AIMS: Bone Marrow Concentrates (BMC) applied to treat several osteo-articular pathologies had reported positive clinical outcomes at short- to medium-term follow up. However, the diversity of indications reported and the lack of consensus describing the formulation of BMC make it difficult to circumscribe basic processing variables to generate them. We analyzed the influence of processing different Bone Marrow (BM) volumes over the formulation and deliverable cell dose of BMC.

METHODS: Main cellular populations were characterized in BMC manufactured and applied for autologous use during the same surgical procedure. To do this, Flow Cytometry and Fibroblastic Colony Forming Units (CFU-Fs) assays were used.

RESULTS: Cell concentration of aspirates was not statistically influenced in the range of volumes analyzed. Consequently the quality of BM seems to be conserved in the range of volumes assayed. By using the protocol described, the quality of BMC traditionally defined as CFU-F/mL did not differ in the range of volumes assayed whereas total dose of CFU-F and other differentiated cells effectively changed.

CONCLUSIONS: The volume of BM defines cellular doses arriving to the patient with statistically significant differences. Parameters currently used to describe the quality of BMC as CFU-F/mL appear to be directly influenced by working volumes and thus total cellular doses applied might better characterize these products. Finally, since it is uncertain what cells within BMC form part of its active substance, the quantification of main cell subsets could be helpful to better understand where, when and how these medicinal products work.

Key words: Bone Marrow Concentrate; Cellular dose; Mesenchymal stromal cells; Point of care Processing

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**INTRODUCTION**

Intraoperative delivery of bone marrow concentrates (BMC) for other functions than hematological reconstitution has been harnessed as an individualized therapy produced and applied during the same surgical procedure to treat diverse pathologies. BMC utilized in such a way have not been specifically regulated in Europe so far[1-7] although they perfectly meet the criteria to be considered as medicinal products.

In this regard, inversely to the developmental pathway of traditional drugs where high quality clinical studies are performed before its accessibility, BMC have fast moved from basic research to clinical practice even at the expense of not truly understanding intimate mechanisms associated to this therapy. On the contrary, based on case reports and short clinical trials, BMC-derived therapies have spread increasingly in the traumatology arena supported basically by observational results reporting safe and successful functional outcomes[3-6]. As a consequence, substantial concepts such as the definition of BMC’s active substance, its mode of action and the dose to be applied for a particular diagnostic remain questions yet to be answered.

One of the most accepted theories concerning BMC mode of action is related to the definition of its active substance. In this regard Mesenchymal Stromal Cells (MSC) has been suggested to play a major role in BMC’s reported therapeutic effects. Thus, it has been proposed that once BMC is applied, damaged tissue is restored by direct repairing of its structures by cell replacement[14,16-17]. However, recent experimental results point towards paracrine effects as the underlying mechanism of action behind MSC[12-13]. More importantly, other differentiated cells administered along with MSC in BMC with known physiological paracrine activities might also be involved in the mode of action of this cytotherapy[14-15]. This hypothesis really shifts the initial and somehow dogmatic belief about MSC as the unique active substance in BMC opening the possibility to include other cells as therapeutic drivers.

BM concentration is possibly the simplest strategy to obtain deliverable MSC although the fact that centrifugation procedure results in the concentration of other types of cells and platelets which are simultaneously applied into the patient is usually underestimated.

The resulting cocktail of living cells is usually seen as simple blood but is actually a natural combination of cells from different lineages generating growth factor able to drive and modulate physiological regeneration processes. Therefore, many of the components within BMC have the potential to play a role by direct action stimulating endogenous resident cells or even as ancillary cells supporting the MSC’s mode of action[14,16-17].

Here our main objective was to analyze how processing different BM volumes influenced the deliverable cellular dose and composition of BMC. To better define these medicinal products applied to the patients, main cellular populations were included in the description of BMC’s formulation.

**MATERIALS AND METHODS**

**Bone Marrow Aspiration**

Bone marrow (BM) aspirations were performed under local anesthesia from the posterior iliac crest by placing the patient in prone position. Standard aseptic procedures were followed in the operating room after obtaining informed consent. Briefly, after anesthetizing the puncture site down to the periosteum with a 3 mL analgesic shot a small incision was performed in the epidermis to facilitate direct access to the bone. There after a bone perpendicular insertion in the spongy bone with a beveled needle (BMB surelock. TSK Laboratory) was followed by a 3 - 4 mL rapid aspiration with a 10 cc syringe. The needle was then reoriented by a 90 degree rotation to a different depth or to a new and separate site of aspiration in order to minimize BM dilution with peripheral blood and another 3-4 mL aspiration was performed. BM was harvested from multiple sites until syringe was full with typically 2-3 aspirations. Syringes were changed at each set of aspirations and fully flushed with anticoagulant medium before use. Both crestes were used for aspiration, two surgeons performed BM harvest simultaneously and the total volume obtained was pooled in a blood transfusion bag with anticoagulant solution containing Phosphate Buffered Saline (PBS.Invitrogen) and 50 IU of sodium heparin per mL (Chiesi). At the end of the procedure light pressure was applied to minimize bleeding followed by skin sutures and occlusive dressing.

