Original Article

Cells Remain Viable When Collected With an In-Line-Suction Tissue Collector From Byproducts of Anterior Cruciate Ligament Reconstruction Surgery

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Purpose: To investigate the viability of cells collected with an in-line-suction autologous tissue collector from the tissue byproducts of arthroscopic anterior cruciate ligament (ACL) reconstruction, to characterize cells from different tissue types, and to identify mesenchymal stem cells. **Methods:** Patients aged 14 to 50 years with ACL injuries requiring arthroscopic reconstruction surgery were offered enrollment and screened for participation. In total, 12 patients were enrolled in the descriptive laboratory study. Arthroscopic byproduct tissue was collected with an in-line-suction autologous tissue collector from 4 intraoperative collection sites for each patient: ACL stump, ACL fat pad, notchplasty debris, and tunnel drilling debris. All tissue samples were digested using collagenase, and the derived cellular populations were analyzed in vitro, characterizing cellular viability, proliferative potential, qualitative multipotent differentiation capacity, and cell-surface marker presence. **Results:** An equivalent mass of arthroscopic byproduct tissue was taken from each of the 4 intraoperative collection sites (1.12-1.61 g, $P = .433$), which all showed an average viability of at least 99.95% and high average total nucleated cells ($\geq 1.37 \times 10^7$ cells/mL). No significant differences in collected mass ($P = .433$), cellular viability ($P = .880$), or total nucleated cells ($P = .692$) were observed between the 4 byproduct tissues. The byproduct tissues did exhibit significant differences in monocyte ($P = .037$) and red blood cell ($P = .038$) concentrations, specifically with greater values present in the ACL stump tissue. Cells from all byproduct tissues adhered to plastic cell culture flasks. Significant differences were found between colony-forming unit fibroblast counts of the 4 byproduct tissues when plated at 10^6 ($P = .003$) and 10^3 ($P = .016$) cells as the initial seeding density. There was a significant relationship found between both the starting concentration ($\chi^2 = 32.7$, $P < .001$) and the byproduct tissue type ($\chi^2 = 30.4$, $P < .001$) to the presence of $\geq$80% confluency status at 10 days. Cells obtained from all 4 byproduct tissues qualitatively showed positive tri-lineage (adipocyte, osteoblast, chondroblast) differentiation potential compared with negative controls under standardized in vitro differentiation conditions. Cells derived from all 4 byproduct tissues expressed cell-surface antigens CD105+, CD73+, CD90+, CD45−, CD14−, and CD19− (>75%), and did not express CD45 (<10%). There were no statistically significant differences in cell-surface antigens between the four byproduct tissues. **Conclusions:** This descriptive laboratory study demonstrated that cells derived from arthroscopic byproduct tissues of ACL reconstruction remain viable when collected with an in-line-suction autologous tissue collector and these cells meet the ISCT criteria to qualify as mesenchymal stem cells. **Clinical Relevance:** It is known that viable mesenchymal stem cells reside in byproduct tissue of anterior cruciate ligament reconstruction surgery (ACLR). Practical methods to harvest these cells at the point of care require further development. This study validates the use of an in-line-suction autologous tissue collector for the harvest of viable mesenchymal stem cells after ACLR.

As the orthopaedic community has considered cell therapies to adjunct current practice, attention has been turned to the most appropriate, convenient, and cost-effective method to harvest and deploy autologous tissues, including sources of resident mesenchymal stem cells (MSCs). MSCs reside in the knee within synovial tissue, adipose tissue, cartilage tissue, anterior cruciate ligament (ACL) injury effusion

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fluid, and the ACL stump. Cells from these sources have shown promise regarding proliferative potential, multipotentiality, and the ability to improve healing.[1-10] However, not all stem cells are MSCs. MSCs differ from embryonic stem cells, which have the capacity to differentiate into any cell in the human body. The International Society for Cellular Therapy (ISCT) has proposed 3 discrete criteria to define and qualify cells as MSCs: adherence to plastic, specific surface antigen (CD105+, CD73+, CD90+, CD45−, CD34−, CD14− or CD11b−, CD79alpha− or CD19−, and human leukocyte antigen class II−) expression, and multipotent differentiation potential (adipocytes, osteoblasts, chondroblasts).[11] MSCs are not progenitor cells, which are the earliest generation of cells that are produced from stem cells such as MSCs. Progenitor cells become progressively less capable of self-renewal and differentiation as they reproduce and become more specialized.

Harvesting MSCs from the knee using a variety of tissue sources has become a method of interest.[7,9,10] When considering which tissues house MSCs, not only the number of cells should be considered but also the viability, proliferative potential, and differentiation capacity of the cells. During arthroscopic anterior cruciate ligament reconstruction (ACLR) surgery, injury effusion fluid, stump tissue, synovial tissue, and fat tissue found inside the knee are removed for visualization and working purposes. Devices such as the GraftNet (Arthrex, Naples, FL) have been developed to harvest tissue typically classified as byproduct waste through a shaver and suction tubing system. This allows for filtered and sterile autologous tissue collection that can be redeployed to the patient during surgery. However, it is uncertain whether the cells contained within these byproduct tissues remain viable and how many, if any, MSCs are contained within them.

The purposes of this study were to investigate the viability of cells collected with an in-line-suction autologous tissue collector from the tissue byproducts of arthroscopic ACLR, to characterize cells from different tissue types, and to identify MSCs. We hypothesized that (1) cells would remain viable when collected from 4 distinct sites of the knee that are considered arthroscopic byproducts during ACL reconstruction and (2) harvested cells would meet the ISCT criteria of MSCs.

Methods

Participants

This was a prospective benchtop study with no clinical follow-up. Institutional review board approval was obtained (number 1554613-1, Baptist Hospital Institutional Review Board). Enrollment started August 2021 and completed in November 2021 and was executed in the outpatient setting at the primary institute (Andrews Research & Education Foundation). Patients who were scheduled to have ACLR by one of the investigating physicians were screened for participation in the study by members of the research team. Exclusion criteria included patients requiring ACL and posterior cruciate ligament combined surgery, patients with a history of an autoimmune disease, diabetes, a blood/clotting disorder, or a history of previous surgery on the injured knee. Benchtop analysis was completed in December 2022, and final data analysis was completed by April 2023. No changes were made to methods nor outcome measures after trial commencement. However, the inclusion age range was changed several times at the request of the primary investigator: from 14-40 to 22-40 years (April 2021), 22-40 to 18-40 years (August 2021), and 18-40 to 14-50 years in (September 2021). Additionally, the blood draw procedure was removed, and REDCap was added as a form of electronic informed consent. Finally, the enrollment goal was lowered from 30 to 12 based on preliminary data, and the 4 byproduct tissues being analyzed were specified in the protocol upon sponsor request in November 2021.

Intraoperative Tissue Harvest

The surgical procedures were independently performed by 3 surgeons (A.W.A., S.E.J., R.V.O.). All participants underwent the standard-of-care arthroscopic ACL reconstruction procedure. Approximately 1 mL of tissue was collected intraoperatively at the time of the preparation phase of arthroscopic ACLR surgery with the GraftNet in-line-suction autologous tissue collector (Arthrex). The tissue collection device was used in-line with an arthroscopic shaver or in isolation with suction (with tibial tunnel drilling) to collect resected tissue. Four separate collectors were used for each patient to obtain arthroscopic byproduct tissue from the following 4 distinct sites: the ACL stump, ACL infrapatellar fat pad, cartilage debris during notchplasty, and bone debris during tunnel drilling. Tissue taken from these 4 sites was reflective of tissue considered waste during standard-of-care ACL reconstruction. The tissue collections were then transferred to preweighed, sterile 50-mL conical tubes on the back table for transport. Immediately after collection, the sample tubes were labeled, loaded into a biohazard bag, and transported to a biologic laboratory within the Andrews Research & Education Foundation (AREF).

