Single- Versus Multiple-Site Harvesting Techniques for Bone Marrow Concentrate

Evaluation of Aspirate Quality and Pain

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Background: Bone marrow concentrate (BMC) is growing in popularity as an alternative treatment option in orthopaedics. The regenerative capacity of BMC has been linked to the number of mesenchymal stem cells (MSCs) present in the graft at the time of its clinical application. MSC counts in bone marrow aspirate (BMA) are affected by harvest technique, but controversy exists over which aspiration method optimizes cellular yield while taking patient comfort and risk into consideration.

Purpose: To compare a single- versus multiple-site bone marrow aspiration technique to determine which would generate a sufficient volume of high-quality BMA for concentration into a BMC graft. The level of pain experienced by the patient was monitored, since patient comfort should be included in the determination of a safe and effective aspiration technique.

Study Design: Controlled laboratory study and cohort study; Level of evidence, 2.

Methods: BMC samples from 6 patients were sent to an outside source for laboratory analysis. All 6 participants underwent bilateral bone marrow aspiration. Each patient received both techniques at the posterior iliac crest: one side underwent a multiple-site aspiration technique, and the contralateral side underwent a single-site technique with needle redirection. BMA and BMC samples were analyzed for concentrations white blood cells, total nucleated cells, red blood cells, neutrophils, and hematopoietic stem cells. One BMC sample was cultured, and MSC analysis was performed via flow cytometry. All patients underwent monitoring of pain scores during and after the procedure through a visual analog pain scale at 24 hours, 72 hours, and 7 days after BMA.

Results: No significant difference was found between the cell ratios of the single- and multiple-site groups. Both aspiration techniques were found to provide ample colony-forming units without a marked difference in appearance. Additionally, no significant difference was found between groups with regard to MSC numbers. Pain during and 24 hours after the procedure was significantly greater with the multiple-site method than the single-insertion method.

Conclusion: The single-insertion method produced final cellular concentrations and culture results that were not significantly different from those of a multiple-insertion method. Additionally, the single-insertion site technique was significantly less painful to the patient at the time of the procedure as well as 24 hours after aspiration.

Clinical Relevance: The results of this study indicated that a high-quality bone marrow aspirate is possible with a single-stick aspiration method.

Keywords: bone marrow aspirate; knee osteoarthritis; biologic; mesenchymal stem cells; bone marrow concentrate

Cell-based therapies, including those using mesenchymal stem cells (MSCs), represent a promising approach to the repair and regeneration of musculoskeletal tissues. These potential therapies have gained media and patient interest as an alternative to surgical intervention.[24] The potential of an autologous biologic approach to improve a patient's symptoms and function while modifying the underlying disease process enough to slow down its natural progress is enticing. The orthopaedic literature demonstrates the potential of MSCs with successful application in cartilage disorders, fracture nonunion, and osteonecrosis.[7,8,10,11,13,14,24,28,31] Tissue engineering, in which stem cells can be synthesized with the use of bioactive molecules and scaffolds, is also a rapidly evolving field.[15]

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Adult stem cells of mesenchymal lineage have been identified in various sources, such as bone marrow, adipose tissue, umbilical cord blood, and synovium.[7] MSCs can migrate toward injured tissue, where they secrete immunomodulatory and trophic mediators.[3] These cells have also been shown to control inflammation, inhibit apoptosis, stimulate endogenous cell repair, and improve blood flow to tissue.[3,28]

Although the optimal source of MSCs has yet to be identified, minimally manipulated bone marrow aspirate (BMA) is acceptable per the United States Food and Drug Administration (FDA).[7,26,31] Bone marrow can be harvested and concentrated in one of many FDA-approved tabletop devices. This process concentrates the buffy coat (containing mononuclear cells) and increases the number of MSCs versus baseline.[13]

The therapeutic potential of bone marrow cells for orthopaedic conditions has drawn considerable interest, as have the methods to best optimize these cellular therapies. The number of MSCs in BMA can be affected by harvest technique and healthy donor variability.[4,14,16,17,20,21] Hernigou et al.[10,11,14] have described a direct relationship between an increased therapeutic effect and the number of progenitor cells in bone marrow concentrate (BMC). It is therefore critical that the MSC content is maximized at the time of the aspiration. An optimized and highly reproducible BMA technique is essential.

