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Bone marrow aspirate clot: A feasible orthobiologic

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ABSTRACT

Musculoskeletal disorders are one of the major health burdens and a leading source of disability worldwide, affecting both juvenile and elderly populations either as a consequence of ageing or extrinsic factors such as physical injuries. This condition often involves a group of locomotor structures such as the bones, joints and muscles and may therefore cause significant economic and emotional impact.

Some pharmacological and non-pharmacological treatments have been considered as potential solutions, however, these alternatives have provided quite limited efficacy due to the short-term effect on pain management and inability to restore damaged tissue.

The emergence of novel therapeutic alternatives such as the application of orthobiologics, particularly bone marrow aspirate (BMA) clot, have bestowed medical experts with considerable optimism as evidenced by the significant results found in numerous studies addressed in this manuscript. Although other products have been proposed for the treatment of musculoskeletal injuries, the peculiar interest in BMA, fibrin clot and associated fibrinolytic mechanisms continues to expand.

BMA is a rich source of various cellular and molecular components which have demonstrated positive effects on tissue regeneration in many *in vitro* and *in vivo* models of musculoskeletal injuries. In addition to being able to undergo self-renewal and differentiation, the hematopoietic and mesenchymal stem cells present in this orthobiologic elicit key immunomodulatory and paracrine roles in inflammatory responses in tissue injury and drive the coagulation cascade towards tissue repair via different mechanisms.

Although promising, these complex regenerative mechanisms have not yet been fully elucidated.

1. Introduction

Musculoskeletal disorders are one of the major contributors to disability worldwide. According to the World Health Organization, the global burden of musculoskeletal disease was the highest contributor to global disability in 2017.[1] Most of the musculoskeletal degenerative conditions are not exclusive to elderly people since young individuals can also be affected by sports injuries. Those conditions may affect bones, joints, and muscles and result in a great economic impact, limiting mobility and causing early retirement and reduced ability to participate in social activities.

Nowadays, the therapies available to treat those conditions comprise non-pharmacological treatments (e.g. manual and

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exercise therapy) and pharmacological interventions (oral, topical and injection of analgesics, non-steroidal anti-inflammatory drugs, and corticosteroids) in an attempt to improve the quality of life of patients. However, these treatments usually have a short-term effect on the reduction of pain and none of them can regenerate any damaged tissue.[2] The use of orthobiologics appears to be a good candidate for these conditions.

One of the first studies to demonstrate the benefits of the use of bone marrow aspirate (BMA) in the musculoskeletal field showed statistically significant difference in posterolateral spine fusion rate in a rabbit model with autologous bone marrow augmentation in comparison with controls.[3] Years later, using the same model, authors added BMA to the fusion site and also demonstrated the positive effects of the bone marrow in situ with statistically significant difference,[4] evidencing its osteogenicity and osteoinductive properties.

Orthobiologics are products that can help in the healing process of orthopedic injuries more quickly and are derived from substances naturally found in the body. Amongst the main biologic products are the platelet-rich plasma - PRP (with [L-PRP] or without [P-PRP] leukocytes), cell allografts and, BMA and bone marrow aspirate concentrate (BMAC).[5,6] The whole body of literature in this regard is rapidly growing and although BMA is the most straightforward product, its mechanisms are not fully understood yet.

BMA is commonly harvested from the iliac crest and is a great source of precursor cells (hemopoietic and mesenchymal stem cells), cytokines and growth factors that help and accelerate the healing process of bones and cartilage.[7] Bone marrow aspirate can be applied after puncture, without processing and avoiding the potential risks of contamination and losing cell viability. Alternatively, it may also be concentrated by centrifugation, therefore being described as bone marrow aspirate concentrate (BMAC).[8] Recently, the use of a new variation of bone marrow aspirate named BMA clot has been described.[9] This product consists of a clot naturally formed from the harvested bone marrow (BM), which possesses all the BMA components retained in a matrix molded by the clot and shows similarities to fracture hematoma that is a highly controlled process, resulting in bone remodeling and healing.[9] Despite its beneficial effects, there is a lack of studies evaluating both *in vitro* and clinical applications of BMA clot.

Therefore, this review aims to elucidate the characteristics found in both BMA and hematoma fracture and its application in the clinical scenario.