Tissue Processing

Tissue samples were processed following Good Laboratory Practices within the laboratory immediately after transport. All 50-mL conical tubes were weighed and a final collected sample weight was determined by comparing final weight to the empty weight of the conical tube. A collagenase type 1 enzyme (Worthington Biochemical Corporation, Lakewood, NJ) was

made up to 1 mg collagenase/1 mL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) before being used to enzymatically digest the tissue samples at a concentration of 1 mL of collagenase per 0.1g of tissue. The samples were then incubated and rocked in their respective 50-mL conical tubes with collagenase for 18 hours at 37°C and 5% CO_2. After digestion, room temperature phosphate-buffered saline (PBS) was added to normalize all sample volumes to 35mL before filtering each sample through a sterile 70-μm filter and into a new 50-mL conical tube. All samples were then centrifuged at 1500 RPM for 5 minutes. The supernatant was gently decanted into a waste container and 10 mL of room temperature PBS was added to resuspend the pellet before centrifuging all samples again at 1500 RPM for 5 minutes. The supernatant was gently decanted into a waste container, completing the washing of the samples. Four milliliters of warm DMEM/F-12 complete media (10% fetal bovine serum and 1% penicillin/streptomycin) was added to resuspend the pellet.

On the day of collection, a portion of all samples was prepared for testing with a complete blood cell (CBC) count, colony-forming unit fibroblast (CFU-F) assay, and confluency assays. A portion of all samples was cryopreserved for later evaluation of tri-lineage differentiation assays and flow cytometry. For cryopreservation, a portion of samples was centrifuged at 1500 RPM for 5 minutes before gently decanting the supernatant and adding 1 mL of cold (2-8°C) CryoStor (STEMCELL Technologies, Vancouver, British Columbia, Canada) cryopreservation medium (10% dimethyl sulfoxide) to resuspend the pellet. The resuspended samples were then transferred to sterile cryogenic vials and allowed to cool to 2-8°C before being placed in a controlled-rate freezer to slowly freeze the samples to −80°C at a rate of −1°C/minute. The frozen samples were then placed in a −80°C freezer for up to 1 week before being transferred to a long-term liquid nitrogen cryogenic storage vessel.

Tri-lineage differentiation assays and flow cytometry analysis were performed at a later date. For these experiments, cryogenic vials were removed from the storage vessel and wiped down with 70% isopropanol before gently swirling in a 37°C water bath for 1 to 2 minutes to allow for rapid thawing. After thawing, the samples were transferred to a sterile 50-mL conical tube and mixed with 9 mL of warm DMEM/F-12 complete media before being centrifuged at 1500 RPM for 5 minutes. The supernatant (containing the CryoStor) was gently decanted into a waste container and 4 mL of warm DMEM/F-12 complete media was added to resuspend the pellet.

Tissue Characterization

In total, 20 μL of each sample was aliquoted into microcentrifuge tubes and mixed with AO/PI Viability Stain (Nexcelom, Lawrence, MA) before being analyzed with an automated cell counter (Nexcelom) to determine the number of total nucleated cells (TNC) and sample viability. Cell viability was reported as the percentage of living cells divided by total cells (%) by the automated cell counter. An additional 50 μL of each sample was aliquoted into microcentrifuge tubes for CBC analysis using an XN-350 automated hemacytometer (Sysmex, Kungsbacka, Sweden).

Cell Culture

CFU-F assays were performed as one measure of proliferative potential. Using the aforementioned total nucleated cell count, each of the samples were aliquoted and diluted to concentrations of 10^6, 10^5, 10^4, and 10^3 cells. DMEM/F-12 complete media was added to all diluted samples to reach a final volume of 4 mL before being plated onto Nunc 25-cm^2 cell culture flasks (Thermo Fisher Scientific, Waltham, MA). Cells were incubated for 10 days at 37°C and 5% CO_2. The media was replaced every 2 to 3 days until the incubation time was complete. After 10 days, the media was gently decanted before rinsing each flask with ice-cold PBS and fixing the cells for 10 minutes with ice-cold 70% methanol. After gently decanting the methanol, the cells were covered with 0.5% crystal violet in 95% ethanol and incubated at room temperature for 1 hour. The crystal violet was gently decanted, and the cells were rinsed 3 times with ice-cold PBS to remove residual dye. Cells were allowed to dry at room temperature for at least 24 hours before being visualized using an EVOS M5000 fluorescent microscope (Thermo Fisher Scientific). After visualizing morphology, colonies were counted by 3 independent researchers using a cutoff of "50-cell or above" for being counted as a colony. An average of the 3 observations was calculated for each sample to minimize counting error.[12,13]

Confluency assays were performed as an additional measure of proliferative potential during the CFU-F assay procedure. The confluency of each sample, at each dilution concentration, was monitored over the 10-day period. When the media were replaced, images were taken to track confluency over time. Before and after crystal violet staining for the CFU-F assay procedure, all samples were checked for at least 80% confluency regardless of CFU-F count (with "Yes" signifying that samples reached at least 80% confluency at day 10).

Qualitative adipogenic, osteogenic, and chondrogenic biochemical differentiation assays were performed using StemPro Differentiation Kits (Gibco, Bristol, RI) per manufacturer's instructions. After an initial expansion with DMEM/F-12 complete media in Nunc 25-cm^2 cell culture flasks, the cells were rinsed with PBS and removed from the flask using TrypLE Express Enzyme (Gibco). TrypLE Express is suggested to have a decreased

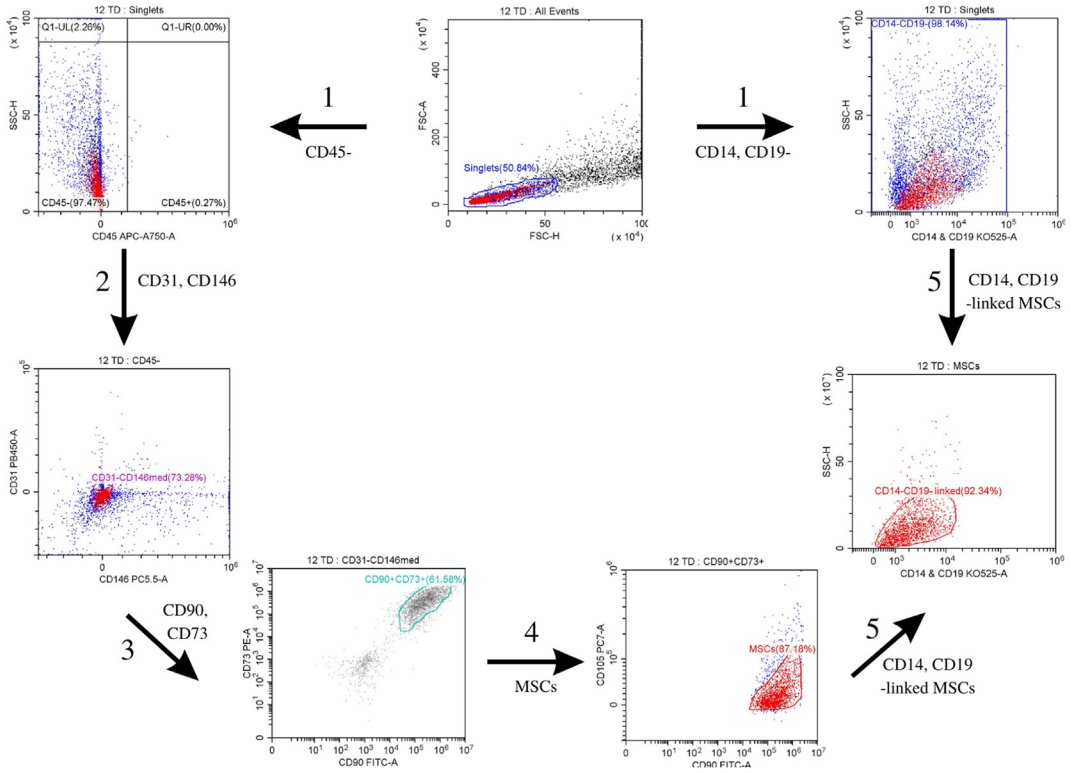

Fig 1. A representative gating strategy for CD45, CD146, CD31, CD90, CD73, CD105, CD14, and CD19. These gates define the area of the scatter plot that should be occupied by MSCs (based on fluorescence intensity) and excludes other types of cells and cellular debris. The x and y axes represent the concentration of a particular antibody that is involved in that specific gating process. The cells within the gate were analyzed as % MSC-specific, whereas all cells on the scatter plot were analyzed as % Total. This gating strategy was recommended by the kit manufacturer (Beckman Coulter, Inc., Indianapolis, IN) and was used in this study. (MSC, mesenchymal stem cells.)

