Platelet-Rich Plasma Devices Can Be Used to Isolate Stem Cells From Synovial Fluid at the Point of Care

Eric A. Branch, M.D., Andrea M. Matuska, Ph.D., Hillary A. Plummer, Ph.D., Robert M. Harrison, and Adam W. Anz, M.D.

Purpose: To assess whether point-of-care devices designed for collecting cellular components from blood or bone marrow could be used to isolate viable stem cells from synovial fluid. **Methods:** Male and female patients older than 18 years old with either an acute, anterior cruciate ligament (ACL) injury or knee osteoarthritis (OA) with a minimum estimated 20 mL of knee effusion volunteered. Ten patients with an ACL injury and 10 patients with OA were enrolled. Two milliliters of collected synovial effusion were analyzed and cultured for cellular content. The remaining fluid was combined with whole blood and processed using a buffy-coat based platelet-rich plasma (PRP) processing system. Specimens were analyzed for cell counts, colony-forming unit (CFU) assays, differentiation assays, and flow cytometry. **Results:** ACL effusion fluid contained 42.1 ± 20.7 CFU/mL and OA effusion fluid contained 65.4 ± 42.1 CFU/mL. After PRP processing, the counts in ACL-PRP were 101.6 ± 66.1 CFU/mL and 114.8 ± 73.4 CFU/mL in the OA-PRP. Cells showed tri-lineage differentiation potential when cultured under appropriate parameters. When analyzed with flow cytometry, >95% of cells produced with culturing expressed cell surface markers typically expressed by known stem cell populations, specifically CD45−, CD73+, CD29+, CD44+, CD105+, and CD90+. **Conclusions:** Multipotent viable stem cells can be harvested from knee synovial fluid, associated with an ACL injury or OA, and concentrated with a buffy coat–based PRP-processing device. **Clinical Relevance:** PRP devices can be used to harvest stem cells from effusion fluids. Methods to use effusion fluid associated with an ACL injury and OA should be investigated further.

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Clinicians and scientists have become interested in improving or augmenting the healing potential of orthopaedic tissues with cellular therapies. Stem and progenitor cells are present in multiple musculoskeletal tissues, including the synovium and synovial fluid.[1-7] Cells mobilize from the synovium and fat pad to the synovial fluid in the settings of osteoarthritis (OA), anterior cruciate ligament (ACL) rupture, and meniscus tears.[1-3,8-19] In animal models, synovial-derived cells have been harvested, expanded in culture, and injected into injured joints with encouraging results for cartilage and meniscus.[8,9,20] Synovial fluid present within knee effusions associated with ACL ruptures or knee OA is a substance that is routinely discarded during arthroscopic knee surgeries and intra-articular knee injections. As such, it represents a potential source of autologous cells.[17,21,22]

Point-of-care autologous products are becoming more frequently used in orthopaedics, with platelet-rich plasma (PRP) being the most proven.[23-25] The production of PRP requires point-of-care devices that use centrifugation of blood creating fluid gradient layers, allowing selection of a layer for therapeutic use. PRP systems can be divided into plasma-based and buffy coat–based systems.[26,27] The principles used to obtain PRP products from peripheral blood can also be used on other bodily fluids that contain cells. While the creation of PRP products using whole blood has been well studied, expansion into harvesting cells from other fluids, such as synovial fluid, combined with blood at the point of care is a relatively novel idea.[28,29]

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Address correspondence to Adam W. Anz, M.D., Andrews Research & Education Foundation, 1020 Gulf Breeze Parkway, Gulf Breeze, FL 32561. E-mail: anz.adam.w@gmail.com

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The purpose of this study was to assess whether point-of-care devices designed for collecting cellular components from blood or bone marrow could be used to isolate viable stem cells from synovial fluid. We hypothesized that synovial fluid could be mixed with whole blood, and a buffy coat–based PRP processing device could be used to concentrate cells within the product.