2. Bone marrow - BM

The bone marrow is an anatomical location within the central cavities of long and axial bones filled with a semi-solid tissue that contains many different cell types.[10] The cell population found in the BM can be classified as non-hematopoietic types (such as osteoblasts, endothelial cells, pericytes, adipocytes and Schwann cells) and hematopoietic types (monocytes, osteoclasts, megakaryocytes, lymphocytes, and neutrophils) with each one having an important role in the regulation of the BM niche.[11] Additionally, two types of adult stem cells are present in the BM: hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). These cell populations have the ability of self-renewal and can give rise to different types of mature cells.[12]

The HSC population encompasses adult progenitor stem cells responsible for the formation of all blood cells, including erythrocytes, leukocytes, and platelets. They are found within the BM in a very small number (approximately 1 HSC to 10.000 BM mononuclear cells).[13] The MSC population is another type of adult stem cells defined by a set of different markers that have high plasticity and can give rise to different mesodermal lineages such as osteoblastic cells, chondrocytes, and adipocytes. They are also found in a small number within the BM and their quantity decreases with aging.[14] It is also interesting to note that there is crosstalk between the two populations, since it has been demonstrated that HSC can guide mesenchymal differentiation towards osteoblastic lineage.[15]

The adult stem cells and other populations contribute to the secretion of a large number of cytokines and growth factors that will assist in the control of the BM microenvironment such as transforming growth factor (TGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stromal cell-derived factor-1 (SDF-1), fibroblastic growth factors (FGF), among others.[16] Collectively, the cells and these soluble mediators cooperate for the success of treatments based on the use of BMA, even though the literature is still quite scarce in regard to the BMA content and its effects.

In a recent prospective study, a characterization of growth factors, cytokines and chemokines showed the difference between BMA, leukocyte-rich PRP (LR-PRP), leukocyte-poor PRP (LP-PRP) and BMAC content.[17] Although BMA carries a lesser amount of growth factors like PDGF and TGF-b in comparison with other biologics, along with BMAC it exhibited a greater concentration of IL-1Ra when compared with LR-PRP and LP-PRP, and lesser concentrations of catabolic proteases, including MMP-1, MMP-2, MMP-3, and MMP-12. These proteases are reported to downregulate collagen types I and III, although they also appear to be involved in the recruitment and mobilization of some progenitor cells.[18]

IL-Ra is a competitive antagonist that binds to IL-1B and IL-1a isoforms of cell surface receptors, thereby inhibiting IL-1 catabolism and its inflammatory effects.[19] IL-1Ra represents a possible target to reduce matrix degradation, since IL-1B reportedly induces MMP-3 and TNF-a gene expression, prostaglandin E2 release, chondrocyte apoptosis and inhibition of collagen deposition.[20]

Over the past decade the emphasis has shifted toward harnessing the BM ability to produce factors and cytokines that stimulate innate tissue repair and modulate inflammation and immune responses rather than just cell replacement where damaged tissue could be readily renewed. In this context, one major rationale for BM use is its paracrine immunomodulatory effect.[21] MSCs contained in BM are able to reduce inflammatory Th1, decreasing inflammatory cytokines produced by T cells such as IFN-gama, and increase Tregs and Th2 responses, by producing IL-10, IL-4 and IL-5. When BM cells interact with dendritic cells (DC), there is a decrease in proinflammatory mature DC type 1, with decrease in TNF-α and IL-12 by these local cells, and increase in DC type 2, with increased expression of the anti-inflammatory interleukin IL-10. When BM cells interact with natural killer cells there is a decrease in the expression of IFN-gama and its subsequent inflammatory cascade by T cells. The same occurs when local macrophages interact with transplanted cells. There is a decrease in the proinflammatory M1 phenotype and an increase in the anti-inflammatory M2 phenotype, with increased PGE2, TSG-6 and IL-1RA (anti-inflamatory cytokines). MSCs of the BM can also reduce the secretion of antibodies from B cells and inhibit bacterial growth by a direct or indirect mechanism.[22] Those are the reasons why BM products are now being studied in the treatment of auto-immune diseases, such as rheumatoid arthritis, and against extracellular pathogens, including Gram-negative bacteria, mycobacteria and fungi.[23]

3. Bone marrow aspirate - BMA

BMA contains all those cell groups and soluble factors previously mentioned that propagate its success in bone, cartilage, and soft-tissue healing.[24] This product has been indicated as an alternative or adjunct treatment for several conditions such as bone

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cysts and/or defects, joint arthritis, ligament tears, osteonecrosis of the femoral head, delayed union/non-union and joint fusion.[25]

