Reliable assessment of bone marrow and bone marrow concentrates using automated hematology analyzer

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Aim: A limiting factor in advancement of bone marrow based cell therapies is the lack of characterization of cell products delivered to patients. **Methods:** Using an automated hematology analyzer that can be implemented in clinical setting, the composition of bone marrow aspirates (n = 17 patients) and bone marrow concentrates (n = 12 patients) were assessed. ICC estimates were calculated for measuring reliability. **Results:** Bone marrow aspirates assessment resulted in excellent reliability for determining white blood cells (ICC – 0.96; 95% CI: 0.92–0.99), red blood cells (ICC – 0.9; 95% CI: 0.77–0.96), platelets (ICC – 0.93; 95% CI: 0.85–0.97) composition. Bone marrow concentrate assessment resulted in excellent reliability for determining white blood cells (ICC – 0.97; 95% CI: 0.93–0.99), platelets (ICC – 0.95; 95% CI: 0.89–0.99) and moderate reliability for red blood cells (ICC – 0.66; 95% CI: 0.36–0.87) composition. **Conclusion:** Modern automated hematology analyzers could assist to better characterize the cell therapy products to provide reliable and consistent outcomes.

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Over the past decade, there has been increasing interest in cell-based therapies for the treatment of musculoskeletal conditions [1–5]. In part, this interest has been created by promising basic science and early clinical results, as well as by deceiving marketing claims [6,7]. In the case of knee osteoarthritis, one of the conditions most often treated with these therapies, there are discrepancies between what is being marketed versus the current evidence-based landscape [7]. In this setting, probably the most frequently offered cell-based therapies in orthopedics are bone marrow derived treatments [3]. This is probably due to the fact that bone marrow aspirates (BMA) can be obtained and processed by density separation to obtain bone marrow aspirate concentrates (BMC) as one of the few cell-based treatments that can be used in accordance with US FDA regulation [21]. The physical process of centrifugation (density separation) has been considered as minimal manipulation by the US FDA; therefore, BMC for injection qualifies as a Category 1 ‘non-HCT/P’ because the cell product involves a specifically exempted product (bone marrow), in an autologous use, used for a homologous purpose, and is not combined with other articles [8].

Despite its widespread use, one of the limiting factors in the advancement of bone marrow derived therapies in orthopedics is the lack of characterization of final products delivered to patients. Correspondingly, the clinical literature regarding the use of BMC is highly heterogeneous and inconsistent in the reporting of these therapies [5,9]. In a systematic review on the use of BMC for musculoskeletal conditions, out of 46 clinical studies identified, none provided a comprehensive reporting of the BMC preparation protocol that would allow the preparation method to be accurately reproduced. Furthermore, there was no consensus regarding a standardized reporting method for describing the composition of the starting material (BMA), or the composition of the processed BMC product that was used therapeutically [9].

Bone marrow is a complex concoction of various cell types including white blood cells (WBC) subtypes, red blood cells (RBCs), platelets, hematopoietic stem cells, connective tissue progenitors (CTPs) and endothelial progenitor cells (EPCs). Previous reports suggest that approximately only 0.08, 0.007 and 0.001–0.01% nucleated cells in bone marrow are hematopoietic stem cells [10], EPCs [11] and CTPs [12,13], respectively. The rest of the cells in bone marrow (>99%) are nonprogenitor cells – WBCs, RBCs and platelets. Although it may be the CTPs in BMA or BMC that play a direct role in connective tissue repair, their homing and reparative activity is hugely influenced by WBCs, RBCs, platelets and the growth factors and cytokines released by these cells [14]. Chaput *et al.* suggested that outcomes of cervical spinal fusion rates were highly dependent on the ratio of MSCs to other cellular bone marrow components [15]. King *et al.* suggested that WBC concentrations in BMC are directly correlated with intraleukin-1ra, anti-inflammatory cytokine involved in tissue healing process [16], Serbest *et al.* suggested that lower platelet activation was associated with better bone healing [17], Bastian *et al.* has shown that high neutrophil concentration leads to lower extracellular matrix mineralization [18], and RBC concentrations have also been correlated with bone formation [19]. In addition to complex concoction and understudied correlations between each component, there is huge patient-to-patient variability. Therefore, it becomes essential to systematically characterize the composition of BMA and BMC in a clinical setting to allow correlation of the therapy delivered to patients with the subsequently chosen clinical outcome [9].

