



A single step, centrifuge-free method to harvest bone marrow highly concentrated in mesenchymal stem cells: results of a pilot trial

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Abstract

Purpose The aims of the present study were: (1) to characterize the bone-marrow aspirate (BMA) obtained with a centrifuge-free process, employing a dedicated aspiration device; (2) to test the in vitro efficacy of BMA in a model of cartilage inflammation; and (3) to report the preliminary clinical results in a small cohort of patients affected by knee OA.

Methods Ten patients (4 M, 6 W; mean age: 51.9 ± 9.2 yy) affected by mild to moderate unicompartmental knee OA (KL grade 2–3) were treated by intra-articular and subchondral injections of BMA obtained by a centrifuge-free process. To evaluate the effectiveness of the device in harvesting mesenchymal stem cells (MSCs), samples of the obtained BMA were tested by flow cytometry before and after subculture; BMA ability to counteract inflammation was also tested in an in vitro model of cartilage cell inflammation, evaluating the expression of *MMP1*, *MMP3*, *TGFβ* and *TIMP-1* by real-time PCR. Patients were also evaluated up to two years' follow-up by using: VAS for pain, IKDC-subjective and KOOS scores.

Results The laboratory analysis showed that BMSCs accounted for 0.011% of BMA cells, similar to what had been expected in native bone marrow. The paracrine activity of BMA was able to reduce in vitro the catabolic response of human chondrocyte, as shown by the decrease in metalloproteases concentration and increase in anti-inflammatory mediators. Moreover, the clinical evaluation showed significant improvements in all scores adopted, with stable results up to two years.

Conclusion The present data showed the effectiveness of the study device to harvest pure bone marrow with minimal peripheral blood contamination. The relevant content of MSCs resulted in the ability to counteract the catabolic cascade through a paracrine action. The clinical outcomes in patients affected by unicompartmental knee OA were encouraging in terms of pain reduction and functional improvement up to mid-term evaluation.

Keywords Bone marrow aspirate · Knee osteoarthritis · Mesenchymal stem cells · Bone marrow harvesting

Introduction

Bone marrow aspirates (BMA) and concentrates (BMC) have been employed as ortho-biologic agents for the treatment of several musculoskeletal disorders, including osteoarthritis (OA) [1–3]. Their mechanisms of action are based on their content in mesenchymal stem cells (MSCs), haematopoietic stem cells (HSCs), platelets, and growth factors [4]. These elements, especially MSCs and growth factors, showed anti-inflammatory and pro-regenerative activities, through the activation of several pathways acting on cell trophism, proliferation, differentiation, apoptosis, and senescence [5]. Indeed, bone marrow-derived mesenchymal stem cells (BMSCs) have been reported to be the main effectors of BMA/BMC clinical effects, either by direct differentiation into terminally differentiated cells or by their paracrine

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action. These actions allow the restoration of tissue homeostasis and the promotion of tissue healing and regeneration [6]. Examples of their clinical application, both through injections or as an augmentation during surgery, have been reported in several publications, where the beneficial effects of bone marrow have been shown [7, 8]. In addition, also the application of cultured BMSCs has been proposed as a treatment option for musculoskeletal disorders [9, 10]. Nevertheless, the findings are somewhat conflicting, possibly due to the lack of standardization in the indications to treatment and product preparation. Indeed, differently from cultured BMSCs, the cell content in BMA/BMC depends on patient's characteristics, as well as on the harvesting site and preparation procedure [11, 12]. In fact, bone marrow obtained from the iliac crest demonstrated a higher content of BMSCs compared to that collected from tibial or femoral condyles [13]. In addition, the harvesting of small quantities of bone marrow from different sites improves the ratio between proper marrow cells and peripheral blood cells [14]. Peripheral blood contains an extremely low amount of MSCs compared to bone marrow [15], and thus MSCs concentration would be reduced if a relevant quantity of peripheral blood is collected together with BMA. Consequently, many procedures for the production of BMC include a centrifugation step in order to concentrate BMSCs. These procedures allow to collect BMSCs and HSCs at a similar concentration compared to the one expected in native bone marrow, i.e., approximately 0.01% and 1% of total nucleated cells, respectively [16–18]. A new device has been developed to avoid the use of centrifugation steps: Marrow Cellution™ (Aspire Medical Innovation, Munich, Germany). This device allows the collection of small volumes of bone marrow from multiple sites through a single access, thus reducing patient's discomfort, donor site morbidity and operative time [19].

The main aims of this study were the characterization of BMA obtained by Marrow Cellution™ and the evaluation of its efficacy in an *in vitro* model of cartilage inflammation. Moreover, this manuscript reports the clinical outcomes of an observational pilot study on the use of BMA obtained by this device in a small cohort of patients affected by mild to severe osteoarthritis.