potential for cellular damage, so it was used over traditional trypsin. For adipogenic and osteogenic assays, cells were quantified using the automated cell counter and re-seeded in triplicate to 12-well cell culture plates. The seeding density for adipogenic cells was $1 * 10^4$ cells/cm^2 and osteogenic cells was $5 * 10^3$ cells/cm^2. Each sample was allowed to grow within DMEM/F-12 complete media until 80% confluency was reached, as recommended by the manufacturer for differentiation assays. Media was changed every 3 days. Upon reaching 80% confluency, the DMEM/F-12 complete media was removed and gently rinsed with PBS before being replaced with Differentiation Basal Media with Supplement as part of the StemPro differentiation kits for adipogenic and osteogenic cells. Adipogenesis and osteogenesis continued for 21 days, with media being changed every three days. At 21 days, adipogenic cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 minutes, rinsed twice with deionized (DI) water, and stained with 0.5% Oil Red O solution for 30 minutes. The cells were rinsed 3 final times with DI water before being visualized under a microscope at 10× objective. Oil Red O staining indicates an accumulation of lipids accompanied by a visual lipid droplet, which is a defining biochemical feature of adipocytes. At 21 days, osteogenic cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 minutes, rinsed twice with DI water, and stained with 2% Alizarin Red S solution (pH 4.2) for 30 minutes. The cells were rinsed 3 final times with DI water before being visualized under a microscope at 10× and 20× objective. Alizarin Red S staining indicates an accumulation of calcium, which is a defining biochemical feature of osteoblasts.

For chondrogenic cells, micromass cultures were prepared by removing the cells with TrypLE Express Enzyme, centrifuging them at 1500 RPM for 5 minutes, and resuspending the cells in a minimum amount of DMEM/F-12 complete media. The cells were counted using the automated cell counter and a cell seeding density of $1.6 * 10^7$ viable cells/mL was prepared by centrifugation. Then, 5 µL droplets of the cell solution was added in triplicate 25-mL conical tubes and allowed to incubate at high humidity for 2 hours. After 2 hours, the cultures were topped off with Basal Media with Supplement as part of the StemPro differentiation kit for chondrogenic cells. Chondrogenesis continued for 21 days, with media being changed via centrifugation for 5 minutes at 1500 RPM every 3 days. At 21 days, the

Table 1. Demographics of Participants

Characteristic*	Overall, N = 10*	Sex		*P* Value†
		Male, N = 6*	Female, N = 4*	
General demographics				
Age at surgery, y	25.21 ± 10.13	24.92 ± 12.08	25.65 ± 8.00	.8
Weight, kg	78.60 ± 14.21	79.23 ± 12.42	77.65 ± 18.62	.8
Height, cm	177.27 ± 7.56	180.76 ± 4.93	172.05 ± 8.38	.2
BMI	25.30 ± 5.87	24.47 ± 4.33	26.55 ± 8.28	>.9
Injury details				
Time between injury and surgery, d	43.20 ± 42.66	29.83 ± 18.80	63.25 ± 63.07	.5
Mechanism of injury				.5
Athletic	7 (70%)	5 (83%)	2 (50%)	
Leisure	3 (30%)	1 (17%)	2 (50%)	

BMI, body mass index; SD, standard deviation.
*Mean ± SD; n (%).
†Wilcoxon rank sum test; an asterisk.

media containing the cells was gently mixed and transferred to a 12-well cell culture plate. Media was then removed, and the cells were rinsed gently with PBS, fixed with 4% paraformaldehyde for 30 minutes, rinsed twice with DI water, and stained with 1% Alcian Blue in 0.1 N HCL solution for 30 minutes. The 3-dimensional chondrocytes were rinsed 3 final times with DI water before being visualized under a microscope at 20× objective. Alcian Blue staining indicates an accumulation of glycosaminoglycans, which is a defining biochemical feature of chondrocytes.

Flow Cytometry

Multicolor flow cytometry was performed on a CytoFLEX V0-B5-R3 Flow Cytometer (Beckman Coulter, Inc., Indianapolis, IN). The DURAClone SC Mesenchymal Tube kit (Beckman Coulter, Inc.) was used during the process of flow cytometry. This kit provided a panel of eight cell-surface antigen markers, CD90, CD73, CD146, CD105, CD45, CD31, CD14, and CD19, which allowed for the identification of MSCs per the ISCT guidelines. Gating was completed using the VersaComp Antibody Capture Beads (Beckman Coulter, Inc.) and the compensation kit provided with the DURAClone SC Mesenchymal Tube. The kit provided a specific gating strategy to evaluate each antigen marker, which was used and is provided in Figure 1.

Before the operation of the flow cytometer, the cultured sample was prepared by transferring 100 µL of the sample into one of the tubes contained within the DURAClone SC Mesenchymal Tube kit. The tube was then vortexed for 8 seconds, and then placed in a dark corner away from direct lighting for 15 minutes to incubate at room temperature. Following this, the tube was removed from the corner, 2 mL of VersaLyse (Beckman Coulter, Inc.) solution was added, and the mixture was vortexed again for 8 seconds. The tube was then placed back into the dark corner, at room temperature, for 10 minutes. After incubation, the tube was centrifuged at 150*g* for 5 minutes, and the supernatant was aspirated from the tube. After aspiration, 3 mL of PBS was added to the tube and then vortexed for 8 seconds and centrifuged at 150*g* for 5. The supernatant was aspirated from the tube and a final 500 µL of PBS was added. The tube was then vortexed and run through the flow cytometry protocol after completion of the daily quality control and MSC-specific gating strategy.

Statistical Analysis

Calculations, tabulations, and visualizations of descriptive statistics (means, standard deviations, and ranges) were performed in R using RStudio and the R packages: readxl, tidyverse, gt, gtsummary, and patchwork.[14-19] Demographic differences between the sexes were evaluated using the Wilcoxon rank sum test. Tissue and cellular differences between the byproduct tissue sites, including mass, TNC, viability, CBC, proliferative potential (CFU-F and confluency), and cell-surface markers results were compared using nonparametric methods, specifically the Kruskal–Wallis rank sum test, followed-up with post-hoc testing to determine group differences using the Wilcoxon rank sum test. Parametric testing was not appropriate in this study due to the small sample size and violation of the parametric assumptions, namely normality and homogeneity of variance. Finally, χ^2 tests for independence were used to determine the association between qualitative data measures (e.g., tissue type and confluency, starting concentration and confluency).