Methods

Participants

Institutional review board (Baptist Hospital, Pensacola, FL) approval was obtained for both ACL injury and OA patient groups (numbers 1157964 and 1032661). Enrollment was discussed with male and female patients, older than 18 years old, presenting to the primary institution over a period of 24 months for treatment of either acute, magnetic resonance imaging–documented ACL injury or patients with knee OA who elected for PRP injection therapy. Further inclusion criteria included a minimum estimated knee effusion of at least 20 mL by clinical examination. Patients were excluded if time between injury and ACL surgery was greater than 5 weeks. The cutoff time frame was determined based on previous effusion fluid analysis. Injury greater than 5 weeks old, or patients with resolved effusion were assumed to have normalized effusion quantity and cellular content; therefore, the cut off was less than 5 weeks.[17] Additional exclusion criteria included previous knee aspiration of the injury effusion, any signs indicating local or systemic infection, a history of immunosuppression, or a history of chronic steroid use. Before participation, the approved procedures, risks, and benefits were explained to all patients and written informed consent was obtained. A total of 10 patients with ACL injury–associated knee effusion and 10 patients with OA-associated knee effusion was enrolled. This study focused on ACL injury and OA-associated effusion fluid because both are readily encountered in the orthopaedic clinic, represent pathologic inflammatory states, with disposable fluids, have low morbidity in the collection process, and previous study been shown to possess cellular content associated with multipotent potential. A total of 20 patients was chosen because previous studies regarding stem cell quantification have found significance with cohorts ranging in size of 5 to 15.[30-32] A power analysis was impossible to perform before the study, as the number of cells mobilized with knee injury has not been previously evaluated, and therapeutic dosage of stem cell technologies has not been established.

Fluid Collection and PRP Processing

Fifty-two milliliters of whole blood was collected from the patient's forearm following standard venipuncture technique in a 60-mL syringe preloaded with an 8-ml anticoagulant citrate dextrose solution. Five milliliters of anticoagulated whole blood was set aside for complete blood count (CBC) analysis and culture. Standard sterile knee aspiration methods were used, and the effusion was aspirated from the superior lateral aspect of the knee joint into a 60-mL syringe (performed by senior author, A.W.A). From the collected synovial effusion, 2 mL was set aside for CBC analysis and culture, and the remainder was combined with the 55 mL of blood. The combined effusion–whole blood solution was processed to prepare a PRP using preprogrammed settings for the total volume of combined input fluid for a buffy coat–based PRP processing system (Angel; Arthrex, Naples, FL) creating a platelet-poor plasma (PPP) and a buffy coat–based PRP product. The PRP system used for this study uses automated valve actuation for sequential aspiration of plasma, platelet, white blood cell (WBC), and red blood cell (RBC) layers based on the wavelength of the product. The percentage of RBCs collected in the PRP syringe is determined by the hematocrit setting selected, therefore giving the ability to modulate platelet, leukocyte, and RBC content. A hematocrit setting of 15% was chosen to maximize capture volume from the platelet and WBC layer, at the expense of a slightly increased final RBC content. This setting isolates cells from a deeper portion of the buffy coat, which would result in capturing more hematopoietic progenitor cells total. Sample aliquots for analysis were placed in sodium heparin coated vacutainers for transport and overnighted to a research laboratory for analysis.

CBCs and Colony-Forming Units (CFUs)

The samples, including the unmixed effusion fluid, unmixed whole blood, PPP, and PRP output, were analyzed. Cell count differential was assessed via an automated CBC (XE-5000; Sysmex, Kungsbacka, Sweden). Fold changes in PRP fractions were calculated by dividing the concentration of a particular cell type in the final PRP product by the respective concentration in unprocessed whole blood. A set volume (200 μL and 100 μL) of each fraction was plated in duplicate, and cultures using complete Dulbecco's Modified Eagle Medium/F12 were carried out for 10 days at 37°C/5% CO_2. After the culture duration, samples were rinsed with phosphate-buffered saline (Gibco, Carlsbad, CA), fixed in ice cold methanol, and stained with 0.5% Crystal Violet (Sigma-Aldrich, St. Louis, MO). Plates were washed with deionized water and allowed to air dry before counting. Colonies greater than 1mm in diameter containing at least 50 cells were counted as a CFU.

Flow Cytometry and Differentiation

Flow cytometry and tri-lineage differentiation potential were used to confirm the stem cell phenotype of plastic adherent cells cultured from a subset of effusion fluid and PRP fractions. Multicolor flow cytometry was

Table 1. Patient Demographics

	ACL	OA	*P* Value
Age, y	18.8 ± 3.3	55.6 ± 14.4	<.001
Sex	3 F/6 M	2 F/8 M	N/A
Fluid volume, mL	36.0 ± 13.5	21.5 ± 9.8	.045

NOTE. Age = mean ± standard deviation; fluid volume = mean ± 95% confidence interval.

