Safety and Feasibility for Pediatric Cardiac Regeneration Using Epicardial Delivery of Autologous Umbilical Cord Blood-Derived Mononuclear Cells Established in a Porcine Model System

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Key Words. Safety • Autologous umbilical cord blood • Congenital heart disease • Porcine • Right ventricle • Intramyocardial delivery

ABSTRACT

Congenital heart diseases (CHDs) requiring surgical palliation mandate new treatment strategies to optimize long-term outcomes. Despite the mounting evidence of cardiac regeneration, there are no long-term safety studies of autologous cell-based transplantation in the pediatric setting. We aimed to establish a porcine pipeline to evaluate the feasibility and long-term safety of autologous umbilical cord blood mononuclear cells (UCB-MNCs) transplanted into the right ventricle (RV) of juvenile porcine hearts. Piglets were born by caesarean section to enable UCB collection. Upon meeting release criteria, 12 animals were randomized in a double-blinded fashion prior to surgical delivery of test article (n = 6) or placebo (n = 6). The UCB-MNC (3 × 10^6 cells per kilogram) or control (dimethyl sulfoxide, 10%) products were injected intramyocardially into the RV under direct visualization. The cohorts were monitored for 3 months after product delivery with assessments of cardiac performance, rhythm, and serial cardiac biochemical markers, followed by terminal necropsy. No mortalities were associated with intramyocardial delivery of UCB-MNCs or placebo. Two animals from the placebo group developed local skin infection after surgery that responded to antibiotic treatment. Electrocardiographic and echocardiographic assessments did not reveal arrhythmias in either group throughout the 3-month study. The safety profile establishes the foundation for cell-based therapy directed at the RV of juvenile hearts and aims to accelerate cell-based therapies toward clinical trials for CHD. Stem Cells Translational Medicine 2015;4:195–206

INTRODUCTION

Several types of congenital heart disease (CHD), particularly those with univentricular hearts of right heart ventricular morphology, are characterized by an increased pressure and right ventricular workload. The development of right ventricular dysfunction and cardiac failure is responsible for persistent morbidity in children affected with these cardiac malformations, who require lifelong surveillance for declining function that may lead to cardiac transplantation. The study of stem cell strategies to address heart failure has advanced steadily over the past decade, predominantly in the adult population. There is expanding clinical experience with first generation cell-based therapies to mitigate the effects of ischemic and nonischemic heart diseases in adults that involve the administration of both autologous and allogeneic bone marrow-derived stem cells (BMSCs) [1–6]. These studies have provided evidence that validates the overall safety and feasibility of direct injection into the left heart muscle in adult cardiac disease.

However, clinical experience with cell-based therapy for cardiac disease in children is limited to a few case reports [7–9]. To date, no large clinical trials have been reported using any type of stem cells to augment CHD management in the pediatric setting. The most recent publication from Rupp et al. in 2012 [8] reported the safety and feasibility of the intracoronary autologous BMSC administration in nine children with end-stage heart failure following analogous protocols established in the adult experience. Cell-based administration was associated with improved cardiac function and clinical stability with no procedure-related unexpected adverse events.

The ideal source of stem cells for cardiac regenerative purposes is dependent on the
individual clinical scenario that includes logistical challenges of collection and processing of the cells, as well as clinical delivery to the cardiac tissue in complex CHD. Umbilical cord blood (UCB) was prioritized herein according to the readily available source of stem cells that contains both hematopoietic and nonhematopoietic tissue precursors. Given the high incidence of prenatal diagnosis of CHD, this source of autologous stem cells was practical to process in the time frame necessary for add-on clinical procedures in early postnatal life. UCB is rich in multipotent stem/progenitor cells with enhanced potency for angiogenic and myogenic differentiation and proliferative characteristics [10–12]. UCB cells have been standardized therapeutic agents in patients suffering from major hematological disorders since 1988 [13]. Subsequently, multiple groups have been pioneering human umbilical cord blood-derived mononuclear cells (UCB-MNCs) in preclinical experimental approaches for myocardial repair and regeneration [14–19]. Collectively, these studies suggest a cell-mediated improvement in the ejection fraction, wall motion, and cardiac contraction, along with a lowered left ventricular end-diastolic pressure. Increases of capillary density were associated with reductions in infarct size, number of apoptotic cells, and left ventricle remodeling [18, 20, 21]. Additionally, intramyocardial delivery of UCB-MNC in an ovine model system suggests a therapeutic benefit cell-based therapy in the right ventricle (RV) [22, 23].