**Bone Marrow Concentration Process**

Bone marrow concentrations (BMC) were obtained by centrifugation at 500 g during 10 minutes at room temperature. Apart from the centrifugation step, the entire process was done in a laminar flow BioII/A hood (Cellgard480.Nuaire) placed at the point of care.

Briefly, BM aspirates were transferred to 15 mL sterile plastic tubes and a 4 to 10 mL sample was drawn for initial cell count and microbiological monitoring. After tubes were centrifuged, the supernatant and most part of the plasma fractions were separated, the buffy coat was recovered along with the immediate layer of red blood cells and placed in a separate sterile tube. Finally, BMC were dispensed in sterile syringes and a final sample of 1 to 5 mL was obtained from each product to proceed to microbiological test and cellular characterization.

BM volumes previously described to give rise to therapeutic doses of MSC range from 55 to 500 mL. Since large BM volumes are relatively complex to manage in the setting of an operating room and are usually processed by semiautomatic devices not available to all surgery teams, we evaluated the impact of three BM volumes (60 mL; 90 mL and 120 mL) easily manageable in a standard bench top centrifuge.

BMC were prepared as indicated and injected during the course of 109 surgeries. A great proportion of BMC (71%) were dedicated to treat two or three application sites during the same surgery including hips, knee joints ankles and wrists. Hips received BMC volumes ranging from 16 to 20 mL, knee joints received 7 to 8 mL and ankles and wrists had 4 to 8 mL of BMC.

All patients were fully informed with respect to the clinical protocol, cell processing details and associated risks. All patients signed informed consent form previously approved by the Ethics Committee at Hospital Quiron (Barcelona).

**Cellular Characterization**

Nucleated cell concentration and viability were determined by flow cytometry in a FacScalibur cytometer (Becton Dickinson). A Single platform, lyse and no wash CD45/CD34/7AAD ISHAGE protocol was applied[18-19]. Briefly, 25. ul of cells were stained in a tube with 10 ul of each FITC- CD45 and PE- CD34 conjugated antibodies (BD Bioscience) and incubated during 15 minutes at room temperature in the dark. Afterwards 1 mL of red blood lysis buffer and 10 ul of 7AAD were added and incubated for 10 additional minutes. Finally, 25 ul of control count fluorospheres were added to the tube and samples were gently mixed and analyzed by flow cytometry (FACScalibur.BD Biosciences). Mononucleated (MNC) and Polyomorphonucleated cells (PMNC) were defined by forward and
side light scatter characteristics. Platelets were measured by using an automated hematology analyzer (ACTDiff, Beckman Coulter).

**CFU-F Assay**

Quantification of Mesenchymal stromal cells (MSC) was performed by means of Fibroblastic Colony Forming units (CFU-F) assay in all cases as previously reported \(^{36}\). Briefly, CFU-F were determined by plating \(5 \times 10^6\) living total nucleated cells (TNC) per \(cm^2\) in 6 well dishes per triplicate. Cell cultures were spanned for 7-10 days using basic culture medium (DMEM, Gibco) supplemented with human serum previously validated for clinical expansion of MSC. Cultures were washed three days after initial seeding and after the time of culture hematoxin stained colonies were counted under optic microscope (DM IL LED, Leica Microsystems). Colonies containing more than 20 cells were counted and CFU-F frequency was defined per \(1 \times 10^6\) TNC. CFU-F frequency was then used in combination with absolute numbers of TNC to calculate total dose of MSC applied along with each bone marrow concentrate (BMC). CFU-F/mL values were calculated by dividing calculated total dose of CFU-F per volume of recovered BMC after centrifugation.

**Statistical Analysis**

Cellular dose and concentration values from non related samples were compared using a non-parametric Mann-Whitney U test (IBM SPSS statistics software version 12.0). Data was considered significantly different when \(p < 0.05\).