Results

Samples from a total of 12 subjects were collected; however, samples from 2 of these subjects was used during the development of tissue-processing procedures. A total of 10 participants were included in this

Table 2. Characterization of Tissue Samples

Characteristic	Tissue				
	Stump, N = 10*	Fat Pad, N = 10*	Notchplasty, N = 10*	Tunnel Drilling, N = 10*	*P* Value†
Sample viability					
Tissue mass, g	1.61 ± 0.87	1.49 ± 1.04	1.12 ± 0.97	1.33 ± 1.29	.433
TNC, cells/mL	$1.41 \times 10^7 \pm 7.40 \times 10^5$	$1.37 \times 10^7 \pm 4.64 \times 10^5$	$1.57 \times 10^7 \pm 5.94 \times 10^5$	$1.65 \times 10^7 \pm 6.78 \times 10^5$	.692
Cell viability, %	99.98% ± 0.06%	99.95% ± 0.11%	99.98% ± 0.06%	99.97% ± 0.09%	.880
CBC					
WBC, K/μL	6.47 ± 16.05	3.38 ± 6.86	0.52 ± 0.78	1.42 ± 2.18	.115
NEU, K/μL	9.10 ± 18.60	4.65 ± 7.21	0.61 ± 0.71	1.57 ± 1.30	.573
Number of no reads	5	5	7	6	
LYM, K/μL	0.87 ± 1.39	1.07 ± 1.57	0.12 ± 0.11	0.24 ± 0.31	.292
Number of no reads	5	5	7	6	
MONO, K/μL	0.84 ± 1.37	0.40 ± 0.42	0.17 ± 0.10	0.09 ± 0.05	.037‡
Number of no reads	5	5	8	6	
EOS, K/μL	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.03	0.12 ± 0.11	.080
Number of no reads	5	5	8	6	
BASO, K/μL	0.11 ± 0.14	0.05 ± 0.05	0.08 ± 0.03	0.07 ± 0.05	.645
Number of no reads	5	5	8	6	
PLT, K/μL	8.40 ± 18.01	2.10 ± 4.65	1.50 ± 2.64	4.40 ± 4.72	.087
RBC, M/μL	0.03 ± 0.07	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.01	.038‡

NOTE. "Number of no reads" samples where characteristics were not present or too small to be measured.

BASO, basophil; CBC, complete blood count; EOS, eosinophil; LYM, lymphocyte; MONO, monocyte; NEU, neutrophil; PLT, platelet; RBC, red blood cell; SD, standard deviation; TNC, total nucleated cell count; WBC, white blood cell.

*Mean ± SD.

†Kruskal–Wallis rank sum test.

‡Indicates significant differences among tissue types at $P < .05$.

study (n = 10). This included 6 male participants (60%) and 4 female participants (40%). The age at surgery for men with a mean ± (SD) was 25 ± 12 years and for women 26 ± 8 years (Table 1). There were no statistically significant differences in general demographics nor injury details between sexes.

Tissue mass (g) collected from surgery, TNC per milliliter, supernatant after collagenase digestion, and viability of cells contained within the supernatant after collagenase digestion are presented in Table 2. For the stump, the mean ± (SD) mass was 1.61 ± 0.87 g, with mean ± (SD) TNC of $1.41 \times 10^7 \pm 7.40 \times 10^5$ cells/mL and viability mean ± (SD) of 99.98 ± 0.06%. For the fatpad, the mean ± (SD) mass was 1.49 ± 1.04 g, with mean ± (SD) TNC of $1.37 \times 10^7 \pm 4.64 \times 10^5$ cells/mL and viability mean ± (SD) of 99.95 ± 0.11%. For the notchplasty debris, the mean ± (SD) mass was 1.12 ± 0.97 g, with mean ± (SD) TNC of $1.57 \times 10^7 \pm 5.94 \times 10^5$ cells/mL and viability mean ± (SD) of 99.98 ± 0.06%. For the tunnel drilling debris, the mean ± (SD) mass was 1.33 ± 1.29 g, with mean ± (SD) TNC of $1.65 \times 10^7 \pm 6.78 \times 10^5$ cells/mL and viability mean ± (SD) of 99.97 ± 0.09%. No statistically significant difference was found between the 4 byproduct tissues for mass ($P = .433$), TNC ($P = .692$), or cell viability ($P = .880$). CBC results are also included in Table 2, which showed significant differences in monocytes ($P = .037$) and red blood cells ($P = .038$) between the byproduct tissues. Specifically, the stump byproduct tissue showed significantly greater concentrations of monocytes, mean ± (SD) 0.84 ± 1.37 K/μL, compared with tunnel drilling debris, mean ± (SD) 0.09 ± 0.05 K/μL ($P = .045$). The stump byproduct tissue also had an elevated amount of red blood cells, mean ± (SD) 0.03 ± 0.07 M/μL, compared with notchplasty debris, mean ± (SD) 0.00 ± 0.00 M/μL ($P = .05$).

The results of the proliferation assays are reported in Table 3 and Figure 2. Cells from all byproduct tissues adhered to plastic cell culture flasks. Assays were started at different concentrations of cells (i.e., varying seeding density), from one thousand cells per plate to one million cells per plate, to evaluate proliferative potential (i.e., how readily cells would grow upon assay). For assays starting with one million cells, a statistically significant difference was found for total CFU counts between the 4 byproduct tissues ($P = .003$), specifically with significantly greater CFU counts observed in the notchplasty debris mean ± (SD) 29.07 ± 51.12 compared with the stump and fat pad byproduct tissues mean ± (SD) 0.10 ± 0.32 for both ($P = .038$). For assays starting with one hundred thousand cells, no statistically significant difference was found for total CFU counts between the four byproduct tissues ($P = .092$). For assays starting with ten thousand cells, no statistically significant difference was found for total CFU counts between the four byproduct tissues ($P = .083$). For assays starting with one thousand cells, there was a statistically significant difference found for total CFU counts between the 4 tissues ($P = .016$), specifically with significantly greater CFU counts

Table 3. Cellular Proliferation, as Measured by Both Colony-Forming Unit Fibroblast (CFU-F) Averages and Confluency Counts, for Each Arthroscopic Byproduct Tissue

	Tissue				
Characteristic[l]	Stump, N = 10*	Fat Pad, N = 10*	Notchplasty, N = 10*	Tunnel Drilling, N = 10*	*P* Value†
CFU-F					
(starting concentration of cells)					
10^6 Cells	0.10 ± 0.32	0.10 ± 0.32	29.07 ± 51.12	5.57 ± 12.39	.003‡
10^5 Cells	0.13 ± 0.32	3.17 ± 8.77	23.40 ± 45.65	6.70 ± 15.51	.092
10^4 Cells	10.67 ± 18.30	19.70 ± 37.59	23.20 ± 35.89	10.07 ± 20.26	.083
10^3 Cells	38.43 ± 30.53	39.07 ± 36.45	16.67 ± 29.37	7.20 ± 17.91	.016‡
Confluency (starting concentration of cells)					
10^6 Cells					<.001‡
Yes	8 (80%)	9 (90%)	2 (20%)	1 (10%)	
No	2 (20%)	1 (10%)	8 (80%)	9 (90%)	
10^5 Cells					<.001‡
Yes	9 (90%)	9 (90%)	2 (20%)	1 (10%)	
No	1 (10%)	1 (10%)	8 (80%)	9 (90%)	
10^4 Cells					.3
Yes	2 (20%)	4 (40%)	1 (10%)	1 (10%)	
No	8 (80%)	6 (60%)	9 (90%)	9 (90%)	
10^3 Cells					.4
Yes	0 (0%)	0 (0%)	0 (0%)	1 (10%)	
No	10 (100%)	10 (100%)	10 (100%)	9 (90%)	

NOTE. Both CFU-F and confluency were measured after 10 days of incubation.
CFU-F, fibroblastic colony-forming units; SD, standard deviation.
*Mean ± SD; n (%).
†Kruskal–Wallis rank sum test and chi-squared test for independence.
‡Indicates significant differences among tissue types at $P < .05$.

observed in the stump and fat pad mean ± (SD) 38.43 ± 30.53 and 39.07 ± 36.45, respectively, compared with the tunnel drilling debris mean ± (SD) 7.20 ± 17.91 ($P = .041$).

Cell confluency was evaluated 10 days after plating each of the concentrations mentioned previously and is also presented in Table 3 and Figure 2. If a sample had reached ≥80% confluency by day 10, it was marked "yes," otherwise it was marked "no." Two χ^2 tests for independence were performed to evaluate the effect of starting concentration and byproduct tissue type on confluency status. There was a significant relationship found between both the starting concentration ($\chi^2 = 32.7$, $P < .001$) and the byproduct tissue type ($\chi^2 = 30.4$, $P < .001$) to the presence of ≥80% confluency status at 10 days.