Despite the evidence for efficacy of UCB cells, there are no long-term safety studies that have investigated the comprehensive profile of UCB-MNC transplantation into juvenile cardiac tissue recapitulating the pediatric setting. The experimental approach herein has been conducted to determine the feasibility and long-term safety of autologous cell-based therapy in a double-blinded, large-animal model system with primary outcome focused on electromechanical stability under physiological stress of a juvenile heart. The porcine model system allowed us to develop clinically relevant autologous cell processing of UCB, surgical delivery strategy for the RV, and cardiac imaging modalities calibrated for preclinical validation studies. Collectively, this clinical-grade pipeline has now established the baseline safety profile of autologous UCB-MNCs that demonstrates no measurable toxicity attributable to cell-based cardiac regenerative strategies within the innate physiological challenges of a juvenile/pediatric heart. This pipeline now provides a systematic platform to identify optimal safety profiles of cell-based therapeutics in the pediatric setting and aims to accelerate regenerative strategies for CHD.

MATERIAL AND METHODS

Animals

The care of the experimental animals was in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 86/5-23, National Academy Press, Washington, DC, revised 1996) under the responsibility of Mayo Clinic Department of Comparative Medicine. All procedures were approved by the Institutional Animal Care and Use Committee review board of the Mayo Clinic in Rochester, Minnesota.

Caesarean Delivery and UCB Collection

Synchronized and timed-pregnant sow and gilt pairs were purchased prior to 100 days of gestation. At day 115 of gestation, the gilts were prepared for nonsurvival caesarean section delivery. Midline abdominal incision and electrocautery were used to gain access to the uterus. Individual piglets were delivered through multiple transverse myotomies in the uterus. Sterile umbilical cord clamp (Mabis Healthcare, Waukegan, IL, http://www.mabisdmi.com) was placed within 3–5 cm from the piglet, and the cord was cut. UCB was drained from vein and arteries by gravity into sterile prelabeled 50-ml conical tubes (BD Bioscience, San Jose, CA, http://www.bdbiosciences.com) filled with 10 ml of anticoagulant (citrate phosphate dextrose-adenine) (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Porcine UCB and anticoagulant contained in the tubes were gently mixed to avoid clot formation.

Isolation and Cryopreservation of UCB-MNCs

UCB was processed within 24 hours by diluting with phosphate-buffered saline (PBS) at a ratio of 1:1 and slowly overlaid onto 15 ml of Ficol density medium (1.077 g/ml; GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com) in 50-ml conical polypropylene tubes (BD Bioscience), followed by centrifugation for 30 minutes at 400g. The interfaceuffy layer was carefully collected and pooled into new 50-ml conical tubes. The cells were washed twice with PBS and, after the second wash, were resuspended in 20 ml of PBS. Two-hundred microliters of the cell solution was taken for total nucleated cells (TNCs) and mononuclear cells (MNCs) counting, viability by flow cytometer, and 100 µl for endotoxin testing. The remaining cells were centrifuged for 10 minutes at 500g at room temperature. After centrifugation, the supernatant was removed to a cell pellet and was used for sterility testing. The final cell-based product was cryopreserved in sterile 2-ml cryovials with 10% dimethyl sulfoxide (DMSO) (Cryostor CS-10; Biolife Solutions, Bothell, WA, http://biolifesolutions.com) at a desired cell concentration of 30 × 10^6 MNCs/ml. Equivalent numbers of cryovials were produced at the same time with vehicle control solution (DMSO 10%) plus 10 µl of autologous red blood cells to recapitulate the external appearance of the cell-based product. All vials were stored long term in liquid nitrogen in a dedicated box.

Postprocessing Quality Control Assays

The absolute TNC count and MNC fraction were calculated by the microsphere-based technique using a single-platform flow cytometry. Aerobic and anaerobic microbiologic culture analysis (BD Bactec Peds Plus and BD Bactec Lytic/10 Anaerobic) (BD Biosciences, San Jose, CA, http://www.bd.com) was completed in the Mayo Clinic microbiology laboratory. Endotoxin was monitored using the Endosafe PTS (Charles River Laboratories, Wilmington, MA, http://www.criver.com), which uses Limulus amebocyte lysate kinetic chromogenic methodology to measure color intensity directly related to the endotoxin concentration in a sample (supplemental online data).

Surgical Procedures

All surgery was conducted in a specialized operating room designated for large-animal cardiothoracic surgical procedures. Each animal was sedated with Telazol (tiletamine HCl and zolazepam HCl; Zoetis, Florham Park, NJ, https://www.zoetisus.com) (5 mg/kg...
intramyocardially [IM]) and xylazine (2 mg/kg IM) and premedicated including antibiotics and analgesics (cefazolin [40 mg/kg i.v.] with or without Excede [cefotiofur; Zoetis] [5 mg/kg IM], and buprenorphine SR 0.15 mg/kg s.c.). The experimental animals were endotracheally intubated to protect the airway and allow administration of inhaled 1%–2% isoflurane as needed. During surgery, the animals were monitored by heart rate, blood pressure, respiratory rate, temperature, and continuous electrocardiography (ECG).