ACL, anterior cruciate ligament; F, female; M, male; N/A, not applicable; OA, osteoarthritis.

performed on a CytoFLEX (Beckman Coulter, Brea, CA). All antibodies and DAPI were purchased from BioLegend (San Diego, CA: CD45-PB, CD44-BV785, CD105-FITC, CD90-PE, CD73-APC, and CD29-AF700). FMO-stained and -unstained samples served as gating controls. Gating was based on co-expression of viable CD45 (leukocyte) negative, and CD105, CD90, CD73, CD44, and CD29 positive expression to identify the percentage of mesenchymal stem cells (MSC) as described by International Society For Cellular Therapy standards and other studies.[33]

Osteogenic, adipogenic, and chondrogenic differentiation cultures were performed using StemPro differentiation kits (Gibco) per manufacturer's instructions. To summarize, 10^5 cells were added to 12-well plates to prepare monolayers (osteogenic or adipogenic) or micromass cultures (chondrogenic). Cultures were carried out over 21 days and fixed in paraformaldehyde (Alfa Aesar, Tewksbury, MA) before staining with Alizarin red (Amresco, Solon, OH), Oil Red O in isopropanol (EMS, Hatfield, PA), or Alcian blue (Millipore, Burlington, MA), respectively.

Statistics were performed in Minitab18 (Minitab, LLC, State College, PA). To make single comparisons between ACL and OA groups, when data sets were normally distributed with equal variance, unpaired *t* tests were performed. If data were shown to fail normality via Anderson-Darling and Levene's test, a Mann–Whitney *U* test was performed instead. Where appropriate, nonparametric donor paired comparisons were evaluated using a Wilcoxon signed rank test.

Results

Patient demographics of the 2 cohorts are presented in Table 1. Patient age between the 2 groups varied significantly ($P < .001$), with the average age of patients with OA significantly greater than the ACL injury cohort (55.6 ± 14.4 vs 18.8 ± 3.3 years old). A sample from the ACL group was excluded, reducing the total analyzed ACL group to 9 samples, secondary to failure of centrifugation and gradation of fluid layers during processing. The volume of joint fluid obtained from the ACL-injured knees was significantly greater than the OA knees ($P = .045$). Characterization of the joint fluid fractions from each group (Table 2) demonstrated significantly greater WBC and RBC in ACL compared with OA effusion fluid ($P < .001$, $P < .001$), with cell subtype (neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets), concentrations in both whole blood and PRP not significantly different between cohorts.

The volume of PRP generated in the ACL and OA cohorts was 3.3 ± 0.4 mL and 2.4 ± 0.8 mL, respectively ($P = .016$). The generation of a cell and platelet-enriched product was successful (Fig 1). On average, the PRP was 3.5× and 4.9× PLT over baseline in ACL or OA samples with no significant difference in the fold change of any cellular components detected between cohorts ($P > .05$).

CFU counts are presented in Table 3. Unmixed whole blood and PPP were cultured to evaluate the presence of CFU and none were detected. Sample cultures demonstrated no significant difference in CFU concentration between ACL and OA unmixed synovial fluid samples ($P = .283$), nor ACL and OA PRP product ($P = .713$). Increases in CFU concentration between unmixed synovial fluid samples and PRP product from OA ($P = .014$) and ACL ($P = .076$) cohorts were observed. As a whole, the CFU concentration was

Table 2. Characterization of Fluid Fractions

	Synovial Fluid			Platelet-Rich Plasma		
	ACL	OA	*P* Value	ACL	OA	*P* Value
WBC, k/μL	1.46 ± 0.65	0.17 ± 0.04	<.001	24.94 ± 8.34	35.38 ± 16.64	.225
NE, k/μL	0.34 ± 0.50	NR	N/A	9.36 ± 2.60	12.30 ± 9.63	.775
LYM, k/μL	0.78 ± 0.19	NR	N/A	9.32 ± 5.04	14.56 ± 5.73	.137
MONO, k/μL	0.37 ± 0.13	NR	N/A	4.78 ± 3.20	5.29 ± 2.45	.779
EO, k/μL	0.03 ± 0.03	NR	N/A	0.20 ± 0.16	0.36 ± 0.29	.838
BASO, k/μL (k/μL)	0.11 ± 0.10	NR	N/A	1.10 ± 0.62	2.49 ± 1.07	.023
PLT, k/μL	23.3 ± 10.8	NR	N/A	667 ± 631	717 ± 371	.488
RBC, k/μL	0.68 ± 0.78	0.00 ± 0.00	<.001	3.56 ± 0.68	2.86 ± 0.89	.175
MNC%	78.0% ± 16.4%	69.5% ± 12.0%	.347	51.8% ± 14.0%	59.1% ± 9.8%	.340

NOTE. This table captures the results of the automated cell counter (mean ± 95% confidence interval).