Cells obtained from all 4 byproduct tissues qualitatively showed positive tri-lineage differentiation compared with negative controls under standardized in vitro differentiation conditions (Fig 3). Cells in the positive (+) assays were exposed to pre-made differentiation media that encourages differentiation down a particular cell line (noted on the y axis). Negative (−) controls were exposed to standard growth media and crystal violet staining. After exposure to adipogenesis media, all 4 byproduct tissues experienced morphologic changes consistent with that of adipocytes (i.e., the formation of distinct lipid droplets). Lipid content of these droplets was confirmed by positive staining of the cells using Oil Red O. After exposure to osteogenesis media, all 4 byproduct tissues experienced morphological changes consistent with that of osteoblasts (i.e., the compacting of cells). Calcium content of these cells was confirmed by positive staining of the cells using Alizarin Red S. After exposure to chondrogenesis media, all four byproduct tissues experienced morphological changes consistent with that of chondroblasts (i.e., the formation of 3-dimensional spheroids). Glycosaminoglycan content of these cells was confirmed by positive staining using Alcian Blue. The negative controls served as an undifferentiated comparison group to observe lineage-specific changes in morphology and staining. The cells in the control group were not exposed to differentiation media, and instead were kept in standard DMEM/F-12 complete media. The fibroblastic morphology of the control cells was visualized using Crystal Violet to stain the nucleus of the cells. All participant samples were subject to tri-lineage differentiation assays, but only the results from one participant are shown as an example.

Specific cell-surface antigens were measured by flow cytometry, as shown in Table 4 and Figure 4. A complete count of antigens was measured for each sample before MSC-specific gating (% Total), and immediately after MSC-specific gating (% MSC-specific). The counts were divided by total events to provide a percentage of the overall sample content. Events define a single particle or cell detected by the instrument, which was set at

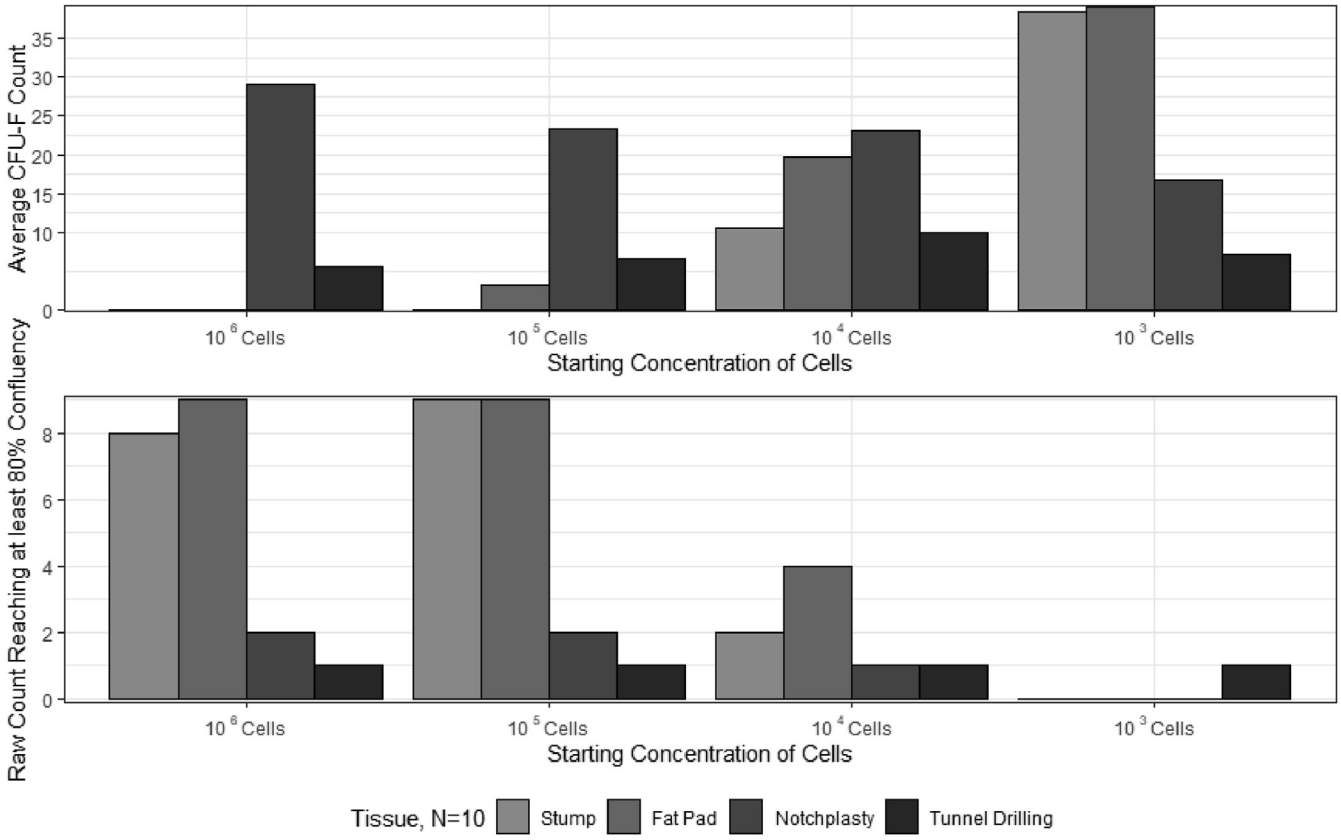

Fig 2. Cellular proliferation, as measured by both colony-forming unit fibroblast (CFU-F) averages and confluency counts, for each arthroscopic byproduct tissue. All 4 tissues (stump, fatpad, notchplasty, and tunnel drilling) were plated at 4 different starting concentrations (10^6, 10^5, 10^4, and 10^3 cells) to evaluate the effect of tissue type and seeding density on proliferation measures CFU-F and confluency.

a limit of 10,000 per sample. (−) indicates that a marker is not present on the cell, (+) indicates that marker is present on the cell. Not all samples had enough cellular content to run flow cytometry and thus only samples with a high-enough cell count (at least 10^6 viable cells) were analyzed. All 4 byproduct tissues expressed high levels (>75%) of % MSC-specific CD105+, CD73+, CD90+, CD45−, CD14−, CD19−, CD31−, and CD146− markers. Additionally, all 4 tissues expressed low levels (<10%) of CD45+ marker. There were no statistically significant differences in cell-surface antigens between the 4 byproduct tissues.

Discussion

The most important finding of this benchtop study is that cells derived from arthroscopic byproduct tissues of ACLR remain viable when collected with an in-line-suction autologous tissue collector and that these cells meet the ISCT criteria to qualify as MSCs, which supported our hypothesis. Viability was assessed in vitro after breaking down the tissue with a collagenase enzyme, which was necessary during processing to free the individual cells. The average cell viability after collagenase digestion was at or greater than 99.95% for all 4 byproduct tissues with high average TNC ($\geq 1.37 \times 10^7$ cells/mL) and no significant differences between the 4 tissues. Importantly, TNC counts included only living cells as differentiated by the addition of AO/PI dye during processing, meaning that if used at the point of care, the cells within these tissues are alive and present in large concentrations. On average, these measures are based on an initial collection of 1.12 to 1.61 g of tissue during surgery. Revisiting the ISCT criteria to qualify cells as MSCs, cells from all 4 byproduct tissues adhered to plastic cell culture flasks, expressed surface antigens (CD105+, CD73+, CD90+, CD45−, CD14−, CD19−) and did not express CD45+, and exhibited qualitative multipotent differentiation into adipocytes, osteoblasts, and chondroblasts.[11] These properties were observed consistently in cells derived from all four arthroscopic byproduct tissues, indicating that MSCs are present in these tissues and could be utilized at the point-of-care.