Telemetry Device Implantation for Continuous Cardiac Monitoring

Piglets that were predetermined to be eligible for the randomization arms of the study had an implantable loop recorder (ILR) (Medtronic, Minneapolis, MN, http://www.medtronic.com) placed 3–5 days prior to thoracotomy. The ILR device ID was linked to the individual piglet. The animals were placed in a sternal position to expose the posterior left paraspinal area, and skin incision at T4 was made 1–2 cm lateral to the spinal process. The ILR device was placed in the desired location subcutaneously, positioned until good signal was achieved, and secured using the sutured tab. ILR data were interpreted by a clinically trained pediatric cardiologist in a blinded fashion, using data directly uploaded into CareLink, a clinically approved device and reporting system by Medtronic. Tachycardia was defined as ≥250 beats per minute for more than 30 seconds, bradycardia was defined as <60 beats per minute for more than 30 seconds, and asystole was defined as a lack of electrical activity for more than 6 seconds. One minute before and after, each episode was recorded and analyzed to determine artifact or clinically meaningful episode of arrhythmia.

Intramyocardial Delivery of Cell-Based Product versus Placebo

Starting with the oldest animals in each cohort eligible for randomization, a lateral right thoracotomy was performed at the T4–T5 intercostal space. The pericardium was reflected to expose the epicardial surface of the RV. Immediately prior to product delivery, each case was randomized in a double-blinded fashion to receive either UCB-MNCs (3 × 10^6 cells per kilogram of body weight) or a comparable volume (0.1 ml/kg of body weight) of a 10% DMSO-based final solution as a vehicle control. The selected product was thawed at 37°C for approximately 5 minutes or until all solids were eliminated within the tube with careful mixing of the vial contents. The product was loaded into 1-ml syringes with an 18-gauge needle and then connected to the delivery device with a 27-gauge needle on flexible tubing. Avoiding major blood vessels and the conduction system, a 2–4-cm-diameter target area was identified on the surface of the RV. Epicardial injections delivered over 20 seconds to 5–15 sites calculated as 0.1 ml per site with a total dose of 0.1 ml/kg of body weight.

Cardiovascular Parameters Assessment

Surface electrocardiograms and echocardiography images were evaluated by an independent contract research organization (QTest Laboratories, Columbus, OH, http://www.qtestlabs.com) in a blinded (treatment vs. placebo) fashion (supplemental online data).

Veterinarian Medical Examination

In addition to research staff measuring and documenting vital signs and cage-side observations (daily observation for adverse events), formal veterinary medical examinations were performed on a predetermined schedule of baseline, 4 weeks post-thoracotomy, and 12 weeks post-thoracotomy beyond standard care.

Pathology and Necropsy

All laboratory samples were analyzed by Mayo Medical Laboratory at defined time intervals. Animals were euthanized 12 weeks after cell/placebo infusion. Necropsy was then performed, and tissues were placed in 10% buffered formalin. Heart tissue was collected from three independent sites within the free wall of the RV (around the injection sites) and the left ventricle, and cardiac weight was recorded. The samples were stained with hematoxylin and eosin, and sections of heart were also stained with Masson’s trichrome. Samples were analyzed in a blinded fashion by a certified veterinary pathologist (Vet Path Services, Inc., Mason, OH, http://www.vetpathservicesinc.com).

CFSE Labeling and Cell Tracking

In a nonrandomized fashion, the cell-based product was thawed at 37°C, washed, and then labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Molecular Probes, Grand Island, NY, http://www.invitrogen.com) following the manufacturer’s recommendations (supplemental online data).

Statistical Analysis

Comparisons between groups were made using Student’s t test. Probability values of less than .05 were considered significant. The data are presented as means ± SD. A customized Medidata Rave database was built to track the health of individual piglets throughout the protocol and ensure strict clinical-grade procedures to certify compliance with double-blinded, randomized study design.