Methods

Patients and samples

This observational pilot clinical study was approved by the IRCCS Humanitas Research Center Hospital Ethics Committee and Internal Review Board (protocol number: 530/18, approved on 18th September 2018) and informed consent was obtained from all patients.

The samples were obtained from the iliac crest of ten patients (mean age: 51.9 ± 9.2 ; mean BMI 24.7 ± 2.8 ; 4 men, 6 women), affected by unicompartamental knee osteoarthritis and enrolled according to the following inclusion criteria: (1) age between 35 and 75; (2) osteoarthritis in the medial or lateral compartment (grade 2 or 3 according to the Kellgren-Lawrence score); (3) MRI evidence of bone marrow lesions (BMLs) in the tibial and femoral subchondral areas of the same compartment; (4) at least four months of persisting pain; (5) failure of at least one previous conservative treatment (including: pharmacological therapy with NSAIDs or analgesics, rehabilitation programs, injective treatments). Exclusion criteria were: (1) Kellgren-Lawrence score 1 or 4; (2) symptomatic patello-femoral disease; (3) lower limb malalignment (varus/valgus $> 10^\circ$); (4) untreated knee instability; (5) history of recent knee surgery (within 12 months from the procedure); (6) rheumatic disease; (7) history of previous or ongoing malignancy; (8) drugs or alcohol abuse history; and (9) BMI < 18 or > 35 .

BMA harvesting procedure

The BMA samples were obtained from each patient using the Marrow Cellution™ System (Fig. 1). With the patients

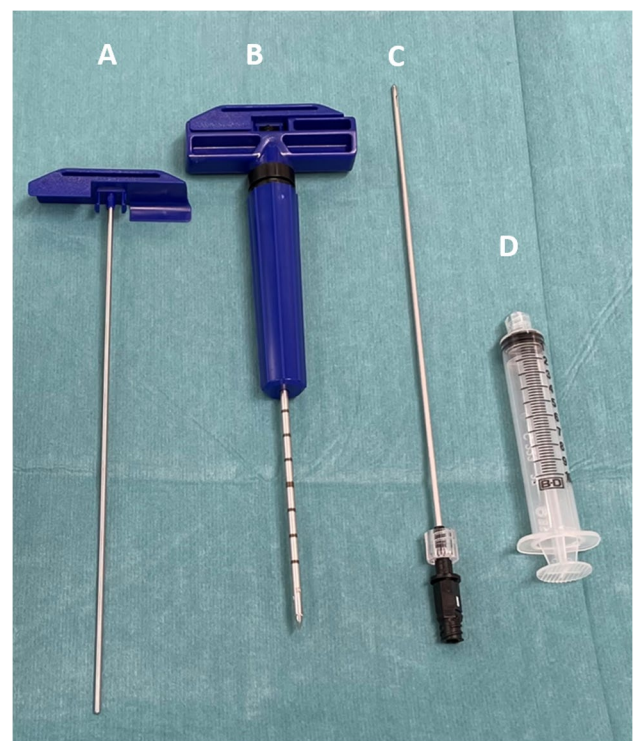


Fig. 1 Marrow Cellution™ System. **A** Blunt stylet; **B** Trocar with a rotating T handle and sharp needle inside; **C** Aspiration cannula with a closed tip and lateral distal fenestrations for BMA aspiration; **D** Luer-lock syringe to collect the final output

lying in supine position, under spinal anaesthesia, approximately 11–12 ml of bone marrow was harvested from the anterior–superior iliac crest.

The device was designed to collect BMA with a high concentration of BMSCs by a centrifuge-free procedure, and consists of a modified trocar with a removable sharp introducer needle used to penetrate the cortical bone wall and reach the marrow cavity. First, the sharp introducer needle is removed and a first aspiration of approximately 2 ml of bone marrow is performed via a luer-lock syringe attached directly to the upper end of the trocar. Then, a blunt stylet is placed to further advance the trocar into the spongy bone and, when the final position is reached, the aspiration cannula is put inside the trocar. Differently from the traditional harvesting system, the aspiration cannula has only lateral holes and therefore aspiration occurs only perpendicularly to and around the channels created by the tip of the device during its insertion. Multiple small aspirations (1 ml each) are performed at various levels of the marrow cavity by gently retracting backward the device, to collect a ready-to-inject final volume of about 12 ml of BMA (Fig. 1).

Treatment and follow-up

All patients were treated at the target knee by subchondral and intra-articular injection of BMA collected as described above. Within a few minutes after BMA collection, trocars were placed under fluoroscopic guidance at the subchondral bone-cartilage interface, and 3 ml of BMA was injected both in the tibial and femoral BMLs. The other 3 ml of BMA was injected into the joint by a lateral superior para-patellar approach. The remaining amount of BMA (approximately 1.5–3 ml) was used for the biologic analyses described below. The patients were discharged the same day of the procedure with the instruction of using two canes with partial weight-bearing for the first two weeks, then progressive weight bearing was allowed. Range of motion and muscle strengthening exercises were started the day after the procedure.