The current literature does not provide any consensus on a specific aspiration needle design or a definitive aspiration speed for bone marrow harvesting.[1,9,17,30,31] A consensus exists that there are more MSCs in the first 10 mL of BMA from 1 site and that the number of cells decreases with increasing volume.[4,17,22] But to obtain sufficient BMA, multiple cortical sticks would be required.

The aim of this study was to compare 2 bone marrow harvesting methods—a multistick with 6 sites along the posterior iliac crest and a single-stick redirect employing aspirates from various depths of a single stick. The resultant BMA and BMC were analyzed for differences in cellular concentration and BMC fold increase. A single randomly chosen BMC sample was cultured, and MSC analysis was performed via flow cytometry. We were limited to the analysis of 1 sample because of budgetary constraints. In addition, we assessed patient pain after the aspiration in both procedures.

METHODS

Experimental Design

This study was performed at a single-center outpatient clinic. Six patients who were scheduled for an elective outpatient percutaneous orthopaedic procedure for knee osteoarthritis were recruited for the study. All patients undergoing the procedure had their bone marrow aspiration and end product placement performed by the same physician—the lead author (K.O.). All patients were fully informed with respect to the rationale of the study and the associated risks, in accordance with an institutional review board–approved protocol and consent form. Based on standard deviations of 5.0 k/μL in white blood cell concentration and 50 k/μL in platelet concentration, sample size analysis revealed that 6 patients were required to detect a statistically significant difference. Inclusion criteria were a diagnosis of Kellgren-Lawrence grade 3 osteoarthritis of the knee. Exclusion criteria were as follows: age <30 or >70 years; the use of oral anticoagulant, nonsteroidal anti-inflammatory, immunomodulatory, or immunosuppressive medications in the 30 days prior to the study; a history of smoking or malignancy; an intra-articular injection of any sort in the 30 days prior to the study; a diagnosis of any autoimmune disorder; and a hemoglobin level <10 g/dL or a platelet count <105 mL.

Percutaneous bone marrow aspiration was performed on the left and right posterior iliac crests and sent for analysis to an outside laboratory. The aspiration method differed from side to side: the single-site (SS) method for 1 iliac crest versus the multiple-site (MS) method for the contralateral side. All patients underwent the procedure on the same day in sequential order, and the side chosen for each method alternated between patients.

Bone Marrow Aspiration

Prior to harvesting, the bone marrow needle assembly, syringes, and filters were washed with heparin. Six 10-mL syringes, each containing 1.5 mL of anticoagulant citrate dextrose solution A (ACD-A), were utilized for the bone marrow aspiration. Each patient was placed in a prone position. The sites for aspiration from the posterior iliac crest were confirmed via ultrasound. The areas of aspiration were sterilely prepared and draped as per standard protocol. A multiport bone marrow aspiration needle was employed for both aspirates, and the total resultant volume was 60 mL (including ACD-A) from each posterior iliac crest. The technique for aspiration from the sites differed as follows.

For the 6 study patients, the bilateral marrow aspiration was conducted per the following groups:

Single-needle insertion with redirection (SS method): One 11-gauge multiport bone marrow aspiration needle was used for a single puncture into the iliac cortex. Once placed, the trocar was advanced 2 cm before the introducer was removed. A 10-mL syringe with 1.5 mL of ACD-A was then attached to the trocar. A total of 8.5 mL of bone marrow was aspirated at this depth as follows: 3 mL was aspirated after trocar removal; then, the needle was rotated 90° clockwise, and an additional 3 mL was aspirated. A final 2.5 mL of aspirate was obtained following another 90° of rotation. The first 10-mL syringe was removed and sterilely capped. The trocar was then pulled back 0.5 cm, and a new 10-mL syringe with 1.5 mL of ACD-A was employed via the same "aspirate-rotate-aspirate" technique, adding an additional 8.5 mL of BMA to the total. This second 10-mL syringe was sterilely capped. Again, the trocar was pulled back another 0.5 cm, and the same "aspirate-rotate-aspirate" technique was employed, adding 8.5 mL of BMA to the total. After 25.5 mL of BMA was