ACL, anterior cruciate ligament; BASO, basophils; EO, eosinophils; LYM, lymphocytes; MNC, mononuclear cells; MONO, monocytes; N/A, not applicable; NE, neutrophils; NR, not reported; OA, osteoarthritis; PLT, platelets; RBC, red blood cell; WBC, white blood cell.

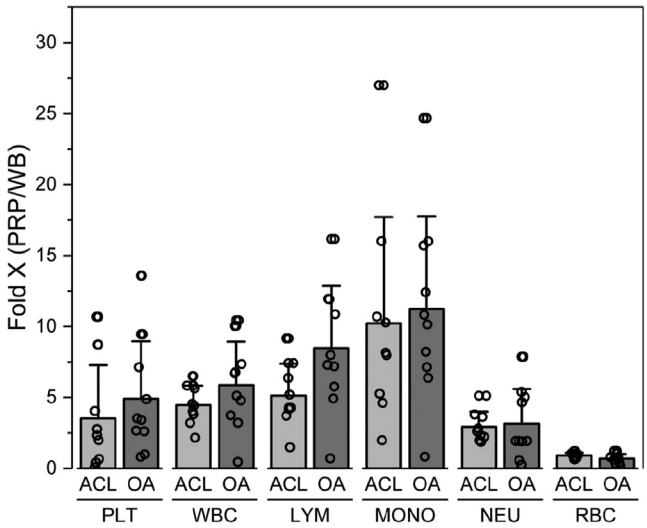

Fig 1. Ability to make PRP when synovial fluid is mixed with blood. Fold changes of cellular components in the final PRP product as compared with unmixed whole blood. There was no difference in any cellular fold changes in PRP produced during the ACL and OA portion of the study. (ACL, anterior cruciate ligament; BASO, basophils; EO, eosinophils; LYM, lymphocytes; MNC, mononuclear cells; MONO, monocytes; N/A, not applicable; NE, neutrophils; NR, not reported; OA, osteoarthritis; PLT, platelet; PRP, platelet-rich plasma; RBC, red blood cell; WBC, white blood cell.)

significantly increased in PRP as compared with unprocessed joint fluid (Fig 2, $P = .032$). Interestingly, it was observed that the colony sizes were typically larger in PRP than in joint fluid samples, although the significance of this is unclear.

Further characterization of the adherent cell population of a single donor demonstrated the majority of cells (>95%) displayed typical MSC markers CD45−, CD73+, CD29+, CD44+, CD105+, and CD90+. When interpreting flow cytometry results, it is important to consider that cells change the expression of cell surface markers depending on the environmental niches they occupy and after culture.[16,34] Positive trilineage differentiation was also confirmed (Fig 3).

Discussion

This study confirmed that stem cells can be obtained and concentrated from postinjury knee effusion and OA effusion at the point of care with a commercially available buffy coat–based PRP processing system without culturing or laboratory processing. Synovial fluid is typically discarded during knee injections and ACL reconstruction. Culture testing revealed the proliferative potential, differentiation assays proved multipotentiality, and flow cytometry confirmed cells expressing cell surface markers typically expressed by known stem cell populations, specifically CD45−, CD73+, CD29+, CD44+, CD105+, and CD90+.[33]

The term MSC was initially used to describe cells obtained from bone marrow that adhered to plastic upon culture and with further culturing produce colonies with phenotypical characteristics of fibroblasts.[4,35-37] Further benchtop work determined that MSCs have multipotent differentiation capacity and the ability to self-renew for multiple generations.[38] Concepts of stem cell function have progressed to not only focus on their ability to reproduce and differentiate but also to exert paracrine effects on surrounding tissues.[39-44] Progenitor cell is another term emerging within orthopaedic vernacular and is defined as a cell type that is more mature and destined for a specific cell line than stem cells. Progenitor cells retain proliferative potential but are believed to have less differentiation potential.[45-47] The identification of progenitor cells in various in vivo locations has also given rise to the term tissue-specific progenitor cell or resident stem cell. The semantics surrounding stem and progenitor cells and how to classify cells with associated healing potential continues to evolve and be a matter of debate.[48-50]