Patients were prospectively evaluated at baseline, six-, 12-, and 24-month follow-up by the Subjective International Knee Documentation Committee (IKDC) score, the Knee injury and osteoarthritis outcomes score (KOOS) subscales, and the Visual Analogue Scale (VAS) for pain.

Cell count and viability

The biological analyses were carried out at the IRCCS Istituto Ortopedico Galeazzi, Milan, Italy. Complete blood cell count was performed to assess the number of red blood cells (RBC) and nucleated cells. The number of nucleated cells, as well as the cell viability, was also assessed by an automatic cell counter (NucleoCounter NC-3000, ChemoMetec) after RBC lysis.

BMA flow cytometry

For each sample ($n = 10$), a volume equal to 3×10^6 nucleated cells was treated with RBC lysis buffer following the manufacturer's instructions (Milteny Biotech, Bergisch Gladbach, Germany), and then suspended in 200 μ l FACS buffer. Then, 1.5×10^6 cells were stained with anti-CD45-PE-Vio770, CD34-FITC, CD271-PE and CD105-PerCP-Vio700 antibodies (Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 minutes at 4 °C, while the remaining cells were maintained unstained as a control. After one wash in FACS buffer, the samples were suspended in 500 μ l FACS buffer and analyzed by CytoFlex flow cytometer (Beckman Coulter, Brea, CA, USA). To score rare BMSC cells, at least 500,000 events were acquired. Data were analyzed with CytExpert software (Beckman Coulter). Hematopoietic Stem Cells (HSC) were identified as CD45 + CD34 + cells, while Mesenchymal Stem Cells were identified as CD45- CD271 + CD105 + cells.

Cell culture and doubling time

An aliquot of each sample ($n = 10$) was cultured up to passage 4 in complete medium (high glucose DMEM, Sigma Aldrich, St. Louis, MO, USA; 10% fetal bovine serum, Hyclone, Euroclone, Milano, Italy; 1% Penicillin–Streptomycin–Glutamine, Thermo Fisher Scientific, Waltham, MA, USA) to allow for doubling time calculation. Samples were initially plated at the density of 50,000 cells/cm²; when cells reached 80% confluence, they were detached by trypsinization, and then plated again at 50 cell/cm² density for four passages. Proliferation was calculated as doubling time (hours per doubling) with the formula:

$$\text{DoublingTime(DT)} = \log(n2/n1) * 3,33/t2 - t1 (n1 = \text{number of cells at seeding}; n2 = \text{number of cells at harvesting}; t2 - t1 = \text{hours between seeding and harvesting})$$

BMSC flow cytometry

BMSCs at passage 4 were detached, suspended in 100 µl FACS Buffer, and incubated with anti-human CD44-PE-Vio-770, CD90-FITC, CD105-PerCP-Vio700, CD73-PE, CD45-PE-Vio770, and CD34-FITC antibodies (Miltenyi) for 30 min at 4 °C. After one wash in FACS buffer, cells were suspended in 100 µl FACS buffer and analyzed by CytoFlex instrument (Beckman Coulter), collecting a minimum of 30,000 events. Data were analyzed with CytExpert software (Beckman Coulter).

Evaluation of the in vitro anti-inflammatory and anti-catabolic potential

Human chondrocytes (hCH) were obtained from surgical waste material resulting from knee replacement surgeries (n. of patients = 3). Material collection was approved by the Institutional Review Board (San Raffaele Hospital Ethics Committee approval, 8 March 2018, number 6/int/2018), and an informed consent was obtained from all the patients. Briefly, cartilage tissue was minced and digested with 0.15% w/v type II collagenase (Worthington, Columbus, OH, USA) for 16 hours at 37 °C, cells were harvested and cultured up to passage 3 in complete medium. To evaluate the paracrine potential of BMA, 5×10^4 human chondrocytes were plated in the bottom portion of a 24-well transwell plate, in the presence or absence of the pro-inflammatory agent IL-1 β (1 ng/ml). For each BMA sample (n = 10), a volume equivalent to 5×10^4 nucleated cells was then added to the top portion of the transwell membrane (pores size 0.4 µm). Equal volumes of PBS were added in control wells instead of BMA. After 48 hours of culture, chondrocytes were harvested and lysed. Then, mRNA was purified using PureLink mRNA mini kit (Thermo Fisher Scientific) according the manufacturer's instructions. 100 ng of mRNA was retro-transcribed to cDNA by iScript (Bio-Rad, Hercules, CA, USA) and then the gene expression of *MMP1*, *MMP3*, *TGF β* and *TIMP1* genes were evaluated using Taqman probes (Thermo Fisher Scientific). Results were normalized on the expression of the housekeeping genes *YWHAZ* and *TBP*. Data are reported as $2^{-\Delta\Delta C_t}$: this value represents the fold change in the relative expression of a specific gene in the sample of interest compared to a control sample. It is calculated by the following formula:

$$2^{-[(C_{t(\text{gene of interest})} - C_{t(\text{housekeeping gene})}) \text{ in the sample of interest} - (C_{t(\text{gene of interest})} - C_{t(\text{housekeeping gene})}) \text{ in the control}]}$$