harvested (with 4.5 mL of ACD-A), the introducer was placed back into the trocar, and the device was redirected 30° laterally and advanced 2 cm. Again, a 10-mL syringe with 1.5 mL of ACD-A was used, and the "aspirate-rotate-aspirate" technique described above was employed, adding 8.5 mL of BMA. The full 10-mL syringe was capped, and a new 10-mL syringe with 1.5 mL of ACD-A was placed. As previous, the trocar was pulled back 0.5 cm, and the same "aspirate-rotate-aspirate" technique provided the final 8.5 mL of BMA. Total volume was 51 mL of BMA.

Multiple-needle insertion without redirection (MS method): One 11-gauge multiport bone marrow aspiration trocar identical to the SS method was used. A total of 6 sites along the posterior iliac crest were used for the harvest. Every site included a separate skin incision and cortical insertion. At each site, one of the 10-mL syringes with 1.5 mL of ACD-A was employed for bone marrow harvest. The initial 8.5 mL of aspirate was obtained from the most medial site; then, each additional skin incision and cortical insertion site were 2 cm lateral to the prior. In all 6 sites, the trocar was advanced 2 cm after placement, and a total of 8.5 mL of aspirate was obtained as quickly as the patient could tolerate. This technique also resulted in a final volume of 51 mL BMA.

Both methods were performed on the same patient. Specifically, the first technique was aspirated from 1 side of the iliac crest, and the second technique was aspirated from the opposite crest. Technique sides were alternated from patient to patient, ensuring no biases from either side.

Preparation of BMC

After uniform mixing of ACD-A, a 2-mL BMA sample aliquot was taken for hematological analysis from both groups (SS and MS). The remaining BMA from both techniques was processed in a separate Arthrex Angel System at a 15% hematocrit setting. The final BMC specimen was analyzed with a final volume of 6.5 ± 0.5 mL. Two Arthrex Angel Systems were used for the processing of the BMA. The systems were alternated between subjects to minimize confounding.

Hematological Analysis, Cell Culture, and Flow Cytometry

BMA and BMC samples were hematologically analyzed (Sysmex XE 5000) for concentrations of white blood cells, total nucleated cells (TNCs), red blood cells (RBCs), neutrophils (NE), and hematopoietic stem cells (HPCs). One BMC sample was cultured in vitro at 37°C with 5% CO_2 in humidified air with MesenPro RS (ThermoFisher Scientific) culture medium. Media was changed every 3 days, and pictures were taken at day 1, day 13, and day 20. At day 20, MSC analysis was performed via flow cytometry with the BD Stemflow MSC Analysis Kit (BD Biosciences) and the CytoFLEX Flow Cytometer (Beckman Coulter). Flow cytometry was used to detect cell surface receptors positive for CD73, CD90, and CD105 and negative for CD34, CD45, CD11b or CD79α, and HLA-DR.

Statistical Analysis

We used t tests ($\alpha = 0.05$) to compare cellular concentrations produced by each technique. For statistical analysis on "fold increase," the formula was as follows: *cellular ratio = cellular concentration of BMC / cellular concentration of BMA*.

Subjective Pain Measures

All 6 patients underwent survey with a visual analog scale (VAS) for pain at the following points in time: immediately after each BMA technique was performed before the patient was repositioned, and 24 hours, 72 hours, and 7 days after the BMA procedure. The first survey was done in the clinic. The rest of the surveys were conducted via telephone at the time points previously outlined.

RESULTS

BMA was harvested from 6 healthy donors, 2 men and 4 women, with a mean age of 47 ± 15 years. Patients were identified at an initial screening visit as good candidates for a percutaneous BMC graft procedure by the lead author. The patients' ages varied from 33 to 68 years. The design of the study was the same for all patients. No complications were experienced in any of the study participants.

