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Quantitative analysis of composite umbilical cord tissue health using a standardized explant approach and an assay of metabolic activity

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Abstract

Background. Umbilical cord (UC) tissue can be collected in a noninvasive procedure and is enriched in progenitor cells with potential therapeutic value. Mesenchymal stromal cells (MSCs) can be reliably harvested from fresh or cryopreserved UC tissue by explant outgrowth with no apparent impact on functionality. A number of stem cell banks offer cryopreservation of UC tissue, alongside cord blood, for future cell-based applications. In this setting, measuring and monitoring UC quality is critical. *Materials and Methods.* UC explants were evaluated using a plating and scoring system accounting for cell attachment and proliferation. Explant scores for fresh and cryopreserved-then-thawed tissue from the same UC were compared. Metabolic activity of composite UC tissue was also assayed after exposure of the tissue to conditions anticipated to affect UC quality and compared with explant scores within the same UC. *Results.* All fresh and cryopreserved tissues yielded MSC-like cells, and cryopreservation of the tissue did not prevent the ability to isolate MSCs by the explant method. Thawed UC tissue scores were 91% (±0.6%; P = 0.0009) that of the fresh, biologically identical tissue. Within the same UC, explant scores correlated well to both cell yield (R^2 = 0.85) and tissue metabolic activity (R^2 = 0.69). *Discussion.* A uniform explant scoring assay can provide information about the quality of composite UC tissue. Such quantitative measurement is useful for analysis of tissue variability and process monitoring. Additionally, a metabolic assay of UC tissue health provides results that correlate well to explant scoring results.

Key Words: *cryopreservation, mesenchymal stromal cells, stem cell banking, umbilical cord tissue*

Introduction

Considerable pre-clinical evidence suggests the safety and efficacy of mesenchymal stromal cells (MSCs) across a wide variety of conditions, due primarily to trophic and immune modulatory effects. It is estimated that more than 450 clinical trials are now evaluating MSCs as part of a therapeutic intervention [1] . MSCs can be found in many different tissues, including bone marrow, adipose tissue, dental pulp and newborn tissues such as umbilical cord (UC) blood, UC tissue and placental tissue [2]. Bone marrow and adipose tissue remain the most often used MSC sources for clinical applications. Yet several groups have observed that MSC prevalence and function are negatively affected by increased age and certain chronic disease conditions [3–5]. In contrast, MSCs isolated from newborn tissue sources, including placental and UC tissue, have demonstrated an enhanced proliferative capacity and in general have had less risk of exposure to virus and toxins compared with their counterparts isolated from aged adult tissues [6]. Thus, preservation of newborn tissues as a source of MSCs may be advantageous for future clinical applications.

Large numbers of MSCs can be consistently isolated from UC tissue, which also serves as a source of other progenitor cell populations [7–12]. Approaches for isolating MSCs from UC tissue include enzymatic digestion and tissue explanting, with recent reports also demonstrating tool-aided mechanical tissue dissociation techniques [2,13]. Compared with tissue digestion or dissociation, isolation of MSCs from UC by an explant approach may minimize cellular stresses and damage that can impact cellular function [2,14–16]. Furthermore, explant isolation results in MSC populations that are more homogeneous than extracts collected by digestion or mechanical dissociation [17,18]. That explanted UC tissues consistently

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produce clonogenic cells and that these cells possess MSC qualities, including morphology, immunophenotype, proliferation kinetics, differentiation potential and immunosuppressive activity, has been well established [6,14,16–21]. It has also been shown that MSCs can be harvested from cryopreserved-then-thawed UC tissues by the explant approach without any obvious functional impact [12,16,22,23].

In recognition of the unique benefits that UC tissue may offer as rich source of MSCs, numerous UC blood banks, including some public banks, have established UC tissue storage programs [13,24]. A popular banking strategy has been to cryopreserve and store the UC tissue as a whole, composite material. This approach minimizes front-end tissue manipulation, reduces processing time and expense and allows for isolation of the progenitor population when the therapeutic need is known. Moreover, it has been proposed that composite UC tissue acts as an ideal and natural storage compartment with maintenance of the stem cell niche [25]. Within the banking setting, it is essential that UC tissue health is maintained throughout the collection, processing and storage steps so that therapeutic cells can be isolated from the tissue when needed. However, unlike the cord blood storage industry, which has been established for over two decades and for which approved transplant indications are defined, the cord tissue storage industry has yet to solidify consensus processing and handling protocols, quality metrics or release criteria, in part due to the complicated nature of assessing composite tissue health.

In pursuing the potential of UC MSCs in regenerative therapies, the research literature has largely overlooked the distinction between UC MSC quality (characteristics such as population doubling time, immunophenotype, differentiation capacity and immunomodulation of cells derived from UC tissue) and UC tissue quality (overall tissue viability and the propensity with which desirable cells are harvestable from the UC tissue). While the former may ultimately dictate use criteria for particular clinical indications, the latter will be essential for the success of a sound banking platform that can support clinical applications by reliably storing and supplying clinical material. Developing baseline expectations for MSC harvests from UC and how various factors, such as donor variation, collection and transport procedures, and processing and freezing protocols impact the ability to harvest MSCs from UC will allow for the optimization and standardization of UC tissue banking. Therefore, it will be important to use assays that can consistently and reliably measure UC tissue health. The current work presents, to our knowledge, the first report of approaches for quantifying the quality of composite UC tissue. We first demonstrate that within a cord blood banking setting, co-collection of UC tissue along with UC blood does not detrimentally impact the quality of cord blood collection. Then, using a standardized UC explant protocol, which measures a tissue's propensity to yield MSC-like cells, and a quantifiable metabolic assay, which indicates a tissue's overall health status, we show that the assays are able to measure and compare the relative quality of UC tissues and identify impacts to UC tissue quality caused by various treatment scenarios. We believe that these approaches represent a starting point for the development of such quality assays as will be required for clinical application of MSCs derived from UC tissue.