The harvest sites of BMA include iliac crest, proximal and distal tibia or calcaneus. However, BMA is commonly harvested from the iliac crest, which allows the collection of a considerable amount of tissue and has a higher number of osteoblastic progenitors compared to tibia and calcaneus.[26] Nonetheless, different harvest techniques result in heterogeneous products concerning the number of progenitor cells[27] and the acquisition of small volumes in different subcortical areas (10 mL) has been demonstrated to be more efficient in the obtention of high numbers of stem cell progenitors.[28] Moreover, harvesting from a single-site with different trajectories show the same cell ratios compared to multiple-site harvest with the advantage of being less painful for the patient.[29]

Bone marrow aspirate has been shown to yield more progenitor cells if harvested from posterior iliac crest.[30] It is up to the physician to use fluoroscope or ultrasound guidance. The authors recommend the use of anatomic landmarks for the average patient and US guidance for larger individuals. For average patients, no more than 90 ml−120ml should be withdrawn. In a large individual, volumes greater than 120−150 ml should be avoided.

Since most MSCs reside in the subcortical areas and pericytes reside around blood vessels, penetrating the bone marrow cavity too deep may not produce highest yields of subcortical and perivascular cells.[30] Some contraindications for BMA harvest should be screened, such as significant anemia, systemic infection, active hematologic neoplasm and patients that are unable to be positioned for the procedure. Some medications should be avoided by at least 4−6 weeks priot to the treatment; in particular, the following examples: prednisone, due to its antianabolic effect; statins, which have a negative impact on cell proliferation; and nonsteroidal antiinflamatory medications because of their interference on platelet aggregation and clot function.[31]

Among the BM-derived therapies for bone nonunion, BMA seems to be very efficient. A meta-analysis performed by Palombella et al. shows that BMA has superior efficacy compared to bone marrow mesenchymal stromal cells cultured for the healing of long bone nonunion.[32] Moreover, this study indicates that the presence of a scaffold positively influences the engraftment of implanted progenitor cells and healing rate. However, none of the studies included in the meta-analysis used a bioscaffold (e.g. fibrin) in association with BMA and despite the clinical use of BMA for orthopedic procedures, very little is known about the use of BMA clot.

4. BMA clot

Due to the presence of megakaryocytes, platelets[33] and coagulation factors, BMA tends to coagulate when collected. To avoid this process, usually, the syringes used in the harvest process are coated with anticoagulant substances such as heparin or acid citrate dextrose solution (ACD).[34] However, it has been discussed that the clot formation could be a favorable outcome in the use of BMA,[9] and from a biological point of view, there is a strong rationale for the use of BMA clot for tissue regeneration strategies. Firstly, the addition of any other products that might interfere in the biological potential is unnecessary and, secondly, in order to obtain clot formation, platelet degranulation is required and that will deliver many osteotropic cytokines and growth factors into the site of injury.

One other important feature of the clot formation is that the fibrinolytic activity that is subsequently created may provide an additional source of angiogenic factors from fibrin splits, and this would be highly beneficial towards the engraftment of the cells.[35]

In a recent laboratory study, in attempts to maximize cell yield, researchers tried to culture both un-clotted BMA and clotted BMA (mechanically cut) and after 15 days of culture, higher growth kinetics of MSCs derived from clotted BMA compared to un-clotted BMA was observed.[36] The potential use of BMA clot in improving chondro and osteogenic DNA content (Sox9 and Runx2 expression) and cell number and ECM accumulation in comparison to other scaffolds has also been demonstrated.[37]

Fibrin is the main component found in the blood clot, bone marrow clot, and fracture hematoma, and it has an important role in hemostasis by serving as a temporary matrix for tissues until their complete remodeling. Also, fibrin interactions with growth factors (e.g. FGF and VEGF) and extracellular matrix proteins creates a scaffold for the cells, stabilizing chondrogenesis by chondrocytes and supporting essential steps of regeneration such as angiogenesis, cell adhesion and bone regeneration, leading to tissue remodeling and healing.[38,39] Moreover, fibrin can act as a delivery agent of bio-active compounds.[40,41]

The use of fibrin clot as a scaffold has demonstrated promising results. *In vitro* experiments showed that MSCs cultured on fibrin gels have a better proliferation potential and are capable of maintaining their osteogenic lineage differential potential compared to MSCs cultured on plastic plates, showing that fibrin matrix could help to maintain stemness.[42] A study by Giannotti et al. reported that the use of constructs built up with fibrin clot and MSC showed successful clinical and functional outcomes in patients with upper limb non-unions, demonstrating effectiveness and safety of fibrin clot as a scaffold for cells on bone healing.[43]

The comparison of fibrin clot from peripheral blood with BM showed that BM fibrin clot has a higher concentration of growth factors (VEGF, SDF-1, and FGF) and a greater potential for osteogenic differentiation and fibroblast proliferation,[44] positioning BM clot as a candidate in regenerative medicine. Supporting this idea, a study by Lim and colleagues in an animal model revealed that the therapeutic potential of autologous bone graft and BMA clot in the repair of ulnar defects are similar between the two treatments, with the advantage of BMA clot being associated with a lower risk of complications.[45] It is worth noting that BMA clot healing process does not involve the resorption of bone graft fragments that may prolong the regenerative cascade as it is the case with bone autograft.