Evaluating the proliferative and differentiation potential of a cell from a given tissue has been a basis of investigating the use of cellular therapies in orthopaedics.[50,51] Proliferative studies have often involved culturing, identifying plastic-adherent cells, and further culturing the adherent cells into colonies, which can be measured as CFUs.[5] Tri-lineage differentiation is often performed on colonies to determine the multipotentiality of a given cell line.[35,44,52-54] Orthopaedic studies often investigate the adipogenic, osteogenic, or chondrogenic potential with orthopedic application in mind.[35,44,52-54] While both stem and progenitor cells have proliferative and differentiation potential, flow cytometry allows for the categorization of tissues into a stem or progenitor classification. The flow cytometry panel CD45−, CD73+, CD29+, CD44+, CD105+, and CD90+ has been described by ISCT standards as well as other studies to reflect stem cells.[33,55] The results of the culturing, differentiation, and flow cytometry of this study suggest that the cells obtained and concentrated from synovial fluid in this study may be referred to as stem cells.

It is important to mention that obtaining tissue at the point of care and preparing with minimal manipulation,

Table 3. CFU Assay

	ACL, CFU/mL	OA, CFU/mL	*P* Value (ACL vs OA)
Synovial fluid	42.1 ± 20.7	65.4 ± 42.1	.283
Platelet-rich plasma	101.6 ± 66.1	114.8 ± 73.4	.713
P value (SF vs PRP)	.076	.014	

NOTE. This table captures the results of the CFU assays (mean ± 95% confidence interval.)

ACL, anterior cruciate ligament; CFU, colony-forming unit; OA, osteoarthritis; PRP, platelet-rich plasma; SF, synovial fluid.

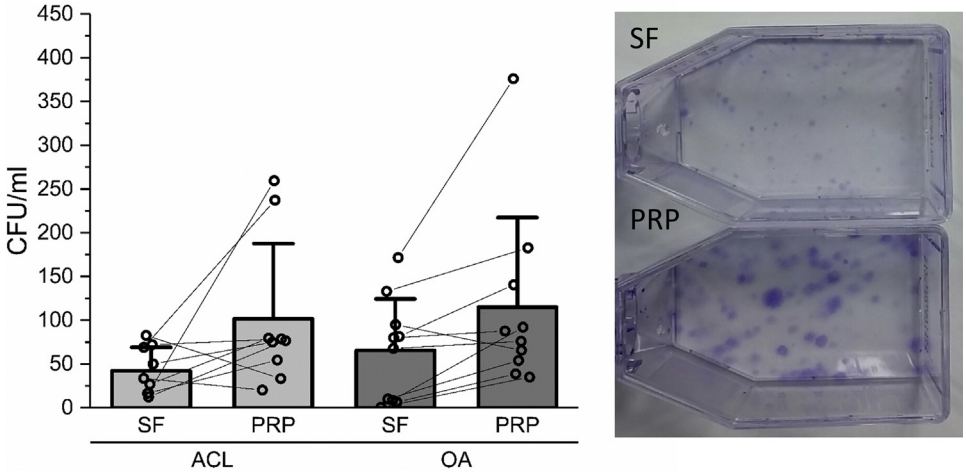

Fig 2. CFU concentration in unprocessed synovial fluid and synovial-mixed PRP. No CFU was detected in whole blood or PPP fractions. There was no significant difference in CFU concentration between ACL and OA fluids. Donor-matched data are connected via lines. The right panel shows the gross morphologic difference in colony size between synovial-mixed PRP and SF, but colonies displayed appropriate fibroblastic morphology under microscopic evaluation. (ACL, anterior cruciate ligament; CFU, colony-forming unit; OA, osteoarthritis; PRP, platelet-rich plasma; SF, synovial fluid.)

as in this study, for homologous use is considered low risk by regulatory agencies.[50] 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 not currently available for patient care in most countries.[50,56] This study used culturing techniques to confirm the presence and concentration of cells harvested from synovial fluid, not with aspirations of injecting cultured cells from this tissue. While cells can be harvested and concentrated with the techniques studied here, it is unclear if the number of cells available with these techniques have any clinical utility to augment healing.