Materials and methods

Cord blood collection unit characteristics

A random sampling of 1000 cord blood collections processed and stored between July 1 and December 31, 2014 was selected for analysis from a larger inventory. All collections were from consenting mothers into gravity bags prefilled with 500 units of lyophilized heparin and transported to a processing facility in Tucson, Arizona. Cord blood was processed on the AutoXpress® Platform (Cesca Therapeutics, Inc.), and post-processing aliquots were assessed for total nucleated cell (TNC) counts on a Sysmex hematology analyzer (model XE-2100L, Sysmex America) and for viability using 7-aminoactinomycin D (7-AAD) staining. CD34+ cell content was enumerated on an FC 500 flow cytometer (Beckman Coulter, Inc.) with the Beckman Coulter Stem-Kit. Birth weights of donors in either collection scenario were not significantly different (data not shown), and cord blood collection volume was normalized by birth weight. Units more than 48 hours old at time of processing or processed as a protocol exception were excluded from the analysis. All units were collected from births >37 weeks gestation, based on self-reported data.

UC tissue collection and cryopreservation

Donated UC tissue units were collected from consenting mothers following either a surgical or vaginal delivery (>37 weeks gestation) and transported to a processing facility at ambient temperatures in a buffered saline solution containing an aminoglycoside antibiotic at typical concentration. Upon receipt, the cords were decontaminated by a stepwise series of immersion rinses, first in Dulbecco's phosphate-buffered saline (DPBS), then in 70% ethanol and finally in DPBS again. After a final DPBS rinse, the cords were segmented into small sections (0.3 cm^3). Cord tissue sections were submerged in a dimethyl sulfoxide (DMSO)-based, clinical-grade cryopreservation solution (CryoStor CS10, BioLife Solutions) prior to