BMA and BMC compositions can be determined in the laboratory; however, the microscopic examination is usually done in a nonautomated, labor-intensive procedure that is dependent on the experience of the reader and the quality of the specimen. Therefore, automated hematology analyzers can address the need for assessment of BMA and BMC composition [20], and can potentially be employed in an efficient and reproducible approach in the clinical setting. Efforts to analyze BMA using automated hematology analyzers have been described, but such functions have remained limited since bone marrow is a heterogeneous tissue containing multiple maturation stages of all lineages of hematopoietic cells [21]. To date, and to the best of the authors knowledge, there is deficiency in validating approaches to assess BMA and BMC in a clinical setting.

In this study, we report on the reliability of using an automated hematology analyzer to assess BMA and BMC concentrate composition including WBCs, RBCs and platelets, with an automated hematology analyzer that can be used in a clinical setting.

Methods

With Institutional Review Board approval, BMA was obtained from 17 healthy volunteer donors (mean age 32 years, range 22–40 years old; 13 males and 4 females). BMA were obtained from the iliac crest of these 17 patients (volume of bone marrow = 2 ml; volume of heparin = 1 ml) using a previously established protocol [22]. BMC was manually prepared with 12 samples. For BMC preparation, the heparinized aspirate was placed in a 50 ml tube, (Falcon 2098) containing 20 ml of α -MEM (Gibco, #11900-073) with 2 U/ml heparin and centrifuged at $400 \times g$ for 10 min. After centrifugation, the buffy coat was manually isolated and suspended in 20 ml of α -Minimum Essential Medium (α -MEM).

Within 1–2 h of collection from the patient, BMA and BMC aspirates were assessed using Sysmex KX-21N™ Automated Hematology Analyzer. A total of 20 μ l of sample was utilized in the pre-dilute mode to obtain eight parameters including: WBC ($1.0\text{--}99.9 \times 10^3/\mu\text{l}$), RBC ($0.30\text{--}7.00 \times 10^6/\mu\text{l}$), hemoglobin ([HGB] 0.1–25.0 g/dl), hematocrit ([HCT]; 10.0–60.0%), platelets ($10\text{--}999 \times 10^3/\mu\text{l}$), lymphocytes absolute content # ($1.0\text{--}99.9 \times 10^3/\mu\text{l}$), neutrophils absolute content # ($1.0\text{--}99.9 \times 10^3/\mu\text{l}$) and mix absolute content # ($1.0\text{--}99.9 \times 10^3/\mu\text{l}$; monocytes, basophils and eosinophils). Each sample of BMA and BMC was vibrated to assure uniform distribution and was sampled and analyzed three-times to allow reliable assessment. In summary using KX-21N system, WBC, RBC and platelets were detected by direct current detection method with coincidence correction. Automatic discriminators separated the cell populations based on algorithms. The intensity of the electronic pulse from each analyzed cell is proportional to the cell volume. The HCT is determined based on cumulative pulse height detection method. The HGB analysis is conducted using a noncyanide method [23].

Statistical analysis

The mean measurements among all the patients were calculated along with standard deviations for the different readings. The repeatability among the three readings was measured using the intraclass correlation coefficient (ICC). The ICC ranges from 0 (perfect disagreement) to 1 (perfect agreement). ICC estimates were calculated with a two-way mixed-effects model for absolute agreement [24]. ICC values ≤ 0.5 are indicative of poor reliability,

Table 1. Repeatability of measurements performed in straight bone marrow aspirate is reported by intraclass correlation coefficient.

Variable	First reading	Second reading	Third reading	ICC (95% CI)
WBC ($\times 10^3/\mu\text{l}$)	32.43 $\pm$ 11.12	33.82 $\pm$ 11.89	33.62 $\pm$ 11.11	0.96 (0.92–0.99)
RBC ($\times 10^6/\mu\text{l}$)	5.13 $\pm$ 1.37	4.79 $\pm$ 1.28	4.71 $\pm$ 1.26	0.9 (0.77–0.96)
HGB (g/dl)	15.63 $\pm$ 3.85	14.43 $\pm$ 3.51	14.18 $\pm$ 3.47	0.87 (0.68–0.95)
HCT (%)	45.19 $\pm$ 11.44	42.27 $\pm$ 10.79	41.51 $\pm$ 10.59	0.9 (0.75–0.96)
PLT ($\times 10^3/\mu\text{l}$)	103.82 $\pm$ 82.35	110.06 $\pm$ 117.55	108 $\pm$ 119.37	0.93 (0.85–0.97)
Lymphocyte ($\times 10^3/\mu\text{l}$)	7.57 $\pm$ 3.19	8.07 $\pm$ 3.62	8.06 $\pm$ 3.4	0.94 (0.87–0.98)
Mix ($\times 10^3/\mu\text{l}$)	2.39 $\pm$ 0.93	2.68 $\pm$ 0.84	3.05 $\pm$ 1.16	0.71 (0.44–0.89)
Neutrophil ($\times 10^3/\mu\text{l}$)	22.22 $\pm$ 8.31	23.2 $\pm$ 9.12	22.24 $\pm$ 8.12	0.97 (0.93–0.99)

The mean values + standard deviation among all the patients (n = 17) are given for each variable.