The media from co-culture experiments were collected for the assessment of the soluble mediators IL-1Ra and IL-6 by ELISA assays (Peprotech, London, UK). The assays were performed according to the manufacturer's instructions, and the ranges of detection were 23–1500 pg/ml and 16–2000 pg/

ml for IL-1Ra and IL-6, respectively. Before the assessment of IL-6, a 1:20 dilution was performed on IL-1 β -treated samples. Samples of BMA incubated with and without IL-1 β in the absence of chondrocytes for 48 hours were also tested to evaluate the contribution of BMA to the production of these cytokines with respect to hCH. All experiments were performed in duplicates.

Statistical analysis

Statistical analysis was performed using Prism v5.0 (Graphpad Software, La Jolla, CA, USA). Descriptive statistics were calculated and numerical variables are reported as mean $\pm$ standard deviation, while categorical variables are reported as counts and percentages (if not otherwise specified). Distribution of data was assessed using Shapiro–Wilk test, and parametric or non-parametric tests were applied accordingly. Paired t-test or one-way ANOVA (or their non-parametric counterparts, Wilcoxon or Friedman tests) with appropriate post hoc tests were used to compare two or more groups differing for a single variable. When two variables were in play, two-way ANOVA with Bonferroni post hoc test were used to evaluate the contribution of all variables. P values < 0.05 were considered statistically significant.

Results

Complete blood cell count and viability

The mean volume available for the analysis was 2.6 ± 1.0 ml. Complete blood cell analysis revealed a total nucleated cell count of $13.6 \times 10^6 \pm 8.1$ cells/ml and a RBC count of $4.0 \times 10^6/\mu\text{l} \pm 1.4$. The mean cell viability was 93.7% (Table 1).

Identification of HSCs and BMSCs in BMA samples

Percentages and counts of HSCs and MSCs in BMA are reported in Table 2. Considering the total amount of nucleated cells, the median percentage of CD45+ cells was 92.8% (interquartile range (IQR): 89.1–96.8%), whereas the median percentage of CD45- cells was 7.2% (IQR: 3.2–10.9%). On this basis, CD45- cells were assessed for positivity to CD34

Table 1 Cell and platelet count in BMA

Nucleated cells ($\times 10^6/\text{ml}$)	13.6 ± 8.1
RBC ($\times 10^6/\mu\text{l}$)	4.0 ± 1.4
Platelets ($\times 10^6/\text{ml}$)	77 ± 33
Cell viability (%)	93.7 ± 5.3

Table 2 HSC and BMSC frequency and count in BMA

Cell type	%	cell/ml
HSCs	$1.2\% \pm 1.0$	$1.9 \times 10^5 \pm 2.5$
BMSC	$0.011\% \pm 0.006$	$1.3 \times 10^3 \pm 0.8$

and CD271/CD105, in order to identify HSCs and BMSCs, respectively. In regard to total nucleated cells, $1.2\% \pm 1.0$ were HSCs and $0.011\% \pm 0.006$ were BMSCs. These percentages correspond to $1.9 \times 10^5 \pm 2.5$ HSCs per ml and $1.3 \times 10^3 \pm 0.8$ BMSCs per ml of BMA.

Cell proliferation and immunophenotype after subculturing

The cells derived from the subculturing of BMA showed a constant proliferation ability up to passage 4. In particular, at passage 2, 3, and 4, the mean doubling time was 16.6 ± 3.8 , 23.7 ± 35.2 , and 23.5 ± 21.6 hours, respectively (Fig. 2A). When observed by optical microscopy, BMA-derived cells formed colonies up to passage 1, showing the typical MSC spindle-shaped morphology (Fig. 2B). After culturing, BMA-derived cells showed the typical BMSC immunophenotype (CD44+, CD90+, CD105+, CD73+, CD45−, CD34−).

Paracrine effect of BMA on human chondrocytes in experimental inflammatory conditions

In the presence of inflammatory conditions in vitro, human chondrocytes (hCH) demonstrated increased expression of the catabolic enzymes metalloproteinases 1 and 3 (*MMP1*, *MMP3*). The use of BMA was able to counteract the catabolic response to the inflammatory stimuli, reducing the expression of *MMP1* and *MMP3* by a mean fold change of

0.82 and 0.73, respectively, compared to the samples treated by IL-1 β -only. No effects were observed on the gene expression of *TIMP1* and *TGF β* (Fig. 3).