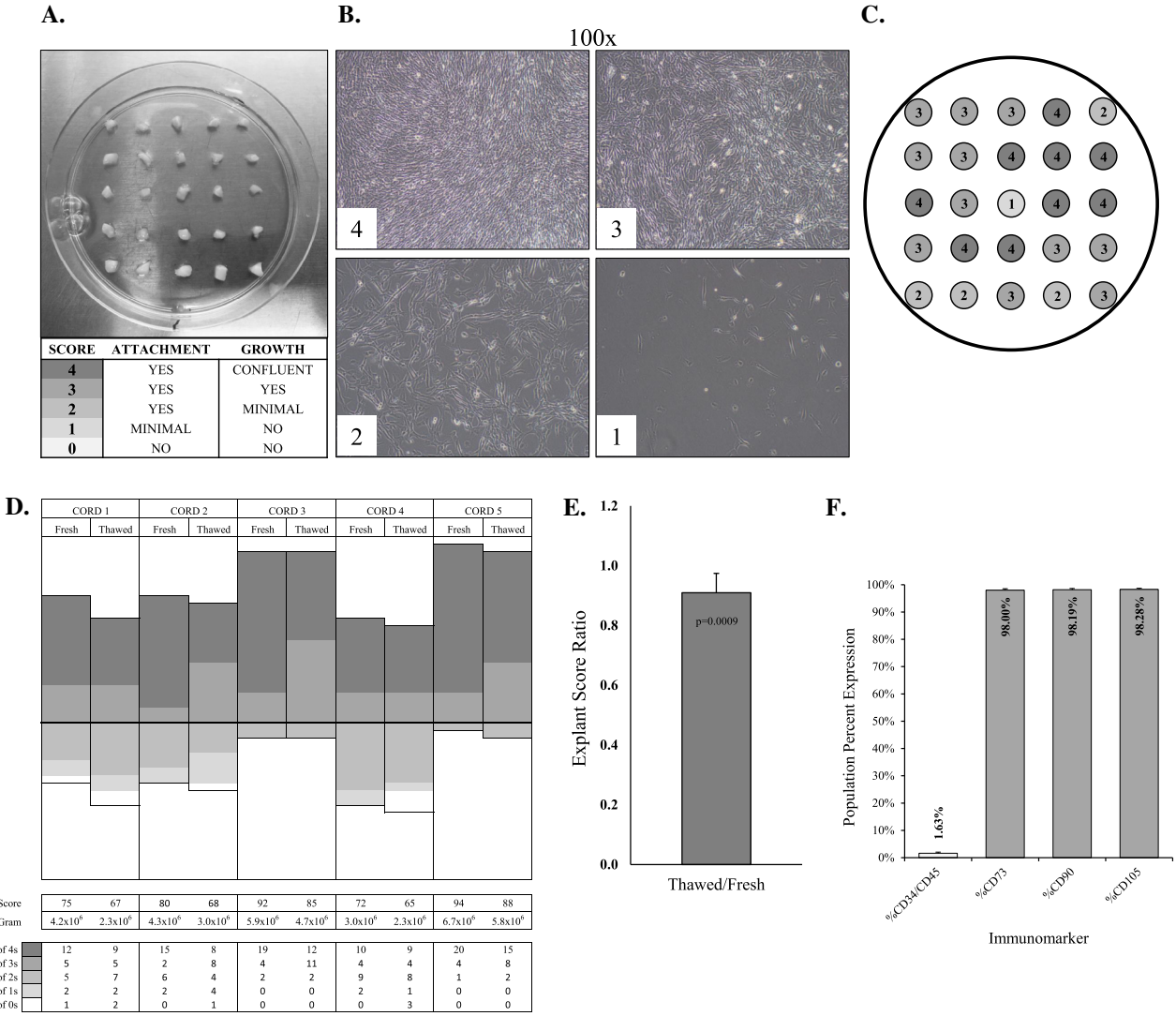

Figure 1. Isolation of MSCs by explant outgrowth in an array amenable to systematic analysis. Example of a 5 × 5 grid of explanted UC tissue pieces from previously cryopreserved tissue plated on a 10-cm cell culture dish. Each of the 25 grid locations was assigned a score on a scale of 0–4 based on degree of cell attachment and cell proliferation (A and B). Depiction of scoring results from a representative plate of previously cryopreserved UC tissue (C). Heat map of location scores per plate comparing outgrowth from explants of representative fresh and frozen UC tissue units (D). Fresh and frozen UC tissue units consistently yield adherent, proliferating cells. Based on explant scores, even fresh cords demonstrate biological variability between units. However, same-unit comparison of frozen to fresh scores shows the ratio is near 1 (n = 10), indicating that fresh and frozen UC tissue is comparable (E). Immunophenotyping confirms the cells isolated from explants of cryopreserved cord tissue express typical MSC markers. Average marker expression of cells isolated from four thawed, explanted UC tissue units is shown (F).

freezing. UC tissue was frozen in a passive controlled-rate freeze device to -80°C, with final storage in a cryogenic dewar (MVE, Pacific Science) at -196°C in the vapor phase of liquid nitrogen.

Isolation of cells by explant outgrowth

After at least 1 month at -196°C, cryopreserved tissue was rapidly thawed at 37°C, taken through a series of DPBS rinses and prepared for plating. Small, uniform tissue pieces were excised from the larger tissue segments using a 4-mm biopsy punch and arranged at regular intervals in a 5 × 5 grid pattern in a 10-cm culture dish (approximately 0.5 g/10 cm dish; Figure 1) treated with attachment substrate (MesenCult-SF Attachment Substrate; Stem Cell Technologies). Grid locations were premarked on the underside of each plate using a template to ensure consistent spacing. Tissue pieces were allowed to rest without medium for 10 min to ensure adherence to the culture plate, then 10 mL of culture medium (MesenCult-XF Medium; Stem Cell Technologies) was added. Tissue pieces did not move or drift from their original placements for the duration of culture. Medium was

exchanged after 7 days of incubation at 37°C and 5% CO_2, at which time the tissue pieces were discarded. On day 14, defined as the end of passage 0 (P0), explants were evaluated and scored.

For comparison of fresh and frozen UC tissue, whole UC units were rinsed and processed as above, and one portion was explanted immediately without cryopreservation while the remainder was cryopreserved then thawed and explanted as described. All fresh-versus-frozen comparisons were, therefore, paired measurements from the same UC unit.

Scoring explants

After 14 days of culture, plates were observed under an inverted microscope (Olympus CKX41; Olympus) at 100× magnification. For each plate, each grid location was observed and assigned a score on a scale of 0 to 4 based on degree of cell attachment and extent of cell proliferation (Figure 1A, 1B and 1C). Overall explant score was the summed score of all 25 tissue piece locations per explant plate. Visual scoring of all plates was performed by the same individual. After scoring each location within the plate, all cells were detached with trypsin and enumerated.

Relative metabolic activity of UC tissue pieces exposed to DPBS, ethanol and cryopreservative

To assess the impact on composite tissue metabolic activity, UC tissues were exposed to DPBS, 70% ethanol or cryopreservation medium (CryoStor CS10), then incubated in the resazurin-based indicator alamarBlue (Thermo Fisher Scientific) as follows (Figure 2A). Donated UCs were collected and decontaminated as described above, then divided into three equal segments. One segment each was placed into a tube containing 40 mL DPBS, 40 mL 70% ethanol or 40 mL CryoStor and left at room temperature for 1 h. Segments were then removed from the tubes and cut into smaller pieces, and 10 tissue samples from each segment were excised with a 4-mm biopsy punch. Each excised tissue piece was placed into a well of a 24-well plate containing 360 µL of medium. alamarBlue reagent was added at 10% total volume to each well, per the manufacturer's instructions, and the plates were incubated at 37°C for 21 h. Wells with no tissue but medium only or medium + alamarBlue were prepared as controls. After 21 h, 100 µL of medium was sampled from each well and loaded into a 96-well plate. Absorbance of samples at 570 nm and 600 nm was measured on a Varioskan LUX multimodal plate reader (Molecular BioProducts, Inc.) and percent reduction of the alamarBlue indicator was calculated from the measured values.

Comparison of metabolic activity and explant growth in same-unit UC tissues exposed to DPBS, ethanol, formalin or aged for 4 days

To compare the relative impact on composite tissue metabolic activity and explant outgrowth measurements, tissue samples from the same UC unit were subjected to a number of potentially quality-impacting treatments (Figure 2B). Donated UCs were collected and rinsed as described above, then divided into five equal segments. For each UC, one tissue segment was placed into a tube containing 40 mL DPBS as a control sample. A second tissue segment was placed into a sterile sample cup containing 60 mL DPBS and left at room temperature for 4 days. Two of the remaining tissue segments were submerged in a bath containing 70% ethanol, and the fifth segment was submerged in a bath containing 10% buffered formalin. After 30 sec, the formalin-treated tissue segment was removed and placed in a tube containing 40 mL DPBS. After 1 min, one ethanol-treated tissue segment was removed and placed in a tube containing 40 mL DPBS. Finally, after 20 min, the second ethanol-treated tissue segment was removed and placed in another tube containing 40 mL DPBS.

For each UC unit, segments from each treatment group were cut into smaller pieces from which 37 tissue samples were excised with a 4-mm biopsy punch. Twenty-five excised tissue pieces were explanted in a 5×5 grid pattern, as described above, while the remaining 12 pieces were placed in groups of two (six replicates) into wells of a 24-well plate containing 360 µL of medium each. After excised tissue pieces from all treatment groups had been added to the well plate, 40 µL of alamarBlue was added to each well and the plate was incubated for 20 h. Wells with no tissue but medium only or medium + alamarBlue were prepared as controls. After 20 h, 100 µL of medium was sampled from each well and loaded into a 96-well plate and absorbance at 570 nm and 600 nm was measured, as above. Tissue explants from each treatment group were scored as described above, and results were compared with alamarBlue assay results.