RESULTS

Experimental Design

Piglets were born at Mayo Clinic from two litters and had autologous UCB collected, processed, and analyzed to achieve predetermined release criteria. When the piglets were ready to be weaned, a trained veterinarian completed a baseline medical exam. The piglets were eligible for this study if they had no active medical conditions, had autologous UCB-MNCs meeting all release criteria, and were 3–4 weeks of age. The four oldest animals (defined by the time of birth) plus four more animals that did not meet cellular release criteria were identified as the nonrandomized satellite group that contained labeled cells to allow for cell-tracking and biodistribution data in parallel to the randomized cohort. The next 13 oldest animals received ILR and had baseline vital signs and echocardiography performed 3–5 days prior to planned thoracotomy. At the time of thoracotomy, planned according to the sequential order of the oldest to youngest animals, 12 healthy piglets were enrolled and randomized once they were successfully intubated; they were monitored and were stable after exposing the cardiac surface via thoracotomy. Preprinted envelopes numbered 1–12 determined, in a double-blinded procedure, the selection into either vehicle control (n = 6) or cell-therapy (n = 6) arms of the study. Following thoracotomy procedure, physiological parameters were monitored until the termination of the study (Fig. 1).
Feasibility of Porcine UCB Collection and Processing

UCB was collected from 26 piglets while the placenta was still in utero. UCB was drained by gravity until there was no further blood flow, between 2 and 7 minutes. UCB volume collected was 20–59 ml (mean = 31.5 ml, SE = 2.03 ml), and none of the samples had signs of coagulation or hemolysis. Transport time was within 2 hours at ambient temperature (∼20°C) (Fig. 2A, 2B). No complications were encountered during UCB collection, and all piglets recovered within hours of delivery. Release criteria were documented with human analogous procedures for cellular composition and viability, microbiologic testing, and endotoxin levels (Fig. 2C, 2D). The resulting products for all piglets born from the two litters are noted in Table 1. Of the 26 piglets born by caesarean delivery, none demonstrated insufficient viability (mean = 92.3%, SE = 0.71%) or endotoxin contamination. TNC counts ranged from $8.9 \times 10^6$ cells to $80.4 \times 10^6$ cells (mean = $40.2 \times 10^6$ cells, SE = $4.68 \times 10^6$ cells) and all animals met the minimum mononuclear cell percentage required in the release criteria (mean = 96.9%, SE = 0.58%) (supplemental online Fig. 1A). Pearson’s correlation shows a positive correlation between porcine UCB volume and postprocessing TNC counts ($R^2 = 0.42$, $p = .0003$) (supplemental online Fig. 1B). Table 1 notes a single UCB unit that was contaminated in the cohort of 26 piglets. Furthermore, two piglets were excluded from randomization because of low TNC.

Figure 1. Study design and time-course data of the porcine safety study. Abbreviations: C-section, caesarean section; ECG, electrocardiography; ECHO, echocardiography; ILR, implantable loop recorder; UCB, umbilical cord blood.
count secondary to low UCB collection volumes. Thus, 92% of piglets achieve predefined cellular release criteria.

Randomization Procedure, Thoracotomy, and Quality Assurance
Six of the original twenty-six animals were not included in the randomization schedule because of failure of meeting health status release criteria (inadvertent injuries inflicted by the surrogate sow resulted in two dead animals, three animals were sacrificed because of large litter size for surrogate mother, and one piglet had an unexplained fever during routine vaccination). Randomization was achieved according to a defined procedure. Immediately after randomization, animals received either UCB cells (3 \( \times \) 10^6 cells per kilogram) or the same volume of vehicle solution (DMSO, 10%) intramyocardially into 5–15 sites of a 2–4-cm-diameter target area of the RV under visualization by open-heart surgery. No complications including mortality were registered caused by the surgical procedure in any of the groups. Identity of each animal assigned to each group was confirmed with postprocedural quality assurance testing of the vials that were not used in the surgical delivery of randomized product. There were no discrepancies noted between the real-time Medidata Rave documentation at the time of procedure randomization and the postprocedural quality assurance testing of the unused frozen products after delivery (Fig. 3).

Safety of UCB-MNC Intramyocardial Transplantation Throughout 3-Month Follow-Up

Clinical Exam
Vital signs did not show sustained differences between placebo and cell-based groups (\( p < .05 \)) (supplemental online Fig. 2). Daily
Cage-side observations revealed five events for two animals, both in the control group. Animal L331 had a necrotic tail the day after thoracotomy that required surgical excision. This was likely because of the routine trauma of living in a pen of littermates and not attributable to any procedure-related activities. The same animal had a pocket abscess noted 7 days after ILR implantation. Antibiotics were given, and draining and cleaning was applied daily. Eventually, the device was displaced from the animal by day 28, and 2 weeks later the device was reimplanted when infection resolved. Finally, animal L375 developed swelling at the thoracotomy site 14 days after surgery that was resolved following standard veterinarian medical care and intramuscular antibiotics. Notably, there were no observations in the cell-therapy cohort noted, after scheduled independent clinical veterinarian examinations. There were no additional medical issues or concerns discovered outside of the daily cage-side observations according to predetermined veterinarian examinations.