The levels of the soluble mediators IL-1Ra and IL-6, possibly involved in the modulation of the inflammatory response, were assessed. IL-1Ra levels were increased in the samples treated with both IL-1 β and BMA, compared to other conditions, and in particular with respect to samples stimulated with IL-1 β only ($p < 0.05$) (Fig. 4A). Concerning IL-6, it was significantly induced by IL-1 β in all conditions, compared to the corresponding non-inflamed samples. Interestingly, even BMA product alone was able to produce IL-6 in response to the inflammatory stimulus, and although in a non-statistically significant manner, the concurrent presence of IL-1 β and BMA elicited an increase in IL-6 content in the samples (Fig. 4B).

Clinical outcomes

The subjective IKDC score significantly improved compared to baseline from a pre-operative mean value of 37.11 ± 13.5 to 65 ± 22.9 at six months ($p < 0.01$), 61.5 ± 12.8 at 12 months, and 64.0 ± 20.1 at 24 months ($p < 0.05$) (Fig. 5A). Regarding VAS, a pain reduction occurred from a basal value of 6.6 ± 1.3 to 4.4 ± 2.9 at six months, 4.3 ± 2.4 at 12 months and 4.1 ± 2.6 at 24 months, with a significant reduction observed at 6 months compared to baseline ($p < 0.05$) and then stable results up to the final evaluation ($p < 0.05$ at 12 months; $p < 0.01$ at 24 months) (Fig. 5B).

A positive trend was also observed in all the KOOS subscales (Fig. 5C). Even in this case, the improvement from baseline to the 6-month follow-up was statistically significant in all the subscales, and the values then remained stable up to the 24 months evaluation.

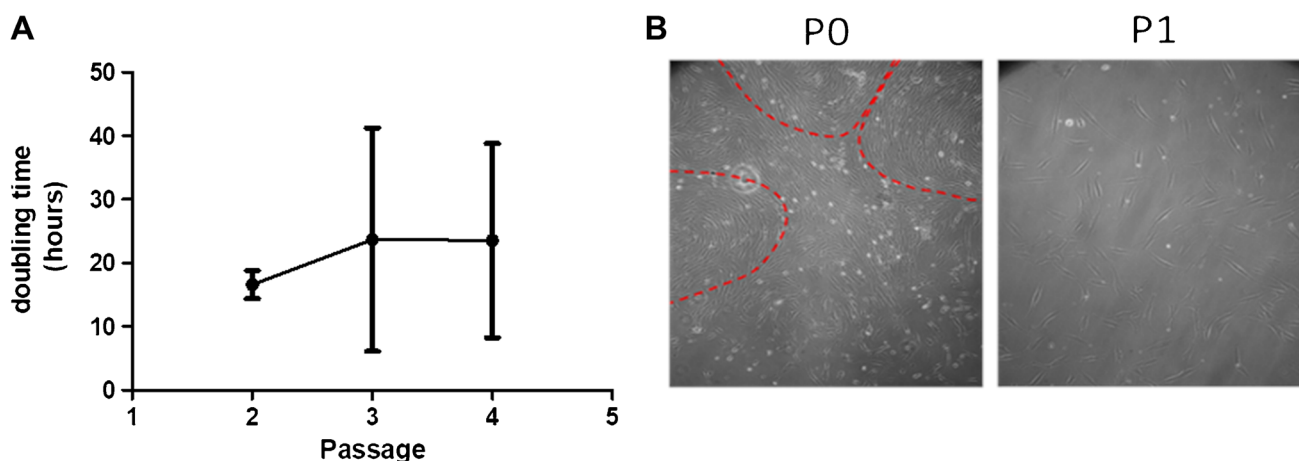


Fig. 2 **A** Doubling time (hours) of BMA-derived cells at passages 2, 3, and 4 ($n = 10$); data are presented as mean $\pm$ SD. **B** Micrographs of BMA-derived cells during subculturing at passage 0 (P0) and passage 1 (P1)

Fig. 3 Gene expression of MMP1 (A), MMP3 (B), TGF β (C), and TIMP1 (D) in human chondrocytes (hCH) in the presence and absence of IL-1 β and/or BMA after 48 h in culture (n=10). *p<0.05, **p<0.01, ***p<0.001 with respect to untreated and unstimulated chondrocytes (hCH). Data are expressed as mean $2^{-\Delta\Delta Ct} \pm SD$