Flow cytometry

Cord tissue cells collected from explants at the end of P0 were collected for flow cytometry by rinsing with DPBS and harvesting with a 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution. Immunophenotyping was performed with the following mouse monoclonal antibodies: R Phycoerythrin-Cyanin 7 (PC7)-conjugated anti-human CD73 (Becton, Dickinson and Company), R Phycoerythrin-Cyanin 5.(PC5)-conjugated anti-human CD90, R Phycoerythrin (PE)-conjugated anti-human CD105 and a negative marker mix of R Phycoerythrin-Texas

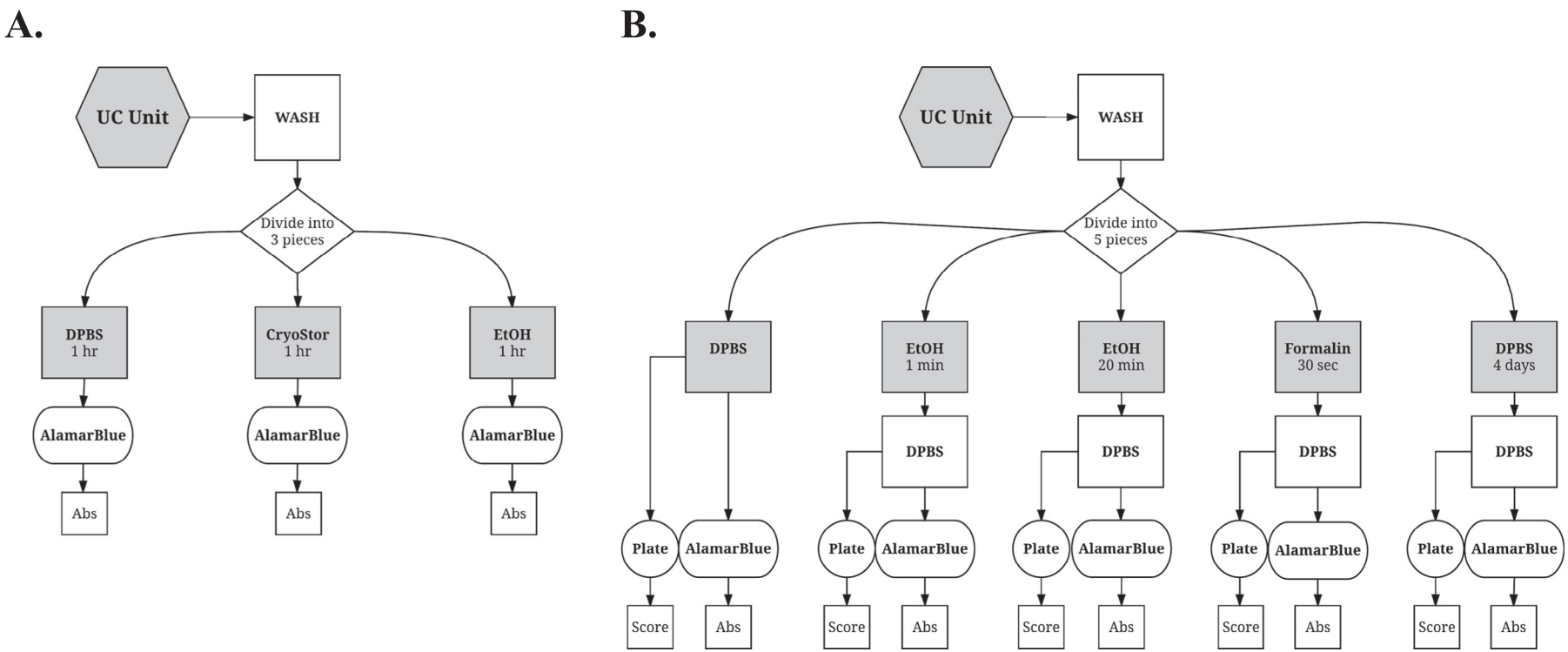

Figure 2. UC tissue sampling experimental design schematics. Schematics for the sampling of UC segments exposed to different conditions followed by assessment of metabolic activity alone (A) or in conjunction with determination of impact on UC quality by the explant outgrowth scoring matrix (B). EtOH, ethanol; Abs, absorbance.

Red-X (ECD)-conjugated anti-human CD34 and ECD-conjugated anti-human CD45. Additionally, cryopreserved cells harvested from P1 cultures were thawed and stained with a broader negative MSC marker mix, including ECD-conjugated CD34, CD45, CD19, CD14 and HLA-DR. Unless otherwise indicated, all antibodies and isotype controls were obtained from Beckman Coulter. All analysis was performed on the FC500 flow cytometer (Beckman Coulter). Cord blood units were analyzed using flow cytometry to determine viability by 7-AAD, total cell content and CD34+ cell content (Stem Kit; Beckman Coulter).

Statistics

Statistical analysis was performed with the use of JMP 8.01 software (SAS Institute) and in Excel (Microsoft). Except where noted, all data are presented as mean +/- standard deviation (SD). One-way analysis of variance (ANOVA) and paired *t*-tests were used to evaluate differences between UC groups, with $P < 0.05$ considered statistically significant. A Student's *t*-test was used to identify statistically significant differences between unit characteristics of cord blood collected with tissue and cord blood not associated with a cord tissue collection.