Clinical Biochemistry, Hematology, and Coagulation

Blood samples were obtained according to predefined schedule to document the natural trends in the placebo control group and compare with the cell-based treatment group. There were insignificant sporadic values that were different between the two groups, yet no clinical chemistry values represented any evidence of systemic toxicity (Fig. 4A; supplemental online Table 1). Because the animals received either cell-based therapy or placebo injection, all randomized pigs were also monitored for cardiac damage caused by the 10–15 injections of 0.1-ml volume each by measuring plasma levels of cardiac enzymes. Troponin-T levels, <0.01 ng/ml were considered below detectable levels as expected if no significant injury was caused by the injections. A single measurement was reported as being mildly elevated (0.03 ng/ml) in an animal within the control group at 2 weeks with CK-MB isoenzyme concurrently monitored not being elevated. This likely represented an inflammatory signal caused by...

### Table 1. Piglet characteristics and release criteria

| Piglet ID | Date of birth | Gender | UCB collection (time) | UCB Volume collected, ml | TNCs, ×10³ | MNCs, % | Viability, % | Sterility tests | Endotoxin levels, <5 EU/kg | Inclusion criteria | Group of study |
|-----------|--------------|--------|----------------------|--------------------------|-------------|---------|-------------|----------------|--------------------------|-----------------|---------------|
| L323      | May 2, 2012  | F      | 10:03                | 30                       | 31          | 95.3    | 90          | –              | –                        | Yes             | No, D         |
| L324      | May 2, 2012  | M      | 10:05                | 32                       | 26.7        | 97.4    | 95          | –              | –                        | Yes             | Yes CT        |
| L325      | May 2, 2012  | M      | 10:08                | 26                       | 17.3        | 87      | 88          | –              | –                        | Yes             | Yes CT        |
| L326      | May 2, 2012  | M      | 10:10                | 24                       | 15.1        | 99.2    | 91          | –              | –                        | Yes             | Yes CT        |
| L327      | May 2, 2012  | F      | 10:13                | 31                       | 45.3        | 97.9    | 80.4        | –              | –                        | Yes             | Yes CT        |
| L328      | May 2, 2012  | M      | 10:16                | 34                       | 58.3        | 93.5    | 92.5        | –              | –                        | Yes             | Yes R         |
| L329      | May 2, 2012  | M      | 10:19                | 24                       | 45.7        | 99.1    | 90.4        | –              | –                        | Yes             | No, C         |
| L330      | May 2, 2012  | M      | 10:21                | 40                       | 66.7        | 95.4    | 90          | –              | –                        | Yes             | No, D         |
| L331      | May 2, 2012  | F      | 10:23                | 31                       | 70.3        | 99.8    | 93.1        | –              | –                        | Yes             | Yes R         |
| L332      | May 2, 2012  | F      | 10:27                | 33                       | 51.5        | 99.5    | 93.5        | –              | –                        | Yes             | Yes R         |
| L333      | May 2, 2012  | F      | 10:29                | 33                       | 66.9        | 89.8    | 96.8        | –              | –                        | Yes             | Yes R         |
| L334      | May 2, 2012  | M      | 10:33                | 29                       | 60.2        | 97.2    | 92.5        | –              | –                        | Yes             | Yes R         |

**First litter**

| Piglet ID | Date of birth | Gender | UCB collection (time) | UCB Volume collected, ml | TNCs, ×10³ | MNCs, % | Viability, % | Sterility tests | Endotoxin levels, <5 EU/kg | Inclusion criteria | Group of study |
|-----------|--------------|--------|----------------------|--------------------------|-------------|---------|-------------|----------------|--------------------------|-----------------|---------------|
| L363      | May 11, 2012 | M      | 9:15                 | 20                       | 11.6        | 99      | 95          | –              | –                        | Yes             | No, LCC        |
| L364      | May 11, 2012 | F      | 9:18                 | 23                       | 13.8        | 98.4    | 97          | –              | –                        | Yes             | No, LCC        |
| L365      | May 11, 2012 | M      | 9:20                 | 23                       | 8.9         | 97.5    | 95          | –              | –                        | Yes             | No, D         |
| L366      | May 11, 2012 | F      | 9:23                 | 25                       | 9.4         | 99.1    | 94          | –              | –                        | Yes             | No, D         |
| L367      | May 11, 2012 | F      | 9:29                 | 39                       | 10.7        | 99.5    | 93          | –              | –                        | Yes             | No, D         |
| L368      | May 11, 2012 | M      | 9:35                 | 36                       | 25.7        | 97.2    | 90          | –              | –                        | Yes             | Yes R         |
| L369      | May 11, 2012 | M      | 9:40                 | 23                       | 52          | 95.6    | 92          | –              | –                        | Yes             | Yes R         |
| L370      | May 11, 2012 | M      | 9:45                 | 23                       | 48.8        | 98.1    | 88          | –              | –                        | Yes             | Yes R         |
| L371      | May 11, 2012 | F      | 9:50                 | 22                       | 31          | 97.1    | 93          | –              | –                        | Yes             | Yes R         |
| L372      | May 11, 2012 | F      | 9:55                 | 27                       | 20.7        | 96.6    | 91          | –              | –                        | Yes             | Yes R         |
| L373      | May 11, 2012 | F      | 10:01                | 50                       | 78.3        | 96.4    | 96          | –              | –                        | Yes             | Yes R         |
| L374      | May 11, 2012 | F      | 10:07                | 25                       | 26.1        | 98.5    | 90.5        | –              | –                        | Yes             | Yes R         |
| L375      | May 11, 2012 | M      | 10:13                | 57                       | 80.4        | 97.3    | 94          | –              | –                        | Yes             | Yes R         |
| L376      | May 11, 2012 | F      | 10:20                | 59                       | 73.4        | 97.7    | 98          | –              | –                        | Yes             | Yes R         |