## RESULTS

Cellular concentration, viability and total cell dose administered to patients in BMC for autologous use were quantified during the course of 109 surgeries (43 females; 66 males; mean age 48.4 ± 14.5 years). The BMC formulation data were analyzed according to the volume of processed BM and three groups of study were created; 60 mL, 90 mL and 120 mL. For these groups, BM blood from a total of 25, 42 and 42 patients with median ages of 46 (15-75), 49 (18-73) and 51 (19-73) years were included respectively. No adverse events related to the harvest, processing or re-infusion of cellular products was reported and microbiologic quality controls were negative for asperates and concentrated product samples.

Volumes of BM initially obtained for the groups of 60, 90 and 120 mL were 77 ± 9, 103 ± 5 and 139 ± 14 mL respectively. For the same groups recovered buffy coat volumes were 11.4 ± 4 mL, 16.4 ± 4.7 mL and 21.5 ± 5.4 mL respectively. Injectable BMC volumes varied from 4 to 20 mL to adjust them to the available space in application sites including knees, ankles, wrists and hips. Hips received BMC volumes ranging from 16 to 20 mL, knee joints received 7 to 8 mL and ankles and wrists had 4 to 8 mL of BMC. Concentration of total nucleated cells (TNC) and cell viability in BM aspirates were not statistically different among groups. Calculated mean values for cell concentration were 18.4 ± 6.5 \(\times 10^6\); 23.4 ± 10 \(\times 10^6\) and 21.7 ± 8 \(\times 10^6\) TNC/mL for 60, 90 and 120 mL groups respectively with viabilities higher than 90% in all cases. Concentration of CD34\(^+\) progenitor cells did not differ statistically between groups and ranged from \(0.16 \times 10^6\) to \(0.19 \times 10^6\) cells/mL. BM aspirates had platelet counts lower than those reported for peripheral blood ranging from 33 \(\times 10^6\) to 160 \(\times 10^6\) per mL.

The process of centrifugation increased 4.7 ± 1.7 times the TNC concentration and it was not influenced by the BM volume processed. Composition of BM aspirates and BMC were approximately 80% of PMNC, 19% of MNC and 1% of CD34\(^+\) cells in all groups (Table 1). Cell concentration in BMC were 73.7 ± 23.9 \(\times 10^6\) TNC/mL; 106.7 ± 48.5 \(\times 10^6\) TNC/mL and 105.6 ± 47.2 \(\times 10^6\) TNC/mL for the groups of study and these differences were statistically significant between the 60 mL group and the other 2 groups which did not statistically differ from each other. Cell recoveries after centrifugation were 73-81% for TNC, 82-92% for MNC and 79-90% for CD34\(^+\) cells and it did not improve when recovered buffy coat volumes were higher than a 10% of the initially centrifuged BM volume. After centrifugation we obtained 0.8 ± 0.3 \(\times 10^6\), 1.7 ± 0.8 \(\times 10^6\) and 2.2 ± 0.9 \(\times 10^6\) deliverable TNC for the groups of 60, 90 and 120 mL respectively. For these groups, concentration of CD34\(^+\) progenitor cells in BMC were 0.7 ± 0.4 \(\times 10^6/mL\), 0.95 ± 0.6 \(\times 10^6/mL\) and 0.9 ± 0.4 \(\times 10^6/mL\) and total dose of these cells was found to be significantly less for the 60 mL group compared to the other ones which did not differ from one another (Table 1). BMC also contained platelets at a median concentration of 200 \(\times 10^6/mL\) ranging from 78 \(\times 10^6\) to 734 \(\times 10^6\) platelets/mL.

Prevalence of MSC indirectly measured as CFU-F colonies per 106 TNC slightly diminished with higher BM aspirate volumes and were 46.3 ± 33.6, 35.4 ± 22.5 and 31 ± 28 CFU-F/mL for groups of 60 mL, 90 mL and 120 mL. Fibroblastic colony count displayed a wide variability and CFU-F concentration in BMC ranged from 360 \(\times 10^3\) to 13520 CFU/mL. CFU-F per mL was not statistically different between groups of study and mean values varied from 2600 to 2100 CFU-Fs respectively) than when processing 60 mL of BM (3.5 \(\times 10^6\) CFU-Fs). In this sense, having a reference value of 50 CFU-Fs/mL and total dose of these cells was found to be significantly less for the 60 mL group compared to the other ones which did not differ from one another (Table 1). BMC also contained platelets at a median concentration of 200 \(\times 10^6/mL\) ranging from 78 \(\times 10^6\) to 734 \(\times 10^6\) platelets/mL.

### DISCUSSION

The opportunity of using living cells contained in BMC as therapeutic tools is a very attractive approach due to the methodological simplicity and its compatibility with current surgical procedures. However basic practical concepts related to BMC management such as cellular composition, its relationship with the clinical results observed, the frequency of administration or the optimal cellular dose to be applied for a particular diagnostic are not well defined yet.