Results

Collection of UC tissue and cord blood from the same donor

While many stem cell banking institutions are establishing UC tissue storage programs, little research has been published on how the addition of a second collection procedure affects cord blood collection. One potential impact of a dual collection scenario is that a healthcare provider may prematurely terminate a cord blood collection to allow sufficient time for the collection of the cord tissue. We performed a retrospective analysis of 1000 cord blood units collected either with or without UC tissue to determine if there was an impact on unit characteristics important for clinical applications (Table I). No significant difference in the mean collection volume ($P > 0.05$) was observed, nor was there a difference in cord blood cell viability ($P > 0.05$).

Furthermore, TNC count, percent CD34+ cell content and number of CD34+ cells did not differ ($P > 0.05$) between cord blood collected with and without tissue and were within ranges typically reported by family cord blood banks.

Quantitative analysis of explant outgrowth from fresh and frozen UC tissue

To assess UC tissue explants, a scoring matrix that considers both cell adherence to culture plastic and evidence of cell proliferation was developed (Figure 1A, 1B and 1C). For a single explant plate, potential scores range from 0 to 100. Explant outgrowth from 10 UC tissue units cryopreserved for 1 month at -196°C was compared with results for the same cords plated as fresh tissue. Proliferating, plastic-adherent cells with fibroblastic morphology were obtained from all fresh and frozen UC units, achieving successful cell isolation from 100% of UC samples. Tissue pieces were sufficiently spaced so that neighboring colonies remained distinct and did not come in contact or merge with one another. Figure 1D shows representative fresh and thawed explant scores for 5 UC units. The average explant score for fresh UC tissue was 74.4 ± 14.4 and for thawed UC tissue was 67.8 ± 14.6. This difference was small but detectable ($P = 0.0009$), with the average explant score ratio between same-unit thawed and fresh tissue being 0.91 ± 0.06 (Figure 1E). Across all samples, average cell yield at the end of explant culture (P0) was 3.6 ± 1.8 million cells per gram of tissue explanted. The range of differences in explant scores between fresh UC units was 3.9-fold higher than the range of intraunit differences between fresh and frozen samples, highlighting the inherent biological variability in UC units.

Cell populations isolated by explant outgrowth from cryopreserved UCs were positive for the MSC markers CD73 (98.0% ± 0.5%), CD90 (98.2% ± 0.4%) and CD105 (98.3% ± 0.4%) and negative for the hematopoietic markers CD34 and CD45 (collectively, 1.63% ± 0.41%; Figure 1F). P1 cultures exhibited

Table I. Cord blood unit characteristics collected with and without UC tissue.

	Mean normalized collection volume (SD)	Mean postprocessing viable TNC count ($\times 10^6$) (SD)	Mean postprocessing % viability (SD)	Mean postprocessing % $CD34^+$ cells (SD)	Mean postprocessing $CD34^+$ cells ($\times 10^6$) (SD)
Cord blood and UC tissue (n = 428)	0.020 mL/g (0.008 mL/g)	830.67 (438.89)	96.14% (2.19%)	0.38% (0.23%)	3.40 (3.39)
Cord blood only (n = 572)	0.020 mL/g (0.009 mL/g)	861.40 (471.20)	96.09% (1.70%)	0.39% (0.25%)	3.64 (3.88)
P	>.05	>.05	>.05	>.05	>.05

further reduction of negative MSC markers, with CD34, CD45, CD14, CD19 or HLA-DR being collectively present in only 0.14% ± 0.03% of cells.

Correlation of explant outgrowth to metabolic active and cell yield

Resazurin reduction assays are commonly used to estimate metabolic activity of cells, and groups have recently established the use of the assays to monitor three-dimensional culture systems including bioreactors and tissue engineered constructs [26,27]. To determine if the results of the explant scoring system correlated to a quantifiable health and viability measurement, it was first necessary to establish the feasibility of modifying an alamarBlue-based assay for use with composite tissue material. Similarly sized pieces of cord tissue were excised from whole UC units (n = 10) using a biopsy punch and the impact of a 1-h incubation in CryoStor or 70% ethanol was assessed (Figure 2A). As seen in Figure 3, a statistically significant decrease in the relative reduction of alamarBlue indicator was observed for UC tissue pieces exposed to ethanol for 1 h compared with tissue from the same cord exposed to DPBS. In contrast, and as anticipated, no difference was observed for cord tissue pieces exposed to CryoStor relative to the DPBS control.

Having demonstrated the feasibility of the assay approach, UC units (n = 12) were exposed to conditions anticipated to adversely impact cell health, and same-unit tissue was concurrently assessed using both alamarBlue and explant outgrowth assays (Figure 2B). As shown in Figure 4A, the average percent reduction of alamarBlue indicator by

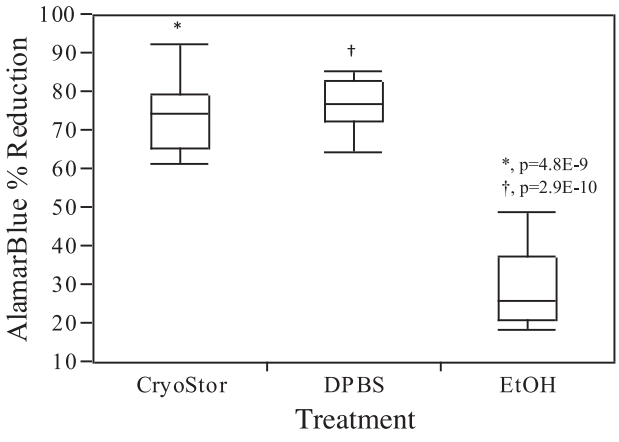

Figure 3. Relative measurement of metabolic activity of UC tissue exposed to cryopreservation medium or ethanol for 1 hour. Metabolic activity was estimated based on reduction of alamarBlue indicator following incubation of segments of the same cord for an hour in CryoStor, DPBS or 70% ethanol. There were statistically significant differences between CryoStor CS10 and ethanol, and between DPBS and ethanol.