**Second litter**

aDiscarded: acute fever after vaccination.
bDiscarded: limping.

Abbreviations: +, blood contamination; –, no blood contamination; C, contamination; CT, cell tracking; D, dead; EU, enzyme unit; F, female; LCC, low cell count; M, male; MNC, mononuclear cell; R, randomization; TNC, total nucleated cell; UCB, umbilical cord blood.
a documented skin incision infection at this time within this animal. Furthermore, no differences were detected in the hematol-
yogy and coagulation analyses between the placebo control and
cell-based treatment groups throughout the 12-week follow-up
study (Fig. 4B; supplemental online Table 2). Also, no abnormal-
ities were observed on the hematology blood smears for the pla-
cebo control and cell-based treatment groups. Overall, there was
no evidence of cardiac injury caused by the injections or biochem-
ical deviation between the two groups as a result of cell-based
treatment during 3 months of follow-up.

Cardiac Electrophysiology Showed No Differences
Between Placebo and Cell-Therapy Groups

As expected, there was wide variation in heart rate and in com-
ponent deflections and intervals in the serial recordings of 60
ECG tracings evaluated qualitatively over this study period, likely
significantly influenced by the body size and lead positions. Nota-
bly, all pigs were in normal sinus rhythm at all times throughout
the 3-month study procedures, without differences between the
placebo and cell-based treatment groups. Overall, there was
no evidence of cardiac injury caused by the injections or biochem-
ical deviation between the two groups as a result of cell-based
treatment during 3 months of follow-up.

Echocardiography Revealed No Cardiac Structure and
Function Changes Between Groups

More than 4,000 echocardiography images from a total of 12
diverse pigs were evaluated both qualitatively and quantitatively.
The quality of the data collection was limited because of the body
size variations range from 5 kg of body weight to 50 kg of body
weight in the study period. Despite the technical limitations of
data collection, data analysis was sufficient to determine that
there were no specific changes in echocardiography images at
any times compared with baseline recordings or between
cohorts. The blinded echocardiography analysis did not reveal
any evidence of structural abnormalities in the cell-based treat-
ment group compared with placebo control group in this study
(Fig. 5). Biventricular wall motion appeared vigorous and uncom-
promised throughout the study. Animal L372 had an apparent
symmetrically thickened left ventricle at baseline (prior to receive
the randomized cell therapy). This was the same animal that had
an asystole episode 12 hours after cell delivery that was self-limiting and went unnoticed until the study was unblinded at the conclusion of data collection. Overall, cardiac imaging demonstrated normal cardiac function in the absence of structural heart disease and provided an age-dependent baseline for future studies.

**Histopathology Findings**

Microscopic histopathological findings correlated with the gross necropsy findings. The occurrence of adhesions of the epicardium, heart surface, right heart wall, pericardium, and thoracotomy site, thickened pericardium, pleural cavity fluid, and

Figure 4. Porcine blood tests at baseline and 2, 4, 8, and 12 weeks after thoracotomy. (A): Clinical biochemistry did not show any difference (\( p > .05 \)) between groups after placebo/cell injection. (B): Umbilical cord blood-derived mononuclear cells transplanted into the porcine right ventricle did not alter either hematological or coagulation parameters. Control and cell-therapy group values are statistically not different (\( p > .05 \)). We established the age-dependent baseline clinical chemistry and hematology values without clinically relevant differences between the groups. Blue curves belong to the cell-therapy group, and orange curves represent the placebo group. Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bb, bilirubin; BUN, blood urea nitrogen; GGT, \( \gamma \)-glutamyl transferase; LDH, lactate dehydrogenase; MCV, mean corpuscular volume; RBCC, red blood cell count; TG, triglycerides.
cells at all time points (Fig. 6C, 6D).