BMA and BMC Cellular Concentration and BMC Fold Increase

The cellular components of the BMA and BMC from both groups were analyzed. Figure 1 shows the cellular ratios of BMC to BMA for all study participants. These ratios were fairly consistent between the SS and MS groups with the exception of the HPC component. There was wide variability in the HPC ratios between donors, as expected. When results were combined, there was no statistically significant difference in the study population as a whole between the SS and MS BMA with respect to RBCs, NEs, TNC count, platelets, or HPCs. The same results were found when the SS BMC was compared with the MS BMC via the Arthrex Angel BMC system at a 15% hematocrit setting and final volume of 6.5 ± 0.5 mL. These findings are demonstrated in Table 1. Also shown in the table are the cellular concentrations for the SS and MS BMA versus the SS and MS BMC. In both aspiration techniques, there was a statistically significant difference in cellular concentration between BMA and BMC.

BMC Cultures and Colony-Forming Units

A comparison of the cellular ratios of each BMC group, SS versus MS, is illustrated in Figure 1. When BMC was compared with its baseline BMA, there was no significant difference between the cell ratios of the SS and MS groups ($P >$ .05 for all groups). As seen in Figure 1, the standard deviation in the HPC ratio was very large for the MS group

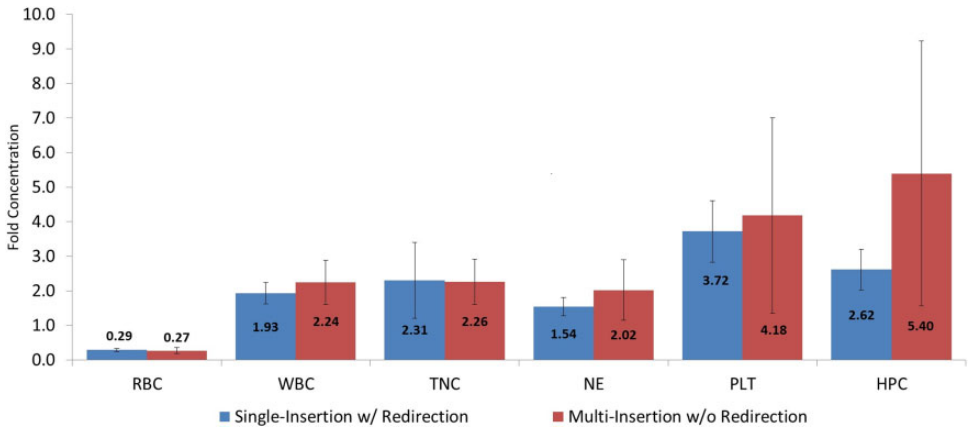

Figure 1. Single- and multiple-insertion cell ratios vs bone marrow aspirate. Error bars indicate the standard deviation (SD) of each cell value averaged amongst all 6 donors, displaying ±1 SD about the mean. HPC, hematopoietic stem cell; NE, neutrophil; PLT, platelet; RBC, red blood cell; TNC, total nucleated cell; WBC, white blood cell.

TABLE 1
Single- vs Multiple-Site Technique: BMA and BMC Cellular Components[a]

	RBCs, $10^6/\mu L$	WBCs, $10^3/\mu L$	TNCs, $10^3/\mu L$	NEs, $10^3/\mu L$	PLTs, $10^3/\mu L$	HPCs, $10^3/\mu L$
BMA						
Single insertion	3.8 ± 0.4	21.3 ± 9.8	22.6 ± 12.7	8.0 ± 4.0	160 ± 44	0.005 ± 0.004
Multiple insertion	3.7 ± 0.4	26.9 ± 12.8	31.3 ± 16.1	7.6 ± 3.4	137 ± 28	0.006 ± 0.005
P value	.674	.415	.323	.856	.305	.710
BMC: Angel 15% HCT						
Single insertion	1.1 ± 0.2	39.4 ± 14.7	44.8 ± 17.3	11.8 ± 4.7	598 ± 208	0.013 ± 0.010
Multiple insertion	1.0 ± 0.3	56.5 ± 19.4	65.2 ± 21.2	16.4 ± 10.2	626 ± 497	0.024 ± 0.014
P value	.512	.116	.098	.339	.901	.148

[a]There was no statistically significant difference between the single- and multiple-insertion techniques in BMA or BMC cellular ratios. BMA, bone marrow aspirate; BMC, bone marrow concentrate; HCT, hematocrit; HPC, hematopoietic stem cell; NE, neutrophil; PLT, platelet; RBC, red blood cell; TNC, total nucleated cell; WBC, white blood cell.