Therapeutic capabilities of BMC have been traditionally related to the concentration of MSC defined as total stromal progenitor counts per volume unit (CFU-F/mL). We observed a high variability among patients regarding this parameter although a mean concentration of

### Table 1. Formulation and cellular doses in BMC.

| Processed BM Volume (mL) | Volume (mL) | TNC \(\times 10^6/mL\) | PMNC \(\times 10^6\) | MNC \(\times 10^6\) | CD34\(^+\) \(\times 10^6\) | CFU-F/10^6 TNC | \(p\) value |
|-------------------------|-------------|-----------------------|-------------------|--------------------|------------------|----------------|-----------|
| 60 mL (n=25)            | 11 ± 4      | 73.7 ± 23.9           | 626 ± 288         | 106 ± 64           | 0.9 ± 4.4        | 46.3 ± 33.6    | 0.024*    |
| 90 mL (n=42)            | 16 ± 5      | 106.7 ± 48.5          | 1364 ± 725        | 301 ± 117          | 15 ± 9           | 35.4 ± 22.5    | \(= 0.604^{**}\) |
| 120 mL (n=42)           | 21 ± 5      | 105.6 ± 47.2          | 1778 ± 830        | 396 ± 143          | 18 ± 7           | 31 ± 28        | \(= 0.604^{**}\) |

Mean ± SD values are shown. †Bone Marrow; ‡Polymorphonucleated Cells; §Mononucleated Cells; CFU-F: Fibroblastic Colony Forming Units. ††TNC: Total Nucleated Cells. *60 mL group vs 90 mL. **90 mL group vs 120 mL group.
3000 CFU-F/mL was maintained irrespective of the BM volume analyzed. Previously reported clinically effective doses of MSC range from 1500 to 9000 CFU-F/mL depending on the diagnostic and the method of BMC delivery\textsuperscript{[5,11,20,21,22,23]} This wide range seems to be related to the huge interpersonal variability of MSC endowment\textsuperscript{[22]} and the different protocols utilized for quantification, but it also might be associated to the different processed volumes of BM and BMC (Table 2). Similarly, another parameter associated to BMC’s healing potential is the number of CFU-F per total nucleated cells which ranges from 25 to 39 CFU-F /106 TNC\textsuperscript{[10,13,12,24]}. We observed a statistically significant reduction of this value when comparing 60 mL to 120 mL aspirated BM possibly due to peripheral blood dilution despite it being aspirationally proportional to the total dose of MSC obtained for the same groups. Consequently, CFU-F/mL and CFU-F/TNC values seem to be directly related to processing variables and perhaps should not be assumed as a benchmark of potency in BMC mainly when comparing products obtained by using different processing protocols.

Beyond concentration or frequency of MSC, total dose of these cells might better define BMC. In this sense, available doses of MSC in BMC have been described to span from $14 \times 10^{6}$ to $3 \times 10^{7}$ in the literature\textsuperscript{[25,20,21,11,18]} (Table 2). We obtained an average total MSC dose ranging from $35 \times 10^{6}$ to $59 \times 10^{6}$ CFU-F with injectable volumes from 6 mL to 20 mL by processing progressive amounts of BM and recovering proportionally higher volumes of buffy coat. This protocol made it possible to adjust BMC volumes to desirable final values in order to fit defined anatomic spaces while maintaining MSC doses with previously reported therapeutic effects.

On the other hand, it is really surprising that while an overwhelming part of the cells within BMC belong to hemopoetic lineages and MSC represent approximately only a 0.003 % of TNC\textsuperscript{[25]}, those blood cells are usually underestimated in terms of its medicinal potential. On the contrary, increasing BM processed from 90 to 120 mL did not statistically change the total cellular doses in BMC. This observation might by relevant because as recently reported in a dose-response analysis\textsuperscript{[10]}, total nucleated cell dose might be an important factor governing clinical outcomes after BMC treatment. Finally, in addition to the mentioned role of those differentiated cells in BMC, CD34\textsuperscript{+} progenitor cells injected along with BMC could also influence the reported BMC therapeutic effects. It is known that BM CD34\textsuperscript{+} cells contain endothelial, hemopoetic and osteoblastic progenitors\textsuperscript{[15]} with described regenerative capabilities mediated by direct differentiation and via paracrine signals\textsuperscript{[34]}. Interestingly, isolated MSC from BM CD34\textsuperscript{+} cells retained unaltered differentiation capabilities\textsuperscript{[18-30]} as those described for cultured expanded MSC\textsuperscript{[17]}. Moreover, as described in a recent pilot clinical trial, purified and locally transplanted CD34\textsuperscript{+} cells promote tissue regeneration and total bone healing\textsuperscript{[28]}. We found that BMC contained total doses of CD34\textsuperscript{+} cells from 7 to $18 \times 10^{6}$ depending on the BM volume initially processed which, in light of their reported paracrine positive effects could also synergistically participate on the BMC clinical outcomes.