...and 1 week (Fig. 6A, 6B). The cell tracking group had the heart, lung, liver, spleen, aorta, and bone marrow evaluated for fluorescence of CFSE-labeled injected cells. No staining for injected cells was observed in any of the animals in any tissues examined, including the step-sectioned blocks from the RV at 24 hours (n = 2) and 1 week (n = 2) postinjection. However, the RV was positive for CFSE-labeled UCB-MNCs 1 hour after cell injection in the 4 animals evaluated. On the contrary, the left ventricle was negative for green cells at all time points (Fig. 6C, 6D).

**DISCUSSION**

The delivery of stem cells directly to the myocardium has safely been performed in a large number of experimental and clinical trials in the left heart ventricle of adult populations [24, 25]. However, congenital cardiac anomalies have not been the target of intramyocardial cell-based cardiac regenerative approaches. Given the critical importance of safety using a novel cell-based product and delivery strategy in a pediatric heart, this long-term safety study was designed and executed as a double-blinded, randomized controlled study in a porcine model system. The specific focus in this study was to evaluate at the pediatric stage of cardiac development the procedural feasibility and long-term risk of adverse effects on cardiovascular parameters, in addition to systemic effects following intramyocardial delivery of an autologous cell-based product. We selected to initially test UCB-MNCs because of the many advantages from an autologous yet available source in CHD. UCB is a well-established source of hematopoietic stem cells for bone marrow transplantation and is emerging as a vital source of nonhematopoietic cells [10] with some similarities with humans. However, this animal model system has some challenges, such as the fact that swine hearts are relatively difficult to interrogate by cardiac echocardiography. These challenges were addressed with the addition of continuous loop records and clinical team members from pediatric electrophysiology and echocardiography. During the 3-month follow-up, the data showed no significant acute or chronic cardiac injury pattern caused by the intramyocardial delivery as measured by cardiac enzyme levels of Troponin-T, unlike previous reports that involved an ischemic cardiac porcine model system [32].

Continuous cardiac monitoring revealed four events in two animals of cell-therapy group. Three of the four events were transient and subclinical asystole in the perioperative period that likely were due to hypersensitivity of anesthesia and vagal stimulation during endotracheal intubation/extubation. Based on time-stamped recordings and medical records, these events were not attributed to cell delivery, and clinically the animals never displayed any health concerns with veterinary care teams monitoring daily. Furthermore, clinical echocardiography data indicated that there were no specific changes in echocardiography images at any time compared with baseline recordings or between the cell-based and placebo test subjects, despite the challenges of echocardiography data collection. One animal in the cell-based treatment group had an apparent symmetrically thickened left ventricle at baseline, prior to randomization and receiving the cell-based product. Interestingly, this was the same animal that had a self-limiting asystole episode 12 hours after surgery and went unnoticed until the study was unblinded. It is
Figure 5. Echocardiography studies show that autologous cell-therapy injection into the porcine right ventricle is safe. (A): Echocardiography images at baseline (M-mode) and at 12-week follow-up (M-mode and two-dimensional echocardiography views [long axis and short axis for the systolic phase]), with a normal electrocardiography record in both study groups. The left column represents the control animal group, and the right column represents the cell-therapy group. The images did not differ between groups. (B–D): There were no significant statistical differences ($p > .05$) between control (blue curves) and cell-therapy group (red curves) at 2, 4, 8, and 12 weeks of follow-up of thoracotomy on the right and left ventricle fractional area change (B), on the left ventricle diastolic area (C), and on the early to late left ventricle filling velocity ratio (D). Abbreviations: Ao, aortic artery; IVS, interventricular septum; LA, left atrium; LV, left ventricle; PW, posterior wall; RV, right ventricle.