Upon further characterization of the byproduct tissues, differences in monocyte and red blood cell concentration were observed specifically with significantly

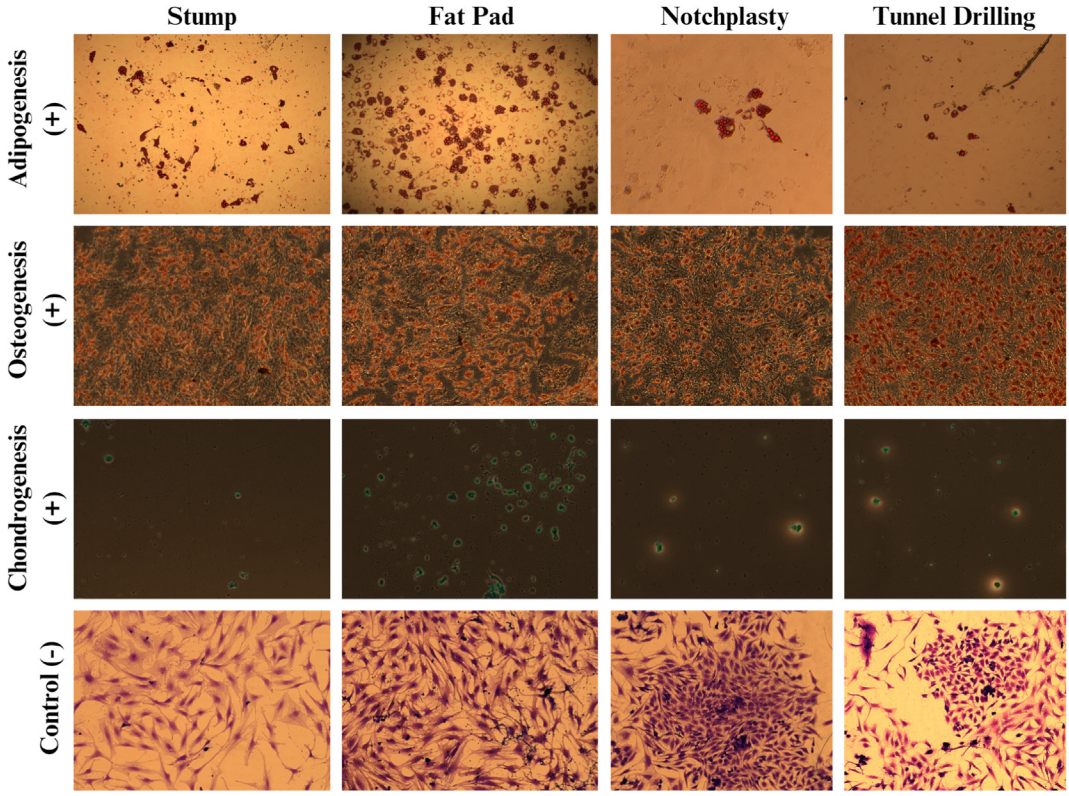

Fig 3. Representative qualitative tri-lineage differentiation assays (adipogenesis—10× objective, osteogenesis—20× objective, and chondrogenesis—20× objective) for each of the 4 byproduct tissue sites (ACL stump, ACL fatpad, notchplasty debris, tunnel drilling debris). Cells in the positive (+) assays were exposed to pre-made differentiation media that encourages differentiation down the cell line listed on the y axis. Negative (−) controls were exposed to standard growth media and crystal violet staining (10× objective). The negative controls served as an undifferentiated comparison group to observe lineage-specific changes in morphology and staining. For adipogenesis, Oil Red O staining indicated an accumulation of lipids accompanied by a visual lipid droplet. For osteogenesis, Alizarin Red S staining indicated an accumulation of calcium accompanied by visual compacting of the cells. For chondrogenesis, Alcian Blue staining indicated an accumulation of glycosaminoglycans accompanied by visual formation of 3-dimensional spheroids. In comparison, the control cells were observed as distinct fibroblasts using Crystal Violet, which was used specifically to stain the nucleus of the cells. All participant samples were subject to tri-lineage differentiation assays, but only the results from one participant are shown.

higher concentrations of both present in the ACL stump. Monocytes are a type of leukocyte that are commonly known as precursors to macrophages which play a vital role in the body's immune response to infections. During injury, inflammatory cytokines are known to recruit monocytes from the peripheral blood stream into injured tissues where they can differentiate into macrophages to aid in healing. Of the four byproduct tissues in this study, the ACL stump is the only tissue that is derived directly from the native, injured ACL. The other 3 tissues, the infrapatellar fatpad, notchplasty debris, and tunnel drilling debris, are derived from structures surrounding the ACL that are not directly injured as part of ACL rupture, which may cause them to have less of an inflammatory response and thus less monocyte and red blood cell concentration. The 4 byproduct tissues also differed in proliferative potential, as measured by average CFU-F counts and confluency during cell culture. The literature is not consistent regarding seeding densities of MSCs, so this study completed all cell culture experiments at four different starting concentrations: 10^3, 10^4, 10^5, and 10^6 cells.[7,20] The CFU-F and confluency measures revealed several interesting findings about the way these tissue-derived MSCs grow. Primarily, cultures at a high seeding density (10^6 or 10^5 cells) only produce CFU-F if the cells are slow-growing, which was the case for both the notchplasty and tunnel drilling debris. In contrast, cultures at a low seeding density (10^4 or 10^3 cells) produce more CFU-F if the cells are fast-growing, which was the case for both the ACL stump and fatpad byproduct tissues. At the same time, cultures at high seeding density (which did not produce CFU-F) preferred to grow confluent monolayers of cells on the bottom of the culture flask, which was the case for the ACL stump and fatpad byproduct tissues. Cultures

Table 4. Specific Cell-Surface Antigens (Ags) for Each Arthroscopic Byproduct Tissue Site, as Measured by Flow Cytometry

	Tissue				
Characteristic*	Stump, N = 5[2]	Fatpad, N = 3†	Notchplasty, N = 5†	Tunnel Drilling, N = 4†	*P* Value‡
Lineage Negative Markers					
CD14− CD19− (% Total)	64.33% ± 23.58%	47.99% ± 26.61%	43.28% ± 25.67%	63.32% ± 17.83%	.3
CD14− CD19− (% MSC-specific)	95.86% ± 2.54%	91.52% ± 7.17%	95.95% ± 2.59%	95.33% ± 2.65%	.8
CD31− CD146− (% Total)	49.73% ± 20.76%	31.41% ± 14.73%	28.92% ± 14.50%	48.14% ± 23.92%	.3
CD31− CD146− (% MSC-specific)	74.61% ± 13.31%	64.79% ± 18.44%	64.44% ± 15.11%	73.40% ± 17.53%	.4
CD45− (% Total)	65.80% ± 22.28%	49.89% ± 25.29%	45.63% ± 23.69%	64.01% ± 18.71%	.3
CD45− (% MSC-specific)	98.66% ± 0.80%	92.56% ± 9.49%	96.95% ± 1.51%	96.94% ± 2.32%	.2
Lineage positive markers					
CD45+ (% Total)	0.40% ± 0.34%	2.55% ± 4.28%	0.95% ± 1.02%	0.97% ± 1.31%	.8
CD45+ (% MSC-specific)	0.84% ± 1.05%	6.13% ± 10.42%	2.27% ± 2.01%	1.75% ± 2.66%	.5
CD90+ CD73+ (% Total)	41.44% ± 20.50%	25.82% ± 15.24%	22.52% ± 12.11%	41.41% ± 27.80%	.3
CD90+ CD73+ (% MSC-specific)	82.40% ± 8.83%	78.23% ± 19.30%	78.73% ± 12.17%	82.37% ± 17.21%	>.9
CD105+ CD90+ (% Total)	38.56% ± 21.05%	24.77% ± 14.60%	21.17% ± 11.68%	39.07% ± 27.93%	.3
CD105+ CD90+ (% MSC-specific)	91.35% ± 4.66%	95.80% ± 1.03%	93.83% ± 3.36%	92.58% ± 4.65%	.6

MSC, mesenchymal stem cells; SD, standard deviation.

*Events, a single particle or cell detected by the instrument, which was set at a limit of 10,000 per sample; Total percentage, lineage marker events (negative or positive) / total events; MSC-specific percentage, lineage marker events (negative or positive) / events within the MSC-specific gate.