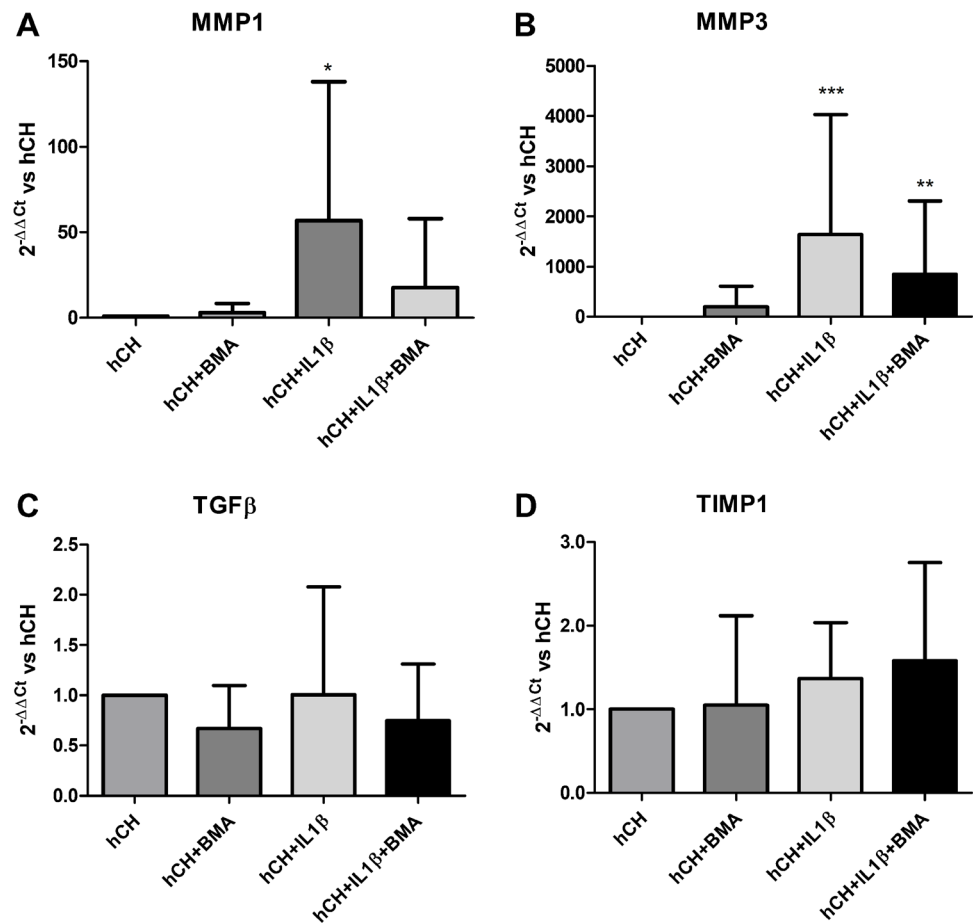
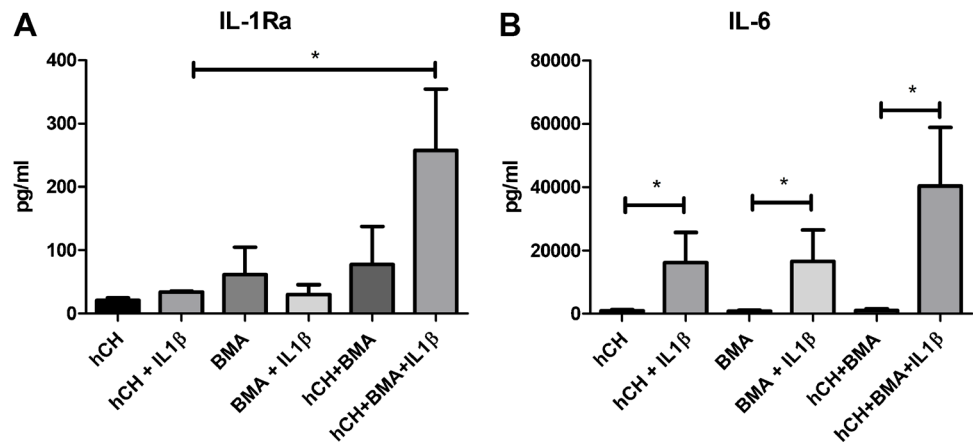


Fig. 4 Content of the soluble mediators IL-1Ra (A) and IL-6 (B) in the culture media of hCH and/or BMA in the presence/absence of the inflammatory stimulus (IL-1 β) (n=10). Data are expressed as mean $\pm$ SEM. *p<0.05