Figure 6. Microscopic images of the porcine right ventricle (RV) postmortem 3 months after placebo and cell infusion and autologous umbilical cord blood mononuclear cells (UCB-MNCs) tracking images in the porcine heart after CFSE labeling. (A): Light photomicrograph of RV cardiac tissue area of placebo/cell injection stained with H&E. (B): Trichrome staining 12 week post-thoracotomy (magnification, $\times 50$). H&E staining did not show any lesions either in the control or in the cell-therapy group. Trichrome staining did not show an increase of tissue fibrosis. Sections of each group did not show any pathological changes in the RV region of the cells transplantation, being comparable with the control group. (C, D): Immunohistochemistry of cardiac tissue after 1 hour of CFSE-labeled autologous porcine UCB-MNCs injected into the RV of a 4-week-old piglet. Heart tissues were stained with CFSE in green, DAPI for cell nuclei are in blue, and MLC2V are in red. The left and right ventricles were analyzed by confocal laser scanning microscopy (magnification, $\times 40$). (C): Photographs of left ventricle show no CFSE-labeled UCB-MNCs. MLC2V staining demonstrates that processed tissue is heart (ventricle). (D): The RV, the site of cell injection, is positive for CFSE-labeled UCB-MNCs (green fluorescence). CFSE staining with DAPI and MLC2V (bottom right panels) illustrates the density of UCB-MNCs in the location of cell administration. Abbreviations: CFSE, 5- (and -6)-carboxyfluorescein diacetate succinimidyl ester; DAPI: 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; MLC2V, myosin light chain 2 ventricular antibody.
not clear whether cardiac hypertrophy could be a risk factor for cardiac ectopy during cell delivery with a single episode; however, this will be continuously monitored in ongoing studies. Supporting the evidence of a lack of adverse outcomes, macroscopic changes noted at the time of scheduled necropsy were consistent with surgical procedure of right thoracotomy causing scarring and fibrosis in the epicardium across all test subjects. The cell-based or placebo injections were not associated with any detectable evidence of cardiac or noncardiac toxicity upon microscopic analysis. Consistent with these findings, Yerebakyan et al. [23] evaluated the efficacy of intramyocardial autologous UCB-MNC transplantation on RV function in a 4-month-old sheep model of chronic RV volume overload. They reported that the area of fibrosis did not show any significant difference between the placebo and stem cell groups, although a detailed analysis of toxicity was not completed in this reported study, and no evidence of acute physiological, biochemical, or electromechanical complications was encountered during the procedure.

Histology analysis of a nonrandomized cohort used to track CFSE-labeled cell product did not reveal significant signal beyond 1 week of intramyocardial delivery. The lack of long-term cellular engraftment was not surprising, because multiple studies have failed to demonstrate long-term engraftment of MNCs in adult studies and a modest ratio of transplanted cells in preclinical studies [22, 33]. Future studies will benefit from optimized protocols for tracking the labeled cells in the setting of autologous cells not requiring immunosuppression therapy. Furthermore, protocols that improve the cellular retention and engraftment may offer significant advantages in the field of CHD regenerative strategies.

CONCLUSION

To our knowledge, this is the first double-blinded, randomized safety study using autologous UCB-MNCs in a large animal model system focused on long-term RV outcomes emulating the pediatric setting. Injection of autologous UCB-MNC into the RV was not associated with any detectable adverse events following this clinical-grade procedure. The final analysis revealed no statistical differences between the cohorts receiving the autologous cell-based product and placebo control injections, and all animals survived the procedures without serious medical complications. This safety study demonstrates that porcine autologous UCB-MNCs can be safely collected and surgically delivered in early stages of development and should establish the foundation to advance new therapeutic modalities aiming toward clinical trials in those affected with CHD. Future dedicated studies will be required to establish a diseased model system to properly monitor efficacy of cell-based therapy in enhancing right ventricular function in a setting to recapitulate CHD.

ACKNOWLEDGMENTS

We thank Adam Armstrong for assistance with endotoxin testing and Diane M. Jech for help with data acquisition of transthoracic echocardiography. This work was supported by the Todd and Karen Wanek Program for Hypoplastic Left Heart Syndrome and the Mayo Clinic Foundation. Wanek Program Porcine Pipeline Group: Sarah L. Edgerton, Scott H. Suddendorf, Steve Krage, Mindy Rice, Joseph A. Rysavy, Joanna H. Powers, Boyd W. Rasmussen, Jennifer M. Miller, Traci L. Paulson, Rebecca K. Lindquist, Chelsea L. Reece, Angela R. Miller, Douglas J. Padley, Mark A. Wentworth, Alexander C. Greene, and Amy G. Andrews with direction from the Wanek Program leadership of Timothy J. Nelson, Patrick W. O’Leary, Timothy M. Olson, and Andre Terzic.

AUTHOR CONTRIBUTIONS

S.C.P. and H.M.B.: conception and design, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.O.: conception and design, collection and/or assembly of data, data analysis and interpretation; S.Y. and B.C.C.: collection and/or assembly of data, data analysis and interpretation; S.L.N.: provision of study material or patients; X.L.: data analysis and interpretation; P.W.O. and A.T.: conception and design; T.J.N.: conception and design, financial support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

B.C.C. has a compensated consultancy with Medtronic. The other authors indicated no potential conflicts of interest.

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