Taken together the results presented here show that processing 60 mL of BM results in statistically significant lower total cellular doses in BMC than those found when processing 90 to 120 mL. On the contrary, increasing BM processed from 90 to 120 mL did not statistically change the total cellular doses in BMC. This observation might by relevant because as recently reported in a dose-response analysis\textsuperscript{[10]}, total nucleated cell dose might be an important factor governing clinical outcomes after BMC treatment. Finally, in addition to the mentioned role of those differentiated cells in BMC, CD34\textsuperscript{+} progenitor cells injected along with BMC could also influence the reported BMC therapeutic effects. It is known that BM CD34\textsuperscript{+} cells contain endothelial, hemopoetic and osteoblastic progenitors\textsuperscript{[15]} with described regenerative capabilities mediated by direct differentiation and via paracrine signals\textsuperscript{[34]}. Interestingly, isolated MSC from BM CD34\textsuperscript{+} cells retained unaltered differentiation capabilities\textsuperscript{[18-30]} as those described for cultured expanded MSC\textsuperscript{[17]}. Moreover, as described in a recent pilot clinical trial, purified and locally transplanted CD34\textsuperscript{+} cells promote tissue regeneration and total bone healing\textsuperscript{[28]}. We found that BMC contained total doses of CD34\textsuperscript{+} cells from 7 to $18 \times 10^{6}$ depending on the BM volume initially processed which, in light of their reported paracrine positive effects could also synergistically participate on the BMC clinical outcomes.

### Table 2 Reported therapeutic Cellular doses in BMC.

| Indication       | BMA Volume | BMC Volume | BMC $\times$ $10^6$ NC / mL | TNC $\times$ $10^6$ | CFU-F $\times$ $10^3$/mL | MSC $\times$ $10^3$/mL | Dose Ref. |
|------------------|------------|------------|-----------------------------|---------------------|-----------------------|----------------------|-----------|
| Bone nonunion    | 300 mL     | 50 mL total| n.r.‡‡                  | 2576                | 312 $\times$ $10^3$ | 37 $\times$ $10^3$ | [5]       |
| Bone nonunion    | 400 mL     | 8 mL       | 113                       | 7300                | 625                   | 312 $\times$ $10^3$ | [21]      |
| Bone defect      | 60 mL      | 8 mL       | 113                       | 904                 | 4600                  | 37 $\times$ $10^3$ | [20]      |
| Osteonecrosis    | 300 mL     | 50 mL total| n.r.‡                    | 870                 | 4000                  | 147 $\times$ $10^3$ | [6]       |
| Osteonecrosis    | 400 mL     | 50 mL total| n.r.‡                    | 1898                | 3579                  | 137 $\times$ $10^3$ | [40]      |
| Cartilage lesion | 60 mL      | 8-6 mL     | n.r.‡                    | 2000                | 3680                  | 184 $\times$ $10^3$ | [9]       |
| Rotor cuff       | 150 mL     | 12 mL      | n.r.‡                    | 904                 | 4300                  | 23-31 $\times$ $10^3$ | [9]       |
| Back disc pain   | 60 mL      | 6 mL       | 121                      | 726                 | 2713                  | 2000 $\times$ $10^3$ | [11]      |

† Bone Marrow Aspirate. ‡ Bone Marrow Concentrate. § Total Nucleated Cells. †† Mesenchymal Stromal Cells. ‡‡not reported.
to MSC, which are a scarce resource, a heterogeneous mixture of cells with known relevant roles in natural regenerative processes is applied within BMC. This particular combination of cells could be part of BMC active substance and consequently its quantification might contribute to better define therapeutic cellular doses for these medicinal products.

ACKNOWLEDGMENTS

The authors wish to thank Dr. José Joaquin Ceron Madrigal, Dr. Joaquim Vives Armengol and Mr. Thomas Oxlee for manuscript review. The authors declare that they have no conflict of interest.

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Peer reviewers: Aaron W James, University of California, Los Angeles, 10833 Le Conte Ave, A3-251, CHS, Los Angeles, California, USA; Jeong Ik Lee, Associate Professor, Regenerative Medicine Laboratory, Center for Stem Cell Research, Department of Biomedical Science and Technology, Institute of Biomedical Science and Technology (IBST), Konkuk University, 143-701, Seoul, Korea.