treated UC tissue pieces was as follows: DPBS (70.4 ± 7.6%), ethanol (EtOH) 1 min (64.9 ± 6.8%), EtOH 20 min (46.7 ± 5.3%), Formalin 30 sec (41.2 ± 9.8%) and aged 4 days (46.2 ± 10.8%). Average explant scores for treated UC tissue were DPBS (85.5 ± 7.2), EtOH 1 min (80.3 ± 8.6), EtOH 20 min (50.6 ± 13.7), Formalin 30 sec (0.0 ± 0.0) and aged 4 days (54.6 ± 19.7). Average cell yields per gram of tissue from explants of treated UC tissue were DPBS ($5.73 \times 10^6 \pm 1.78 \times 10^6$), EtOH 1 min ($5.07 \times 10^6 \pm 1.95 \times 10^6$), EtOH 20 min ($2.06 \times 10^6 \pm 1.64 \times 10^6$), Formalin 30 sec (0.0 ± 0.0) and aged 4 days ($2.48 \times 10^6 \pm 1.96 \times 10^6$). As noted in Table II, within each assay, differences between all treatment groups were significant except for UC tissue exposed to ethanol for 30 min versus tissue aged for 4 days, which did not differ significantly for any assay, and UC tissue exposed to formalin for 30 sec versus tissue aged for 4 days, which did not significantly differ in metabolic activity but did significantly differ in explant score and explant cell yield. While exposure of UC tissue to ethanol for 1 min in addition to the validated 30-sec exposure during the decontamination step of UC tissue processing resulted in a small but significant decrease in the alamarBlue quality measure, alamarBlue results for the validated 30-sec decontamination in EtOH alone did not significantly differ from a 30-sec control wash in DPBS ($P = 0.9463$; data not shown). UC tissue exposed to formalin for 30 sec exhibited measurable, although reduced, metabolic activity but did not produce any cells in explant culture. Excluding Formalin-treated UC samples, percent reduction of alamarBlue correlated positively and linearly to explant score with an R^2 of 0.69 and a correlation coefficient of 0.83 (Figure 4B). Explant assay score was also positively correlated to explant cell yield ($R^2 = 0.85$).

Discussion

The worldwide number of clinical trials evaluating MSC-based interventions in potential regenerative medicine applications continues to increase. Because UC tissue is an abundant source of MSCs that can be collected in a noninvasive manner and preserved at birth, many stem cell banks have established programs to store UC tissue, and it is likely that UC tissue banking will be an important part of the clinical success of UC-derived MSC treatments. UC tissue banking appears to be a particularly good fit for UC blood banks, which are already experienced at preserving stem cells from neonatal sources. Indeed, collection at birth of both cord blood, for its hematopoietic stem cell content, and UC tissue, for its MSC content, is appealing and convenient. However, the relative novelty of UC tissue banking means that a full understanding