(3.840) versus the SS group (0.590); thus, there was no statistically significant difference between the 2 groups.

The BMC from both groups were cultured separately in vitro to determine if the colony-forming units (CFUs) were different in any fashion. Figure 2 represents the BMC cultures for both groups after days 1, 13, and 20. At day 1, both groups showed minimal confluence, and the culture had an abundance of erythrocytes and TNCs. At day 13, very few erythrocytes, nucleated RBCs, and white blood cells were present in the SS and MS groups. In both groups, the MSCs that had adhered to the tissue culture plastic became 30% to 40% confluent and assumed a CFU-like appearance. At day 20, both groups became 80% to 90% confluent on the tissue culture plastic. Therefore, the SS and MS techniques both provided for ample CFUs without a marked difference in appearance.

Flow Cytometry Data for Total MSC Counts

As proposed by the International Society for Cellular Therapy,[5] a panel of markers was used to detect the presence of MSCs. Figure 3 illustrates the concentrations of MSCs in the BMC specimens after 20 days in culture. The mean MSC count was 420 cells in the SS group and 328 cells in the MS group. Thus, there was not a significant difference between the 2 groups with regard to MSC numbers ($P = .609$).

Subjective Pain Scores

Table 2 provides statistical comparison results for the mean VAS pain scores of the SS and MS groups. When the 6 patients' data were combined, there existed a statistically significant increase in pain during the procedure ($P < .001$) and 24 hours after the procedure ($P < .046$) for the MS versus SS group, as seen in Table 2. However, at the 72-hour and 7-day time points, this difference in pain was no longer evident. In 3 patient comparisons, mean scores at all time points showed statistically significantly more pain with the MS method versus the SS.

DISCUSSION

In this study, we found no statistically significant difference in the quality of bone marrow aspirated with small

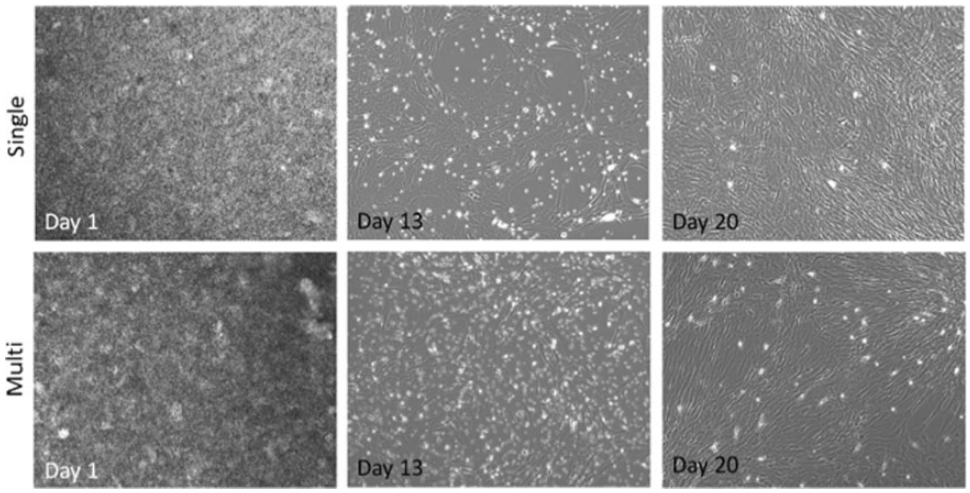

Figure 2. Bone marrow concentrate culture out to 20 days for single- and multiple-insertion techniques.