HCT: Hematocrit; HGB: Hemoglobin; ICC: Intraclass correlation coefficient; Mix: Monocyte, basophil and eosinophil; PLT: Platelet; RBC: Red blood cell; WBC: White blood cell.

Table 2. Number of measurements (percent of total samples) that were invalid in straight bone marrow aspirate are reported below.

Variable	First reading, n = 17(%)	Second reading, n = 17(%)	Third reading, n = 17(%)	Overall, N = 51 (%)
WBC ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
RBC ($\times 10^6/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
HGB (g/dl)	0 (0)	0 (0)	0 (0)	0 (0)
HCT (%)	0 (0)	0 (0)	0 (0)	0 (0)
PLT ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
Lymphocyte ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
Mix ($\times 10^3/\mu\text{l}$)	2 (12)	3 (18)	2 (12)	7 (14)
Neutrophil ($\times 10^3/\mu\text{l}$)	0 (0)	1 (6)	0 (0)	1 (2)

HCT: Hematocrit; HGB: Hemoglobin; Mix: Monocyte, basophil and eosinophil; PLT: Platelet; RBC: Red blood cell; WBC: White blood cell.

values between 0.5 and 0.75 indicate moderate reliability, values between 0.75 and 0.9 indicate good reliability and values greater than 0.90 indicate excellent reliability [24]. The 95% confidence intervals were also calculated for all the ICC estimates. Statistical analysis was performed using Stata Statistical Software: Release 12 (StataCorp, LP, TX, USA).

Results

The use of an automated hematology analyzer for the assessment of BMA resulted in excellent reliability for the assessment of WBC (ICC: 0.96; 95% CI: 0.92–0.99), RBC (ICC: 0.9; 95% CI: 0.77–0.96), PLT (ICC: 0.93; 95% CI: 0.85–0.97; Table 1). Analysis of the composition of lymphocytes (ICC: 0.94; 95% CI: 0.87–0.98) and neutrophils (ICC: 0.97; 95% CI: 0.93–0.99) also had excellent reliability, while mix (monocytes, basophils and eosinophils) had moderate reliability (ICC: 0.71; 95% CI: 0.44–0.89). Additionally in 14% of the readings, the assessment of ‘mix’ resulted in invalid readings (Table 2).

The use of an automated hematology analyzer for the assessment of BMC resulted in excellent reliability for the assessment of WBC (ICC: 0.97, 95% CI: 0.93–0.99), PLT (ICC: 0.95, 95% CI: 0.89–0.99), lymphocytes (ICC: 0.93, 95% CI: 0.83–0.98) and neutrophils (ICC: 0.97, 95% CI: 0.92–0.99) has excellent reliability, mix assessment (monocytes, basophils and eosinophils) had good reliability (ICC: 0.76, 95% CI: 0.47–0.92) and assessment of RBC (ICC: 0.66, 95% CI: 0.36–0.87), HGB (ICC: 0.60, 95% CI: 0.27–0.85), HCT (ICC: 0.57, 95% CI: 0.21–0.85) resulted in moderate reliability (Table 3). Additionally in 17% of the readings, the assessment of ‘mix’ resulted in invalid readings, as well as 11% of the HCT readings (Table 4).

Discussion

Numerous bone marrow derived therapies are being used for musculoskeletal applications without any assessment of their composition at the time of delivery [1,3–5,25]. The quality and composition of BMA has been found to be influenced by a number of factors including patient variables [26,27], aspiration volume per site [28], needle type [29], physician experience level, etc. [30]. Thus it becomes essential to assess the composition and quality of the BMA

Table 3. Repeatability of measurements performed in bone marrow aspirate concentrate is reported by intraclass correlation coefficient.