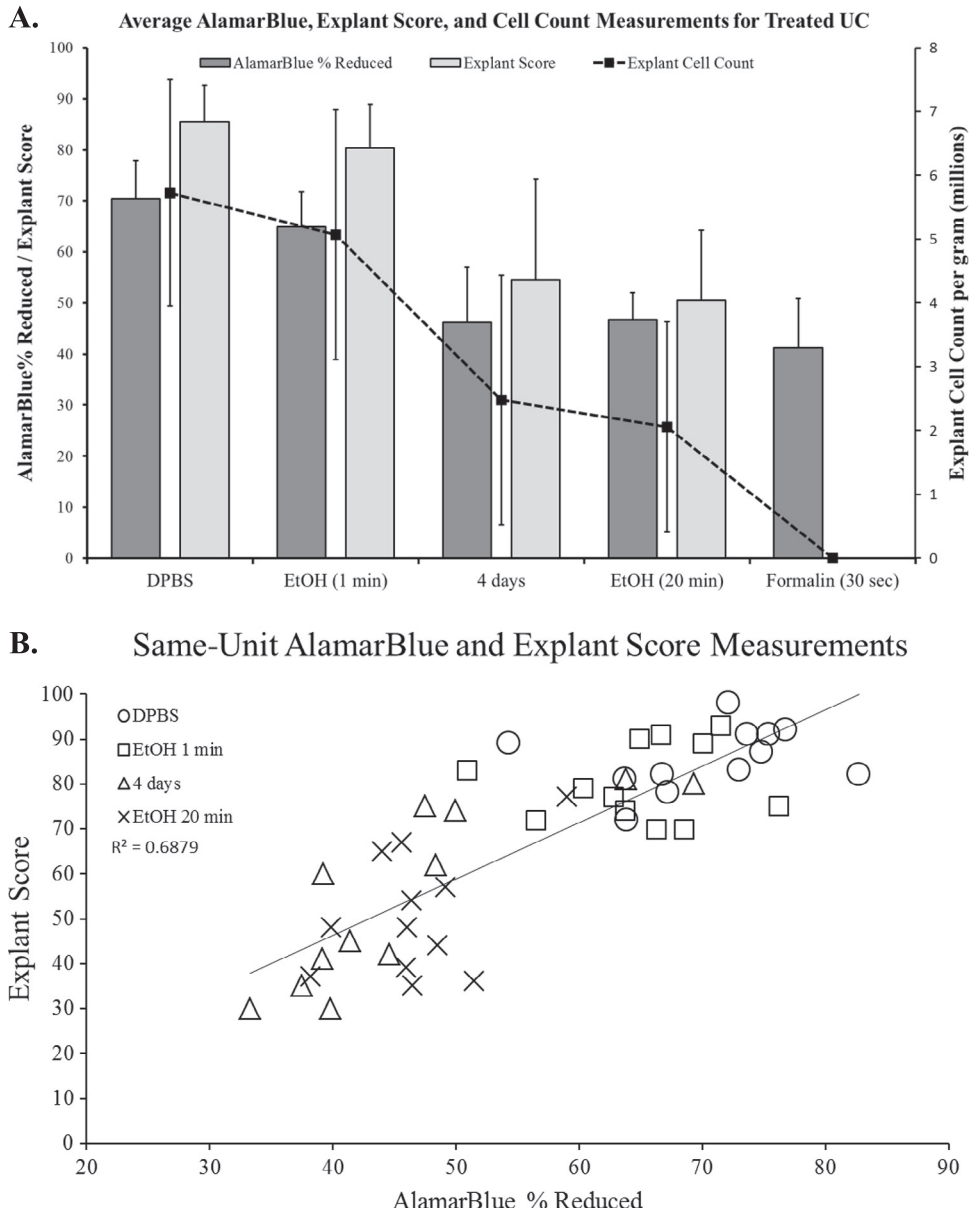

Figure 4. Comparison of metabolic activity, explant score and cell yield of UC tissue exposed to various conditions. Metabolic activity was estimated based on reduction of alamarBlue indicator following incubation of segments of the same cord in potential quality impacting conditions (ethanol, 1 min; ethanol, 20 min; Formalin, 30 sec; DPBS, 4 days) as compared with conditions previously established not to affect tissue quality (DPBS, 1 min). Concurrently, explants of the same cords were prepared, with explant scores and cell yields determined at the end of P0 (A). The reduction in alamarBlue was positively correlated to explant assay score (B).

of how UC tissue can be best collected, handled and stored as well as how UC tissue collection might impact a concurrent cord blood collection is still being developed. In an attempt to address some of these questions, this work (i) shows that collection of UC tissue concurrently with cord blood at birth does not impact the quality of the cord blood collection, (ii) suggests that a serum-free, clinical-grade cryopreservation medium is a suitable storage solution for composite UC tissue, (iii) confirms that MCS-like cells can be consistently isolated from explants of composite UC tissues that have been previously cryopreserved and (iv) demonstrates two approaches for quantifiably characterizing the quality of composite UC tissue, which may be useful in establishing MSC yield expectations and for tuning processes to consistently maximize the yields expected from stored UC tissues.

UC blood banking as an industry has existed for over two decades. More than 200 public and private cord blood banks exist worldwide, and it is estimated that more than 40 000 cord blood hematopoietic stem

Table II. Statistical comparison of alamarBlue, explant score and cell yield measurements for UC tissue under study treatments.

	alamarBlue % reduced				Explant score				Cell yield/g			
	Mean 1	Mean 2	% Mean difference	*P* (paired test)	Mean 1	Mean 2	% Mean difference	*P* (paired test)	Mean 1	Mean 2	% Mean difference	*P* (paired test)
DPBS vs EtOH (1 min)	70.4	64.9	7.81%	.0027[a]	85.5	80.3	6.08%	.0019[a]	5.7×10^6	5.1×10^6	11.44%	.0136[a]
DPBS vs EtOH (20 min)	70.4	46.7	33.66%	8.0×10^{-7}[a]	85.5	50.6	40.82%	1.8×10^{-7}[a]	5.7×10^6	2.1×10^6	64.07%	3.8×10^{-6}[a]
DPBS vs 4 days	70.4	46.2	34.38%	4.8×10^{-5}[a]	85.5	54.9	35.79%	2.1×10^{-5}[a]	5.7×10^6	2.5×10^6	56.69%	7.5×10^{-6}[a]
DPBS vs Formalin (30 sec)	70.4	41.2	41.48%	4.4×10^{-8}[a]	85.5	0.0	100.00%	2.0×10^{-13}[a]	5.7×10^6	0	100.00%	2.5×10^{-7}[a]
EtOH (1 min) vs EtOH (20 min)	64.9	46.7	28.04%	9.1×10^{-6}[a]	80.3	50.6	36.99%	1.3×10^{-6}[a]	5.1×10^6	2.1×10^6	59.43%	1.7×10^{-5}[a]
EtOH (1 min) vs 4 days	64.9	46.2	28.81%	.0003[a]	80.3	54.9	31.63%	2.7×10^{-5}[a]	5.1×10^6	2.5×10^6	51.10%	1.2×10^{-5}[a]
EtOH (1 min) vs Formalin (30 sec)	64.9	41.2	36.52%	2.7×10^{-7}[a]	80.3	0	100.00%	3.0×10^{-12}[a]	5.1×10^6	0	100.00%	2.1×10^{-6}[a]
EtOH (20 min) vs 4 days	46.7	46.2	1.07%	.8589	50.6	54.9	−8.50%	.2522	2.1×10^6	2.5×10^6	−20.51%	.3128
EtOH (20 min) vs Formalin (30 sec)	46.7	41.2	11.78%	.0181[a]	50.6	0.0	100.00%	5.9×10^{-8}[a]	2.1×10^6	0	100.00%	.0012[a]
4 days vs Formalin (30 sec)	46.2	41.2	10.82%	.1996	54.9	0.0	100.00%	1.1×10^{-6}[a]	2.5×10^6	0	100.00%	.0011[a]

DPBS, Dulbecco's phosphate-buffered saline; EtOH, ethanol (70%).
[a]Statistically significant at $P < 0.05$.

cell transplantations have been performed worldwide as part of the treatment of dozens of conditions [24,28,29]. It is essential to confirm that collection of UC tissue with cord blood does not damage the quality of the UC blood unit, especially considering that half of the existing cord blood banks now offer UC tissue banking [24]. The present work reports no significant difference in average UC blood collection volume, viable TNC count or $CD34^+$ content for cord blood units collected along with UC tissue compared with cord blood units collected without a concurrent UC tissue collection. This supports that both UC blood and tissue can be collected at birth without affecting the quality of the UC blood collection, providing an opportunity for storage of multiple, complementary cell resources in a large-scale banking setting.