In this study, culture analysis found on average 42.1 ± 20.7 CFU/mL in the ACL effusion, 65.4 ± 42.1 CFU/mL in the OA effusion fluid, 101.6 ± 66.1 CFU/mL in the ACL-PRP, and 114.8 ± 73.4 CFU/mL in the OA-PRP. These are relatively low concentrations. Other studies that allow for direct comparison include cell counts from bone marrow aspirate as well as injury effusion and arthroscopic byproducts.[17,32,57] Hernigou et al.[32] analyzed cell counts from bone marrow aspirate from the iliac crest for tibial non-unions and found an average of 612 ± 134 progenitor cells/ml (range, 12 to 1224 progenitor cells/ml). Beitzel et al.[57] analyzed cell counts of bone marrow aspirate obtained from the proximal humerus and distal tibia during arthroscopic procedures and similarly quantified 766.3 ± 545.3 progenitor cells/ml from bone marrow aspirate. This current study is similar to a previous study that isolated stem cells from ACL injury effusion and reconstruction byproducts with off-site laboratory processing. That study produced similar counts but with off-site laboratory processing, with effusion fluid containing 135 ± 22.6 CFU-F/mL and by-product tissue 429 ± 99.8 CFU-F/mL.[17] The similar counts suggest that PRP point-of-care processing can be used with similar results to off-site processing of effusion fluid. Comparison of results with bone marrow aspirate studies confirms effusion fluid does not contain as dense of a population of cells with stem potential as bone marrow; however, they appear to be present at a greater frequency among WBC types than bone marrow (in this study an average of 0.02% CFU/WBC for synovial fluid). Clinical application studies have suggested that success is dependent upon the number of stem cells harvested and used, although a threshold value for efficacy has not been defined.[58-62]

One potential confounding variable of this study is the lack of a CFU comparison of non-synovial fluid—mixed PRP to the synovial fluid and synovial fluid-mixed PRP. It is important to note that In previous study demonstrating peripheral blood stem cell harvest, the harvest techniques usually utilize Neupogen (Filgrastim) or other colony stimulating factor pretreatment to increase cell count in peripheral blood before blood draw.[61,62] In addition, a previous study in which PRP was analyzed with similar culturing techniques was unable to identify CFU colonies from PRP samples in 10 healthy volunteers.[63] Therefore, PRP samples were not cultured in this study. However whole blood and PPP were cultured from each subject and analyzed, neither of which produced cultured CFUs.

Variability in quantity and content of resulting PRP products between OA and ACL groups is likely multifactorial. The resulting decrease volume of the final PRP