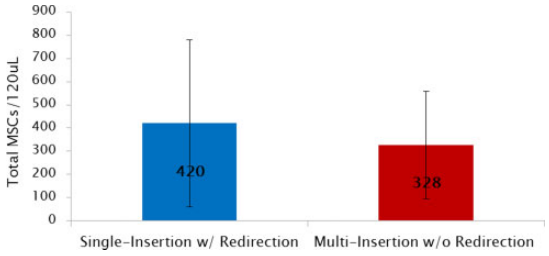

Figure 3. MSC flow cytometry data for single- and multiple-insertion bone marrow concentrate. Total MSCs per 120 μL. The error bars indicate the standard deviation (SD) of each insertion technique averaged amongst all 6 donors, displaying ±1 SD about the mean. MSCs, mesenchymal stem cells.

TABLE 2
Pain Score Comparisons for the Single- and Multiple-Site Groups[a]

Comparison: Duration	*P* Value
Single vs multiple site	
During procedure	**<.001**
24 h postoperative	**.046**
72 h postoperative	.481
7 d postoperative	.959
Mean pain scores at all time points[b]	
Patient 2 vs patient 3	**<.001**
Patient 3 vs patient 4	**<.001**
Patient 4 vs patient 6	**<.025**

[a]Two-way analysis of variance ($\alpha = 0.05$). Boldfaced *P* values indicate statistical significance.
[b]No other patient comparisons were significantly different.

volumes and multiple cortical sticks versus larger volumes with a single stick. We did find a statistically significant increase in patient pain during and 24 hours after an MS aspiration versus an SS aspiration.

Many factors affect the number of MSCs in a bone marrow sample: type of aspiration needle, speed of aspiration, total volume of aspirate, operator capability, size of syringe, and patient variables (eg, age, smoking status, immune status, and various medications).[18,19,22,27,30] The posterior iliac crest is the preferred harvest site, but no one type of aspiration needle has been concluded as being optimal.[6,17,30,31] Two randomized trials showed differing results in MSC content of BMA when comparing slow versus fast aspiration techniques.[1,9] Increasing MSCs is not as easy as merely aspirating large volumes of bone marrow. The inclusion of peripheral blood will increase the number of mononuclear cells harvested but not the amount of MSCs in the final product.[4,12,22,29] In fact, dilution of BMA with peripheral blood may be responsible for the large (up to 500-fold) variability of MSC frequency in aspirates.[4] Multiple studies have supported methods of bone marrow aspiration in which no greater than 10 mL of product is harvested per aspiration site to improve MSC yield.[4,17,22] But most commercial tabletop devices require 60 mL of BMA. To achieve this volume with a multistick low-volume method, a minimum of 6 sites are needed for aspiration. An increase in the amount of bone puncture sites leads to increased potential for patient discomfort, increased procedure time, and a potential increased risk of complications.

In an equine model, Peters and Watts[25] reported that a single sternal puncture in horses, at varying depths with needle rotation, resulted in higher MSC numbers than an SS/depth method. Without biopsy needle advancement, continued aspiration from the same site simply collects blood from the penetrated lacunae versus collecting additional MSCs.[20] Advancing the biopsy needle between aspirations has allowed new bony trabeculae to be penetrated, thus resulting in a higher MSC-containing BMA.

We postulated that the optimal method of aspiration in the human population may involve application of the method that Peters and Watts[25] employed in their equine model. A single cortical puncture site, with small volumes

of bone marrow aspirated at varying depths with needle rotation, may allow the larger MSC yield seen in the small-volume aspirates while minimizing patient discomfort by employing a single cortical stick.