†Mean% ± SD%.

‡Kruskal–Wallis rank sum test.

at low seeding density (which did produce CFU-F) did not grow confluent monolayers of cells. A relationship was identified in this study between the starting concentration (i.e., seeding density) of cells and their observed proliferative potential, as well as the tissue type and their observed proliferative potential. Qualitatively, the capacity of cells derived from each byproduct tissue to undergo adipogenesis, osteogenesis, and chondrogenesis, may be different and may depend on the underlying source of the tissue, which is similar to the findings of other studies of MSC differentiation.[9] These secondary findings may be important to consider when investigating the appropriate therapeutic dose of MSCs to apply at the point of care.

Other studies have investigated MSC content within ACL byproduct tissues. One laboratory study characterized MSCs isolated from ACLR (derived from ACL tissue) and joint replacement surgery (derived from bone marrow) tissues in 5 young (aged 17-27 years) and 6 old (aged 69-79 years), donors.[21] Both groups of MSCs cells performed similarly in terms of proliferative capacity and multilineage differentiation potential (adipogenic, osteogenic, and chondrogenic) regardless of age.[21] Proliferative cultures were seeded at a density of 200 cells per cm^2 in DMEM/F-12 complete medium and 25 cells was the cutoff for a colony, which differed from our study. All samples grew in culture with no age-dependent differences in CFU-F being detected, but 2 samples from old donors did enter growth arrest after approximately 80 days in culture.[21] Notably, it is not mentioned how this study qualified their tissue-derived cells as MSCs.[21] Another laboratory study isolated cells available from byproducts of arthroscopic cruciate ligament surgery, including post-injury effusion fluid (effusion fluid), fat pad and cruciate ligament stump debridement tissue (byproduct tissue), and arthroscopic fluid collected during fat pad and/or stump debridement (byproduct fluid).[7] The study included 22 male and 8 female participants aged 18-57 years old, with 23 isolated ACL injuries, 4 combined ACL and meniscus injuries, 2 combined ACL and medial collateral ligament, and 1 posterior cruciate ligament surgeries. Culturing techniques were similar, but the study used varying seeding densities based on the cell source and counted CFU-F colonies as 20 or more cells.[7] Flow cytometry was not performed so cells were not referred to as MSCs but instead viable stem cells.[7] Cell counts and viability was compared between a small volume and large volume collection method, and the average TNC counts were lower than that found in our study, with mean ± (SD) $6.89 \pm 0.79 \times 10^5$ cells/mL (small volume collection container) and $5.93 \pm 1.86 \times 10^5$ cells/mL (large volume collection container).[7,8] Both of these findings suggest that there may be differences in viability and cell counts based on the intraoperative collection method, as well as variations in cell culture methods.

The application of biologics to enhance ACL graft healing, including platelet-rich plasma, bone marrow aspirate concentrate, and cellular therapies has been examined in conjunction with ACL reconstruction with some treatments being reported to improve graft maturation and clinical outcomes.[22-32] However, these biologic augmentations frequently come at added time

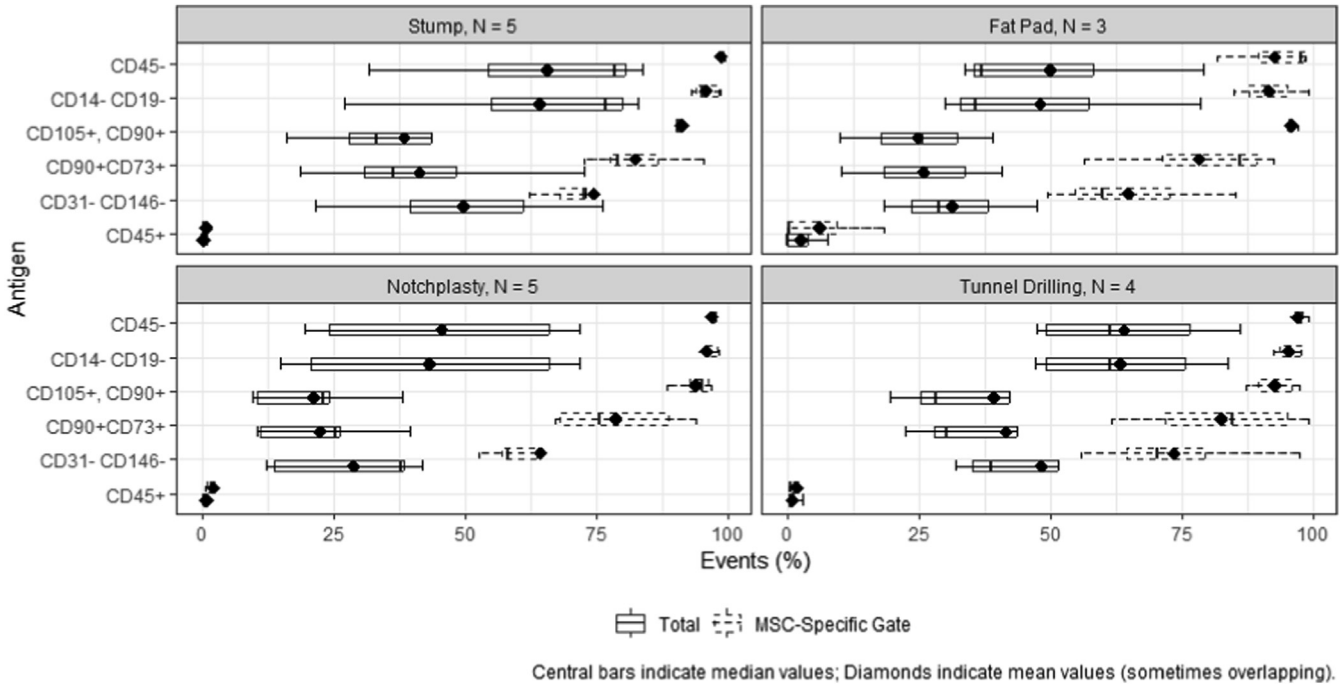

Fig 4. Specific cell-surface antigens (Ags) for each arthroscopic byproduct tissue site, as measured by flow cytometry. Boxplot lines represent the range, whereas the ending brackets represent the minimum (left) and maximum (right) values respectively. The central line represents the median while the central diamond represents the mean. A complete count of antigens was measured for each sample before MSC-specific gating (% Total), and immediately after MSC-specific gating (% MSC-specific). The counts were divided by total events to provide a percentage of the overall sample content. Events define a single particle or cell detected by the instrument, which was set at a limit of 10,000 per sample. (−) indicates that a marker is not present on the cell, (+) indicates that marker is present on the cell. Not all samples had enough cellular content to run flow cytometry and thus only samples with a high-enough cell count (at least 10^6 viable cells) were analyzed. (MSC, mesenchymal stem cells.)

and expense to the reconstruction procedure.[22-32] Thus, the autologous byproduct-tissue-derived MSC sources qualified in this study may serve as a cheaper and faster alternative to the current augmentation options. However, the goal of ACLR is not to induce fat, bone, nor cartilage growth, but instead to replace the damaged ACL with a tendon graft that matures to a new ligament. Thus, future in vitro studies should evaluate the ability for byproduct-tissue-derived MSCs to migrate to the injured graft and assess their ability to differentiate into tendon-like cells (tenocytes) or tissue-specific fibroblasts that may aid in the healing, strength, and longevity of reconstructed ACLs. Future clinical studies are needed to determine the clinical benefit, if any, of the application of these MSCs during ACLR.

Limitations

This study is limited by a small sample size, which may make our results more susceptible to type II error. A sample size calculation was completed, assuming a large effect size ($\eta^2 = 0.25$) in the difference among the tissues in terms of viability percentage, which revealed that n = 12 is needed to ensure 80% power with level of significance of 5%. Thus, this study is underpowered to detect differences in cellular viability percentage. A finite amount of tissue is captured during surgery, which limits the number of analytical tests that can be performed. Some of the samples were not sufficient to run all the tests which further limited the power of some of the outcomes, primarily flow cytometry analysis. Another limitation that must be considered for all laboratory studies of this nature is the variation in culturing techniques, conditions, and the investigators' interpretations of qualitative measures, such as in the observation of differentiation capacity. However, this study attempted to limit bias by having multiple investigators assess qualitative outcomes.