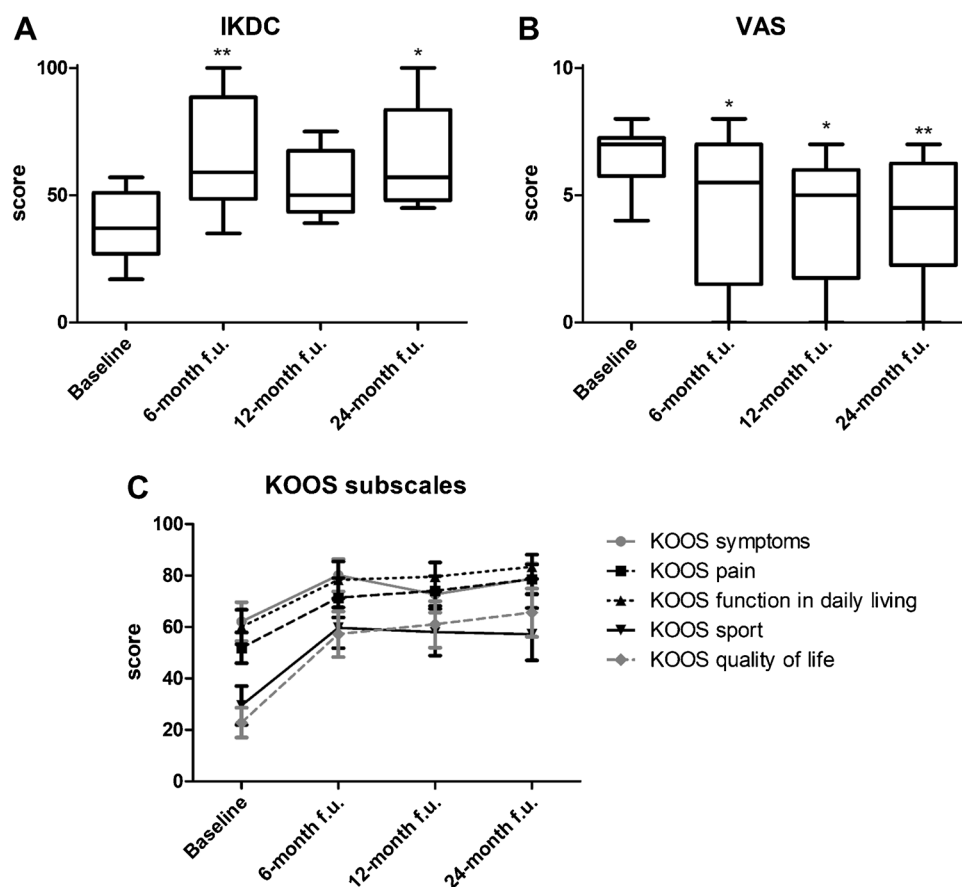


Discussion

This study aimed at characterizing BMA obtained by Marrow Cellution™ device, and at evaluating the clinical outcomes of its use in knee OA. In particular, the experiments performed demonstrated that the content of BMSCs was able to control inflammation and counteract the catabolic cascade triggered by an inflammatory stimulus.

Native bone marrow contains approximately 1% HSCs and 0.01% BMSCs [16–18] with respect to total nucleated cells, and these percentages are very close to those observed in our study samples (1.2% HSCs and 0.011% BMSCs). This demonstrated the efficacy of Marrow Cellution™ in harvesting pure bone marrow, minimizing blood red collection while reducing donor site morbidity and patient discomfort. The cells obtained using this device presented key features of mesenchymal stem cells after subculturing, such as the

Fig. 5 Trend of the subjective IKDC score (A), Visual Analogues Scale (B) and KOOS subscales (C) at different follow-ups (f.u). * $p < 0.05$, ** $p < 0.01$ vs baseline



ability to proliferate in adherence to plastic and the typical MSCs immunophenotype (CD34⁻, CD45⁻, CD44⁺, CD105⁺, CD90⁺) [20]. MSCs are the key elements supporting the clinical use of BMA and BMAC, and the development of devices able to collect MSCs-enriched products is of crucial importance [21]. It is known that several aspects of the bone marrow collection process may influence MSCs content and, therefore, the quality and efficacy of the final product. In particular, a sub-optimal harvesting site, the collection of large volumes per aspiration (> 2 ml), and the use of large syringes (> 10 ml) may result in the collection of peripheral blood rather than bone marrow, thus reducing the overall MSCs content [13, 14]. The advantage of the present device is that, reducing the amount of peripheral blood collected, it allows to obtain a product rich in MSCs without the need of further processing such as centrifugation steps, thus reducing the time interval between harvesting and injection, making surgery faster and minimizing the risk of contamination. Indeed, other systems used to obtain bone marrow-based products usually require concentration steps to reduce the initial large volume of bone marrow and obtain a final output enriched in BMSC. Although a direct comparison between BMA obtained with this method and other BMC products has not been published yet, the existing

data show that the concentration of MSCs per ml is equal or even higher in Marrow Cellution™ samples [22–24]. This is because this device allows the surgeon to aspirate in a measured manner over a large geography inside the marrow space, albeit using a single access and collecting a smaller volume of bone marrow compared to the traditional trocars, thus reducing the invasiveness of the procedure.