In our comparison of an SS aspiration technique with an MS technique, we found that the BMA and BMC cellular ratios of RBCs, NEs, TNCs, platelets, and HPCs were not statistically significant between the groups. The results demonstrated in Table 1 support the large donor variability in the number of MSC in healthy patients seen in previous publications.[4,14,21] Also, the MSC content of the BMC, when assessed by flow cytometry in the SS and MS groups, was increased as suspected, but there was no statistically significant difference between the groups. One donor was chosen for additional cultures to assess the quality and differentiation potential. The evaluation of these cultures showed similar changes from day 1 through day 20. The resultant BMAs and BMCs showed no significant difference in RBCs, NEs, TNCs, platelets, HPCs, MSCs, and CFUs. We therefore concluded that a multistick bone marrow aspiration technique is not superior to a single-stick method with redirection. This conclusion does assume that the provider performing the bone marrow aspiration is experienced in the procedure.

Of interest is the inconsistent findings with regard to the HPC ratio between the study participants. In some donors, there was a higher HPC ratio in the SS compared with the MS site, but in others the results were the opposite. Perhaps this finding supports the notion that neither method of aspiration is superior. Another conclusion may be that HPCs are not the best cellular component to use when quantifying BMC from an orthopaedic perspective. HPCs are used in many studies because of their relative density equivalence to MSCs. Perhaps culturing out BMC specimens and counting MSCs would provide more accuracy than hematological testing of HPCs. But cultures are very expensive and not readily available to many researchers, which limits options.

A few findings deserve further review. One item that deserves clarification is the HPC "fold concentration" seen between the SS and MS groups, as demonstrated in Figure 1. There is no statistically significant difference between the groups, but there was a very large standard deviation in the MS group, which is likely attributed to harvesting from 6 sites versus 1 site. It is reasonable to postulate that, if there exists variability with 1 cortical stick, it would be multiplied by performing multiple sticks. Another finding of interest is that flow cytometry showed a 30% increase in the number of MSCs per 1200 μL with the SS method over the MS one. This finding suggests superiority of an SS approach over an MS one, but our sample size was too small to make any firm conclusions. Further study with a larger donor population may be able to illustrate greater significance.

Although our exclusion criteria included age, the actual patient age range of 33 to 68 years may have led to some confounding based on the known changes in the cellular composition of bone marrow with age. A power analysis was performed to determine that a study size of 6 patients was sufficient, but we acknowledge that including a larger number of patients would have lent more significance to our findings. One example that demonstrates that more participants would have been valuable is the HPC concentrations between the 2 study groups. Although the HPC ratio between the SS and MS groups was not statistically significantly different, it was great enough to postulate that this study may have been underpowered in this single cell type.

What was found significantly different in this study was the pain experienced by study subjects during the multistick procedure and 24 hours after the BMA. The MS technique was shown to be significantly more painful than the SS technique. This finding is supported by previous publications.[1,9] The current study supports these prior publications in the pain experienced at the time of harvest and 24 hours after harvest.

One limitation of our study is the lack of objectivity when reporting pain, since both iliac crest harvests were performed on the same patient. We did specifically ask the patient to rate the pain immediately after completion of each harvest to minimize this risk. Also, during the 24-hour, 72-hour, and 7-day phone surveys, the patients were asked to report the pain on the right side and then the pain on the left side. Of course, our patients were not sedated intravenously or with oral medication. If sedation were provided, it is possible that the difference in pain at the time of the procedure would no longer be statistically significant. Yet, we cannot assume that sedation would lead to improvement in discomfort at 24 hours after the procedure, and sedation would have added risk to the procedure itself.

CONCLUSION

The quality of BMA and its resultant BMC can vary because of numerous factors.[2,23] We have shown in this study that performing a posterior iliac crest aspiration with multiple sticks through the periosteum versus a single stick is not such a factor. Further studies to support this finding would be beneficial in confirming our results. Additional study is warranted to determine if using the same SS technique yet drawing a series of six 10-mL aspirates with a 10-mL syringe has any increase in quality versus using the larger 30-mL syringes. This method would incorporate the findings of previous studies[4,17,22] showing increased MSC yield through small-volume syringes, with our data supporting the benefit of a single-stick technique to minimize patient discomfort. Ultimately, our goal was to determine the best bone marrow aspiration technique for maximizing the quality of BMA while minimizing risk and patient discomfort. Thus, our study, while limited in sample size, does provide information on a bone marrow aspiration technique that may be incorporated into future evidence-based protocols.

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