Further benchtop and clinical studies are needed to determine the potential mechanisms for therapeutic application and clinical benefits, if any, of these MSC-containing tissues. It is important to mention that obtaining tissue at the point of care and preparing for homologous use with minimal manipulation is considered low risk by regulatory agencies.[33] However, obtaining tissue and processing it to alter its relevant structural properties, such as enzymatically digesting or culturing, is considered high risk by regulatory agencies and thus is not currently available for patient care in most countries.[33,34] This study used culturing techniques only to confirm the viability of cells derived from

ACLR byproducts and qualify them as MSCs. Future in vitro studies should attempt to replicate the natural migration patterns of MSCs from their host tissue to the target tissue instead of using an exogenous enzyme like that of collagenase, the use of which is restricted to benchtop work.

Conclusions

This descriptive laboratory study demonstrated that cells derived from arthroscopic byproduct tissues of ACLR remain viable when collected with an in-line-suction autologous tissue collector and these cells meet the ISCT criteria to qualify as MSCs.

Disclosure

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References

1. Kim MJ, Son MJ, Seol B, et al. Generation of human induced pluripotent stem cells from osteoarthritis patient-derived synovial cells. *Arthritis Rheum* 2011;63:3010-3021.
2. Koyama N, Okubo Y, Nakao K, Osawa K, Fujimura K, Bessho K. Pluripotency of mesenchymal cells derived from synovial fluid in patients with temporomandibular joint disorder. *Life Sci* 2011;89:741-747.
3. Suzuki S, Muneta T, Tsuji K, et al. Properties and usefulness of aggregates of synovial mesenchymal stem cells as a course for cartilage regeneration. *Arthritis Res Ther* 2012;14:R136.
4. Harvanova D, Tothova T, Sarissky M, Amrichova J, Rosocha J. Isolation and characterization of synovial mesenchymal stem cells. *Folia Biol (Praha)* 2011;57:119-124.
5. Horie M, Driscoll MD, Sampson HW, et al. Implantation of allogenic synovial stem cells promotes meniscal regeneration in a rabbit defect model. *J Bone Joint Surg* 2012;94:701-712.
6. Matsumoto T, Ingham SM, Mifune Y, et al. Isolation and characterization of human anterior cruciate ligament-derived vascular stem cells. *Stem Cells Dev* 2012;21:859-872.
7. Anz AW, Branch EA, Rodriguez J, et al. Viable stem cells are in the injury effusion fluid and arthroscopic byproducts from knee cruciate ligament surgery: An in vivo analysis. *Arthroscopy* 2017;33:790-797.
8. Mifune Y, Matsumoto T, Takayama K, et al. Tendon graft revitalization using adult anterior cruciate ligament (ACL)-derived CD34+ cell sheets for ACL reconstruction. *Biomaterials* 2013;34:5476-5487.
9. Fu W, Li Q, Tang X, Chen G, Zhang C, Li J. Mesenchymal stem cells reside in anterior cruciate ligament remnants in situ. *Int Orthop* 2016;40:1523-1530.
10. Segawa Y, Muneta T, Makino H, et al. Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. *J Orthop Res* 2009;27:435-441.
11. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8:315-317.
12. Puck TT, Marcus PI. Action of x-rays on mammalian cells. *J Exp Med* 1956;103:653-666.
13. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc* 2006;1:2315-2319.
14. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, 2023. https://www.R-project.org/. Accessed January 23, 2024.
15. _readxl: Read Excel Files_. Version 1.4.2. 2023. https://CRAN.R-project.org/package=readxl. Accessed January 23, 2024.
16. *Welcome to the tidyverse*. Journal of Open Source Software, 2019.
17. _gt: Easily Create Presentation-Ready Display Tables_. 2023. Version 0.9.0. https://CRAN.R-project.org/package=gt. Accessed January 23, 2024.
18. Sjoberg DDWK, Curry M, Lavery JA, Larmarange J. Reproducible summary tables with the gtsummary package. *R J* 2021;13:570-580.
19. _*patchwork: The Composer of Plots*_ 2022, Version 1.1.2. https://CRAN.R-project.org/package=patchwork. Accessed January 23, 2024.
20. Bastos R, Mathias M, Andrade R, et al. Intra-articular injections of expanded mesenchymal stem cells with and without addition of platelet-rich plasma are safe and effective for knee osteoarthritis. *Knee Surg Sports Traumatol Arthrosc* 2018;26:3342-3350.
21. Prager P, Kunz M, Ebert R, et al. Mesenchymal stem cells isolated from the anterior cruciate ligament: Characterization and comparison of cells from young and old donors. *Knee Surg Related Res* 2018;30:193-205.

22. Hexter AT, Thangarajah T, Blunn G, Haddad FS. Biological augmentation of graft healing in anterior cruciate ligament reconstruction: A systematic review. *Bone Joint J* 2018;100-b:271-284.
23. Figueroa D, Figueroa F, Calvo R, Vaisman A, Ahumada X, Arellano S. Platelet-rich plasma use in anterior cruciate ligament surgery: Systematic review of the literature. *Arthroscopy* 2015;31:981-988.
24. Alentorn-Geli E, Seijas R, Martínez-De la Torre A, et al. Effects of autologous adipose-derived regenerative stem cells administered at the time of anterior cruciate ligament reconstruction on knee function and graft healing. *J Orthop Surg (Hong Kong)* 2019;27:2309499019867580.
25. Berdis AS, Veale K, Fleissner PR Jr. Outcomes of anterior cruciate ligament reconstruction using biologic augmentation in patients 21 years of age and younger. *Arthroscopy* 2019;35:3107-3113.
26. Silva A, Sampaio R, Fernandes R, Pinto E. Is there a role for adult non-cultivated bone marrow stem cells in ACL reconstruction? *Knee Surg Sports Traumatol Arthrosc* 2014;22:66-71.
27. Sánchez M, Anitua E, Azofra J, Prado R, Muruzabal F, Andia I. Ligamentization of tendon grafts treated with an endogenous preparation rich in growth factors: Gross morphology and histology. *Arthroscopy* 2010;26:470-480.
28. Baird JPE, Anz A, Andrews J, et al. Cellular augmentation of anterior cruciate ligament surgery is not currently evidence based: A systematic review of clinical studies. *Arthroscopy* 2022;38:2047-2061.
29. Jackson DW, Corsetti J, Simon TM. Biologic incorporation of allograft anterior cruciate ligament replacements. *Clin Orthop Relat Res* 1996;324:126-133.
30. Muller B, Bowman KF Jr, Bedi A. ACL graft healing and biologics. *Clin Sports Med* 2013;32:93-109.
31. Patel TK, Lycans D, Lavender C. All-Inside Allograft ACL reconstruction augmented with amnion, BMC, and a suture tape. In: Lavender C, ed. *Biologic and nanoarthroscopic approaches in sports medicine*. Cham: Springer International Publishing, 2021;17-26.
32. Forsythe B, Chahla J, Korrapati A, et al. Bone marrow aspirate concentrate augmentation may accelerate allograft ligamentization in anterior cruciate ligament reconstruction: A double-blinded randomized controlled trial. *Arthroscopy* 2022;38:2255-2264.
33. Anz AW, Hackel JG, Nilssen EC, Andrews JR. Application of biologics in the treatment of the rotator cuff, meniscus, cartilage, and osteoarthritis. *J Am Acad Orthop Surg* 2014;22:68-79.
34. Chirba MA, Sweetapple B, Hannon CP, Anderson JA. FDA regulation of adult stem cell therapies as used in sports medicine. *J Knee Surg* 2015;28:55-62.