from cryobags⁶. The placentas (1 and 2) processed in the first two manufacturing campaigns using clinical grade manufacturing protocols yielded a total of 4.5×10^9 cryopreserved cells. MSC were released from production¹¹ as 97% viable, Gram stain negative, endotoxin test < 2 EU/mL, sterile on 14 day microbiological culture, with an appropriate cell surface phenotype CD45⁻ and > 85% CD73⁺/CD105⁺ (eg in P2 the results of cell purity were: 0.4% CD45⁺, 95 % CD73⁺/CD105⁺; in P3: 0.0% CD45⁺, 99%CD73⁺/CD105⁺; in P4: 0.0% CD45⁺, 91% CD73⁺/CD105⁺; and in P5: 0.0% CD45⁺, 96% CD73⁺/CD105⁺) demonstrated by flow cytometry, and with normal karyotype^{5, 6, 11}.

A two-stage release process was applied with a set of criteria for each stage. Release from manufacturing included tests performed on each batch of cells immediately following manufacturing of each passage (eg sterility, mycoplasma and endotoxin test, purity, viability, maintenance of normal karyotype). Release from cryopreservation included tests performed on a cryovial corresponding to each bag planned for administration to a patient; the test-vials were thawed several weeks before the planned administration to a patient and sterility, viability and mycoplasma tests were performed.

In both first two manufacturing campaigns, a collagenase I based tissue digestion protocol was utilised. One of the major obstacles in the initial phase of MSCs manufacturing was a lack of cGMP grade reagents or clinical-grade reagents in the cell isolation steps⁶. Hence, the human recombinant DNase was used for digestion in our manufacturing process, which was produced by Roche under the cGMP conditions. It is intended for clinical use in cystic fibrosis treatment^{6, 11}.

Additionally in both our first two manufacturing campaigns the cell preparation (following tissue dissociation and digestion) was initially purified using density gradient centrifugation with Ficoll-Paque Premium™ (GE Healthcare).

This is an alternative to Percoll and it is a clinical-grade reagent^{6, 11}.

Use of media with fetal calf serum for cell expansion may increase the theoretical risk of bovine spongiform encephalitis transmission. The Australian regulatory body for medicines, the Therapeutic Goods Administration, (TGA, equivalent of the Food and Drug Administration [FDA] in the USA and the European Medicine Agency [EMA] in Europe¹⁵) approves in principal the use of fetal calf serum in clinical grade material production – as long as it is sourced from a country free of bovine spongiform encephalitis, such as Australia or New Zealand^{6, 11}. Most of the protocols use 10–20% fetal calf serum (FSC) growth media^{24–28} but serum-free media is a preferred option from the safety perspective.

At this stage our process of MSCs manufacture is still conducted in an “open” system due to the multiple steps required in MSCs extraction and culture. The extended period of cell expansion (up to 6 weeks) introduces the risk of microbial contamination although class II biosafety cabinets and/or clean rooms are utilised,^{6, 11}. Hence, an extensive in-process and end-product testings are utilised prior to release of the cell product for clinical use^{6, 11}.

Secondly, there is consideration of regulatory requirements related to MSCs manufacturing for use in early stage clinical trials. All stages of the manufacturing process were completed in accordance with cGMP principles and internal Quality Management System policies and procedures. A Quality Management System includes cGMP (or equivalent standard)-compliant policies, procedures and extensive documentation applies, but is not limited, to the following: facilities, equipment, materials, staff, monitoring, validation, process change, record generating and record keeping^{5, 6}. Production scientists had to work closely with the regulatory compliance staff to ensure a safe cellular therapy product^{11, 13, 14}. Staff education and ongoing training were crucial components of regulatory compliance. Overall, biologic drugs (also referred to as biologics, biologicals or Advanced Therapy Medicinal Products) regulations have been widely developed by mature regulatory agencies such as the Australian framework administered by the TGA, the European Union’s EMA levied framework and the framework of the United States of America (USA) imposed by the FDA¹⁵. All of these biologic drugs regulations are multilayered and complex^{15, 16}. However, common underlying principles of cGMP, provide a useful guide in each regulatory domain applicable to biologic drugs, including cell and tissue based therapeutics^{15, 16}.

Finally, there are considerations related to the safety, accessibility and usability of human placenta-derived MSCs. Adult MSCs can be derived from different source-tissues and can be expanded in culture while maintaining their characteristics. MSCs are currently extensively used in preclinical and clinical studies, including tissue engineering. There is a considerable promise for the use of MSCs in rebuilding damaged or diseased mesenchymal tissues in different tissue-engineered models^{8–10}. The fact that MSCs secrete a large spectrum of bioactive molecules was intriguing¹⁰. The im-

munosuppressive molecules produced by MSCs, especially those affecting the T-cell immune response, are now considered to be one of the mechanisms by which MSC mediate their therapeutic effect in some disease settings^{18–23}. MSC-secreted bioactive molecules may be able to provide a regenerative microenvironment for a variety of injured adult tissues and limit the area of tissue damage¹⁰. Hence, a number of clinical trials currently use allogeneic MSCs for the treatment of Crohn's disease, myocardial infarcts, graft-versus-host disease, cartilage and meniscus repair, spinal cord injury, stroke and other clinical indications^{18–23}.

The placenta and other pregnancy-related tissues appear an attractive source of MSCs^{7, 11–14, 17, 21, 25, 28}. Cells can be prepared in advance, for a number of patients or clinical trials, on a therapeutic scale manufacturing and cell banking process. Placenta, for example, is normally discarded after the birth of the baby, thus providing a practically unlimited supply of source material. Placental MSCs exhibit the classical MSCs surface phenotype, differentiation potential and have potent immunosuppressive properties^{2, 6, 14, 17}. Placental MSCs have been utilised by a number of other research groups worldwide¹².

Conclusion

Clinical trials using both allogeneic and autologous cells have demonstrated MSCs to be safe and have targeted diseases in areas such as orthopaedic, cardiovascular, degenerative and inflammatory diseases. Phase I clinical trials are a challenging step in this process due to limited funding, laborious and long manufacturing procedures and the need for a multidisciplinary team with a unique skill set. We described a manufacturing protocol for human placenta-derived MSCs that is appropriate for use in a clinical setting, relatively inexpensive and can be relatively easily adjusted to a different set of regulatory requirements, as applicable to early phase clinical trials.

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