In addition to concerns surrounding the risk of xenogenic pathogen exposure, serum products, which are commonly used in tissue and cell culture reagents, often exhibit variability in lot quality. For these reasons, there is an industry-wide effort to reduce the use of serum-containing products in manufacturing processes, especially those of clinical-grade products. In the context of cord tissue banking, there have been reports of technical challenges in isolating cells from UC tissue cryopreserved as a composite material when using a cryoprotectant that includes either human- or fetal bovine–based serum or plasma [30]. Previous reports have found that serum-free, clinical-grade, DMSO-based solutions provide suitable protection during cryogenic storage of composite UC tissue [14,23,31]. In fact, the reports by Shimazu *et al.* suggest that a serum- and xeno-free cryoprotectant is superior to serum-containing cryoprotectant solutions and that prolonged exposure to such a cryoprotectant solution is feasible without notable impact on cell recovery or function [23]. Consistent with these results, we found that an hour-long, room temperature exposure of fresh UC tissue to a serum-free, DMSO-based cryoprotective medium did not reduce the metabolic activity of the tissue. Furthermore, when UC tissue that had been cryopreserved with the serum-free cryopreservation medium was thawed and explanted, all tissues yielded fibroblastic, clonogenic, MSC-like cells. This outcome contrasts with previous studies that have reported an inability to recover viable MSCs from cryopreserved UC tissue when using enzymatic tissue digestion as the harvest technique [32]. Chatzistamatiou *et al.* also reported that cells cryopreserved immediately following isolation by enzymatic digestion failed to grow in culture, with only 10% of units cryopreserved for 1 week or 6 months yielding cells that exhibited growth [30]. Alternatively, allowing the cells to recover in culture medium following enzymatic digestion and prior to

cryopreservation resulted in improved post-thaw recovery. This suggests that enzymatic digestion protocols may increase the sensitivity of isolated cells to cryopreservation and that explant isolation of MSCs from UC tissue may be the preferable method. Our results indicate that cryopreservation of composite UC tissue in a serum-free, DMSO-based cryoprotective medium does not prevent the later collection of MSC-like cells from the thawed tissue by the explant method.

Of the institutions offering UC tissue storage, roughly half cryopreserve the UC tissue as a composite material [24]. The job of isolating and expanding cells from the cryopreserved tissue for clinical use may ultimately fall to one of a variety of entities, including a contract manufacturing organization, the tissue bank itself or a treatment facility. While the responsibility for ensuring that the final, UC tissue-derived cellular product meets all applicable critical release criteria—including identity, safety, purity and potency—may lie with a different entity, the banking institution can monitor the quality of the source UC tissue that it cryopreserves to provide an estimate for post-thaw cell yield. It is, therefore, important to consider approaches for determining the potential progenitor cell yield from a given UC tissue unit at the time of storage. Such quantitative measures of UC tissue quality could also allow for a better characterization of the range of biological differences that exist between UC tissue donors or variables external to the cord blood bank operating environment. Furthermore, changes in tissue quality metrics could identify how particular aspects of processing impact UC tissue quality and could inform process optimization efforts. The approaches presented in this work describe such measurements.

The capacity to produce rapidly proliferative, fibroblastic cell colonies is an established characteristic of explanted UC tissue, and that these cells are MSC in nature is also well accepted. Furthermore, numerous studies report no obvious difference in MSC characteristics, including morphology, immunophenotype and differentiation potential, between MSCs isolated from pre-freeze and post-thaw UC tissues [22,23,25,32,33]. Therefore, we established a standardized approach to preparing and scoring UC tissue explants as a measurement of UC tissue quality. In agreement with the published literature, cells isolated from our explants were plastic-adherent, proliferated rapidly, displayed fibroblastic morphology and exhibited a typical MSC surface marker profile. Explant scores in mild treatment groups, where minimal change in quality would be anticipated (fresh versus thaw and DPBS versus 1 min exposure to EtOH), were much more similar between explants from the same tissue than between tissues from different donors, as demonstrated by average percent differences in scores that were 2.56- and 1.68-fold higher, respectively, between tissues from different donors than between treatment groups for the same tissue. This suggests that UC tissue quality measurements are consistent within a tissue and highlights the inherent, presumably biologic, variability in tissue quality between donors. Measured variability between donors in turn emphasizes the importance of being able to measure UC tissue quality to establish unit-specific expectations for the tissue.

An assay of UC tissue metabolism was also examined as an approach for measuring UC tissue quality. Metabolic scores for UC tissues correlated well to both explant score and enumerated cell yields from UC tissue explants, while providing a result within 1 day as opposed to 2 weeks commonly used for explant-based assays. This rapid quality indicator may allow for assessment of an UC tissue unit's quality status at receipt, prior to any significant processing steps taking place. Both explant and metabolism assays were capable of resolving statistically significant differences between treatment groups where such differences were expected, with the assays resolving statistical differences between groups as small as 6.1% and 7.8%, respectively. Both explant and metabolic scores were predictive measures of a UC tissue unit's ability to produce MSC-like cells. Furthermore, there was less variance in explant and metabolism scores than in overall explant cell yields, which are partially a function of exponential growth.

The methods presented in this work represent a starting point in the standardized analysis of composite UC tissue quality. Although characterization of the identity and properties of MSCs isolated from UC tissue and how processing and handling of UC tissue impacts these properties continues and will be required in preparation for clinical application of UC MSCs, analysis of composite UC tissue quality will be important for the development of best practices in the UC tissue banking industry, which strives to preserve quality material to supply for future clinical therapies.

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