The findings of this study showed that BMA obtained by this device was able to reduce the production of catabolic enzymes by human chondrocytes exposed to an inflammatory environment. The expression of *MMP1* and *MMP3* in chondrocytes was observed to be susceptible to IL-1 β stimulation in vitro [25]. In particular, *MMP1* has been described as a key marker in OA pathogenesis [26], thus the reduction of its expression represents a putative mechanism of action supporting the use of BMA. No effects were observed on the cartilage trophic factor *TGF β* and the metalloproteases inhibitor TIMP1. Nevertheless, the inflammatory stimulus was not able to modify their expression, thus the lack of evidence could be model-specific rather than related to the BMA product action. Other studies showed reduction of catabolic enzymes expression (*MMP1*, *MMP3*, *MMP13*) by chondrocytes exposed to IL-1 β , using either exosomes derived from BMSCs [27] or Platelet Rich Plasma [28, 29].

These observations support the idea that the paracrine action of ortho-biologics is effective in counteracting inflammation through both soluble mediators and extracellular vesicles.

The in vitro model applied in this study is based on a transwell system, thus allowing only a paracrine action by BMA. Interestingly, the direct antagonist of IL-1 β , IL-1Ra [30] was significantly more abundant in the culture medium of samples treated by both inflammatory stimulus and BMA compared to other combinations. This observation suggests that BMA is able to produce immunomodulatory mediators in response to the environment rather than constitutively, thus supporting the idea of an adaptative response to inflammation by the BMSCs contained in the product [25, 31]. The adaptative response is a key element in support of the application of MSCs in regenerative medicine as it underlies the “drugstore” activity of these cells, and highlights the superiority of cell-based products compared to other ortho-biologics containing only platelets and growth factors [32]. Indeed, although the latter products undergo activation in specific contexts [33], they are not capable of an adaptative response to the microenvironment. These observations are confirmed by the increased production of IL-6 in BMA samples alone when stimulated with IL-1 β . Indeed, the direct action of IL-1 β stimulation on IL-6 production is a well-known mechanism [34], and the same was observed when hCH were cultured in the presence of this inflammatory mediator. Despite the IL-6 induction that may be due to the strong inflammatory stimulus chosen for this in vitro model, the prominent role of this mediator in OA progression and initiation [35, 36] would lead to further investigations about the capability of BMA to produce this mediator, in order to understand the possible implications on cartilage catabolism. This effect on IL-6 has been previously observed in other widely-used MSCs-based products [37], and thus it is possible that the clinical relevance of this increment would be negligible.

Looking at the clinical outcomes, significant improvement in pain and functional scores was documented at six month follow-up, lasting up to the final evaluation at two years. These results are in line with those reported concerning the application of BMC in OA [38] and with previous reports on BMA obtained by the same device [19, 39]. Beyond the mere positive clinical outcomes, which should be confirmed in the setting of a randomized controlled trial, the use of the present device could offer some relevant “practical” advantages, i.e., the reduction of surgical time and increased safety due to the fact that the harvested product is ready to be injected and there is no need to process it outside the sterile surgical field, as happens with other kits for BMC production. Furthermore, the possibility of aspirating at various levels within the bone cavity allows to obtain enough BMA with a single puncture, thus avoiding multiple bone penetrations, with an inherent lower morbidity for the patients, quicker times and minimal harvest of peripheral blood.

The present study was mainly designed for the in vitro characterization of BMA obtained by Marrow Cellution™

and we are aware that the clinical observations in such a small cohort of patients bear limitations. Together with the small sample that did not allow for reaching a proper statistical power, the lack of a control group did not allow for correction of the “regression-to-the-mean” effect. Furthermore, another limitation is the lack of objective outcome assessments, such as post-op. MRI imaging to evaluate the effects of BMA on cartilage and bone marrow oedema. Concerning the in vitro functional experiments, the high inter-donor variability limited the possibility to observe a direct, statistically significant difference between the gene expression of inflamed samples in the presence and absence of BMA. Nevertheless, while inflammation resulted in significant enhancement of *MMP1* gene expression compared to unstimulated and untreated samples, the lack of significant difference between untreated controls and inflamed samples treated with BMA is suggestive of the ability of this BMA to counteract the detrimental effects of inflammation. In addition, the concentration of IL-1 β used in this in vitro model may have been significantly higher compared to the physiological microenvironment, thus partially masking the effect of BMA on other markers such as *MMP3*.

Conclusion

The present study demonstrated the effectiveness of Marrow Cellution™ to harvest pure bone marrow with minimal peripheral blood contamination. Its relevant content of BMSCs resulted in the ability to counteract the catabolic cascade by paracrine action, thus providing the rationale for its use to treat OA. The preliminary clinical outcomes were positive, and in line with the results previously observed with other ortho-biological products obtained with more complex and expensive devices and/or techniques.

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Data availability Data are available from the corresponding Author upon reasonable request.

Code availability NA.

Declarations

Ethics approval and consent to participate The pilot clinical study was approved by the Hospital Ethics Committee and Internal Review Board of IRCCS Humanitas Research Center (protocol number: 530/18, approved on 18th September 2018).

Consent for publication NA

Conflict of interest The authors declare no personal conflict of interests. Geistlich Biomaterials Srl provided part of the funding for the present research.

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