Variable	First reading	Second reading	Third reading	ICC (95% CI)
WBC ($\times 10^3/\mu\text{l}$)	6.97 $\pm$ 5.38	6.48 $\pm$ 5.47	6.84 $\pm$ 5.17	0.97 (0.93–0.99)
RBC ($\times 10^6/\mu\text{l}$)	0.36 $\pm$ 0.2	0.3 $\pm$ 0.17	0.27 $\pm$ 0.13	0.66 (0.36–0.87)
HGB (g/dl)	1.11 $\pm$ 0.58	0.91 $\pm$ 0.45	0.8 $\pm$ 0.38	0.60 (0.27–0.85)
HCT (%)	3.4 $\pm$ 1.62	2.84 $\pm$ 1.31	2.44 $\pm$ 1.12	0.57 (0.21–0.85)
PLT ($\times 10^3/\mu\text{l}$)	31.33 $\pm$ 28.88	29.5 $\pm$ 33.56	31 $\pm$ 33.57	0.95 (0.89–0.99)
Lymphocyte ($\times 10^3/\mu\text{l}$)	1.72 $\pm$ 1.09	1.52 $\pm$ 1.11	1.74 $\pm$ 1.19	0.93 (0.83–0.98)
Mix ($\times 10^3/\mu\text{l}$)	0.71 $\pm$ 0.46	0.59 $\pm$ 0.32	0.66 $\pm$ 0.38	0.76 (0.47–0.92)
Neutrophil ($\times 10^3/\mu\text{l}$)	3.95 $\pm$ 2.68	3.88 $\pm$ 3.1	4.1 $\pm$ 3.23	0.97 (0.92–0.99)

Each bone marrow aspirate concentrates sample was obtained by suspending 1–2 ml of buffy coat in 20 ml of α -MEM to perform the assessment. The mean values + standard deviation among all the patients (n = 12) are given for each variable.

HCT: Hematocrit; HGB: Hemoglobin; ICC: Intraclass correlation coefficient; Mix: Monocyte, basophil and eosinophil; PLT: Platelet; RBC: Red blood cell; WBC: White blood cell.

Table 4. Number of measurements (percent of total samples) that were invalid in bone marrow aspirate concentrates are reported below.

Variable	First reading, n = 12 (%)	Second reading, n = 12 (%)	Third reading, n = 12 (%)	Overall, N = 36 (%)
WBC ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
RBC ($\times 10^6/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
HGB (g/dl)	0 (0)	0 (0)	0 (0)	0 (0)
HCT (%)	1 (8)	1 (8)	2 (17)	4 (11)
PLT ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
Lymphocyte ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)
Mix ($\times 10^3/\mu\text{l}$)	2 (17)	2 (17)	2 (17)	6 (17)
Neutrophil ($\times 10^3/\mu\text{l}$)	0 (0)	0 (0)	0 (0)	0 (0)

HCT: Hematocrit; HGB: Hemoglobin; Mix: Monocyte, basophil and eosinophil; PLT: Platelet; RBC: Red blood cell; WBC: White blood cell.

and BMC before injecting into the patient, so we properly document and analyze the affect of various variables influencing cellular therapy outcomes [41]. Efforts to analyze BMA using automated hematology analyzers have been described, but such functions have remained limited since bone marrow is a heterogeneous tissue containing multiple maturation stages of all lineages of hematopoietic cells [21]. Additionally, quantification methodologies need to be compatible with the limitations in the workflow of a clinical setting. Therefore, we proposed to assess the reliability of an automated hematology analyzer that can be used at the point of care, to obtain a broad assessment of nucleated cell counts, platelets and RBCs. Our main finding was that the Sysmex KX-21N Automated Hematology Analyzer can be used to analyze human BMA samples and BMA concentrates in regard to the composition of WBCs, RBCs and platelets with high reliability. We did not encounter measurement with invalid readings in the main parameters of interest (WBC, RBC and PLT; Tables 2 & 4). However, it is important to note that buffy coat (BMA concentrates) assessment was done after diluting the samples by 10- to 20-fold in α -MEM media.