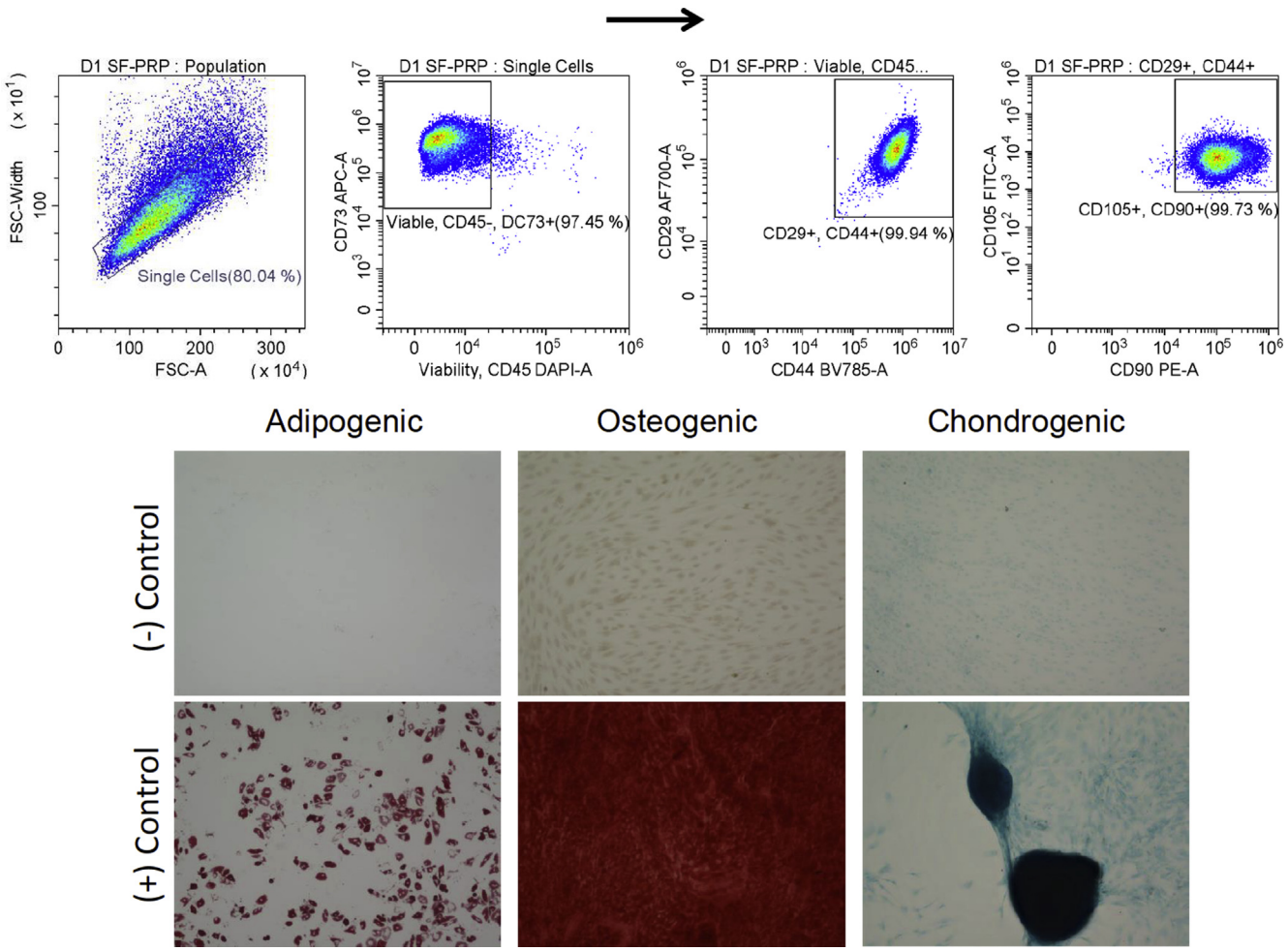

Fig 3. Characterization of adherent cells grown from PRP fraction. Cultured cells expressed MSC markers (CD45−, CD73+, CD29+, CD44+, CD105+, CD90+). Oil Red O (Adipogenic), Alizarin red (osteogenic), and Alcian blue (chondrogenic) staining confirms tri-lineage differentiation potential when cells were cultured under appropriate conditions. Negative controls were cells cultured without differentiation media, while positive controls were cultured with differentiation media.

product in the OA group, 2.4 ± 0.8 mL, compared with 3.3 ± 0.4 mL for ACL ($P = .016$), is likely due to lower whole blood input volume for OA samples. The cellular content demonstrated variability between groups, with greater cell counts in ACL fluid likely attributed to blood infiltration associated with trauma, as demonstrated by higher RBC counts as well. Both ACL and OA synovial fluid cell compositions were highly mononuclear, 69.5 ± 12.0% for OA, and 78.0 ± 16.4% ACL ($P = .347$). Donor variability also likely contributes to the results; however, further investigation would be needed to determine the contribution of disease factors; for example, duration of symptoms, changes in pain, and radiograph severity of disease. In a recent study, synovium-derived MSCs harvested from synovial fluid in hips with OA were compared with hips with femoral acetabular impingement syndrome (FAIS). The hip OA group demonstrated greater colony numbers, greater osteogenic, and adipogenic potentials, but less viability and proliferative potential when compared with synovial fluid from the FAIS group. The FAIS group demonstrated greater chondrogenic potential, therefore implying a correlation with severity of disease.[21]

Limitations

This study is limited by small sample size. A power analysis was impossible to perform before the study, as the number of cells mobilized with a knee injury has not been previously evaluated, and the therapeutic dosage of stem cell technologies has not been established. When looking critically at the OA group compared with the ACL group there are demographic differences; therefore, conclusions between the populations may be susceptible to type II error. However, combining the sample groups allows increased confidence in validating the use of PRP point of care devices for stem cell harvesting and preparation. A limitation of quantification studies involving assays is that the number of CFU progenitor cells present in a particular culture or the average in an individual study is highly

dependent on the culturing techniques, conditions, and investigators' definition of a colony.

Conclusions

Multipotent viable stem cells can be harvested from knee synovial fluid, associated with an ACL injury or OA, and concentrated with a buffy coat–based PRP processing device.

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