For the past decade, researchers have tried various modern automated hematology analyzers such as ADVIA2120i (Siemens), XE-2100 (Sysmex), Cell Dyn 4000 (Abbott Diagnostics), Coulter GEN.S (Coulter Corp), LH750 (Beckman Coulter) to assess BMA [21,31–34]. All these analyzers were mainly developed to assess peripheral blood; however, modifications to sample preparation protocols, including using anticoagulants, appropriate dilutions, homogenization and washing of the cells/cell pellets have enabled these devices to provide reliable, precise and useful information regarding cell composition in BMA and BMCs [21,35–37]. For the first time in this study, Sysmex KX-21N has been used to assess BMA and BMC composition. Although we did not validate it against any existing devices or manual method of counting, we followed the protocols previously established in the lab for preparing the BMA and BMC samples [22,28], and the composition reported here is in the range of some of the previous reports [32,35,38]. Unlike Sysmex XE-2100 instrument, that detects cells based on optical analysis, Sysmex KN-21N uses direct current for RBC, WBC, and PLT counting, thus eliminating the lipid interference, which is a limiting factor in analysis of bone marrow and causes overestimation of marrow total nucleated cell counts [39]. Although analysis of BMA using automated blood cell counters is not completely satisfactory, acceptable results have been

previously reported that are reproducible [1,33,40]. These results have been validated by comparing to microscopic assessments and flow cytometry analysis.

This study is not without limitations. First, we performed an internal validation using only one of the automated hematology analyzer available in the market; therefore, assessing only the precision of the measurements being obtained from that device. Second, we did not compare the BMA and BMC measurements to any other methods of measurement; therefore, we cannot comment on the accuracy of the measurements. This will require further validation. Third, we did not perform differentiation between cells found in bone marrow, such as in a myelogram. However as first step, since hematology analyzers have shown low intra-sample variability, further assessment of BMC quality will be required. Fourth, we only assessed cellular composition of BMA and BMC and did not characterize the concentration of stem and progenitor cells within the samples by *in vitro* colony forming unit assays. Optimal characterization of bone marrow derived cellular therapies should precisely and accurately determine the cell composition of treatment as well as stem cell populations present [1,13,42]. Until the value of these treatments is established, characterizing the composition after processing for each sample is essential, so that one can accurately correlate the composition of the therapy delivered with the obtained outcome [9].

While the use of BMC is promising as a therapeutic approach, the success of BMC procedures differs from patient to patient [3]. It is generally assumed that the composition of BMC will be related to its clinical efficacy. However, the critical quality attributes that are associated with success or failure of BMC use are not yet known. A minimum dataset for reporting on BMA or BMC composition should include the total volume and the concentrations of nucleated cells, platelets, RBCs and stem and progenitor cells (e.g., colony forming units) [9]. The development and use of the first automated hematology analyzers was to provide comprehensive automated assessment of patient's blood sample. Over the years, with improvements in the capabilities and performance of the automated analyzers, their applications have broadened for use in BMA and concentrate analysis as well. Use of such systems for consistent assessment of BMA and BMC will improve reporting methods among various laboratories, as well as provide better correlations to clinical outcomes for musculoskeletal cell-based tissue repairs [43–46].

Conclusion

BMA and BMC concentrate cellular composition can be measured with high reliability in regard to WBC, RBC and platelet composition using an automated hematology analyzer, at the point of care. Modern automated hematology analyzers, widely used for the analysis of peripheral blood samples could assist to characterize these treatments and provide partial quality assurance at the time of treatment delivery. Further research will be required both for internal and external validation.

Future perspective

BMA and BMC are increasingly being used for autologous cell therapies to treat musculoskeletal repairs. However, the efficacy of these cell therapies is low and there is a gap in correlating with patient outcomes. There is a need to consistently measure and report the cell composition of BMA and BMC being injected during the cell therapies for better correlations to patient outcomes. Since manual measurement methods are cumbersome, time consuming and have huge variability based on experience of the counter, automated methods provide a major advantage in all these aspects. In addition, the automated analyzers provide detailed assessment of BMA and BMC composition, as opposed to total nucleated cell counts obtained by manual methods (hemocytometer). As a next step, when BMA or BMC samples are injected in patients being treated for osteoarthritis (knee or hip), we will focus on correlating these automated cell composition measures with the number of stem and progenitor cells in BMA and BMC samples, along with patient reported outcomes and other clinical outcomes (x-ray or MRI), to establish a database that can be used for complete analysis. Based on our enhanced understanding of the complex BMA or BMC concoction, stem and progenitor concentration and performance, we believe that cellular therapy treatments can be more patient specific, and potentially help improve clinical efficacy.

Summary points

- Improvement of cell-based therapy outcomes needs better quantitative assessment of the cell composition resident in the cell products being delivered.
- Human bone marrow aspirate and bone marrow concentrate composition can be determined with high reliability using automated hematology analyzers.
- In addition to assessment of the composition of cell products being delivered, future studies should also focus on *in vitro* assessment of stem and progenitor populations, along with their biological potential to correlate to patient outcomes.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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