Despite the fact of fibrin clot being a good matrix for bio factors and bone marrow aspirate demonstrating great clinical outcomes, there is a lack of clinical studies evaluating the use of bone marrow clot as a feasible and improved technique in regenerative medicine. The physiological aspects of bone marrow clot seem to be close to fracture hematoma, which has a significant role in bone healing.

5. Fracture hematoma and bone healing

The biology of bone healing is a complex process in which bone heals aiming at the balancing of mechanical loads. This well-orchestrated complex process includes three main steps: hematoma, callus formation, and bone remodeling. The callus formation and bone remodeling are the final steps occurring with the purpose of tissue regeneration and property restoration of the preexisting tissue through the promotion of bone union and healing.[46]

In the fracture hematoma, the first step of bone healing has proven to be of great importance in bone remodeling since its removal results in delayed healing or nonunion fracture.[47] The hematoma formation involves many stages such as coagulation (the immediate extrinsic coagulation pathway mainly via tissue factor TF) and then immunological activities (liberation of IL-6 and TNF-α leading to an inflammatory response) with platelet and leukocyte activation (thus driving the production and liberation of growth factors). Ultimately there is migration and differentiation of MSCs and angiogenesis.[48] All these events will induce a formation

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of a scaffold for the MSCs, promoting their migration, proliferation, and differentiation, as well as supporting other biological cascades, ultimately leading to bone formation and remodeling.[49,50]

The fracture hematoma microenvironment includes low pH and oxygen saturation, high pressure and concentrations of calcium, phosphorous and alkaline phosphatase and an inflammatory profile (with high levels of IL-1β, IL-6, TNF-a IL-8, IL-12, M-CSF, MCP-1) in addition to a high concentration of growth factors (especially bone morphogenic proteins – BMPs, osteoprotegerin – OPG, PDGF, TGF, VEGF) extremely important for regenerative functions.[51] The main concept is that, after fracture, coagulation and inflammation take place to form a matrix architecture, with major inflammatory cytokines (TNF-a, IL-1 and IL-6) driving and enhancing the clotting cascades, particularly the extrinsic pathway.

It has been suggested that hematomas with thicker fibers are in favor of cell migration (e.g. MSCs), ingrowth of tubular structures and better nutrient exchange at injured sites.[52] On the other hand, the higher the thrombin concentration, the more resistant to fibrinolysis the clot is.[53] Recent studies have shown that inflammatory cytokines can upregulate the viscoelastic properties of the fibrin clot, making the fibrinolysis process more difficult.[52,51] Activated protein C (APC) and antithrombin (which neutralizes various coagulation enzymes including thrombin, factors Xa and IXa) are two of the main agents responsible for fibrinolysis. Ideally, a fine tune between coagulation, inflammation and fibrinolysis should exist in order to keep an ongoing healing process.[54]

In the context of clots, the inflammatory response and coagulation cascades are quite intertwined in a bidirectional relationship. For instance, IL-6 and IL-1 act as important mediators in the initiation of blood clotting, promoting new platelet production, strengthening thrombogenicity and increasing TF expression in local monocytes and endothelial cells. IL-10, in turn, diminishes coagulation by directly inhibiting the generation of TF on the surface of monocytes *in vitro*, and downregulates fibrinogen mRNA expression.[54] On the other hand, the coagulation factors can induce the release of IL-1, IL-6, CXCL8 and TNF-a by mononuclear and endothelial cells. Fibrinogen itself acts a proinflammatory molecule facilitating IL-1 production and activating NF-kb signal (hallmark of inflammation).[55] An example of this coordination is the change from the macrophages with a pro-inflammatory phenotype (M1 macrophages) that are present in the initial fracture hematoma towards macrophages with the M2 phenotype during the first 3 days of healing.[56]

A recent report by Pountos et al.[57] revealed molecular differences in fracture hematoma and peripheral blood. They found that the concentrations of 33 cytokines were higher in fracture hematoma compared to peripheral blood, where IL-8, IL-11, MMP-1, MMP-2, MMP-3 and IL-10 yielded the highest amounts, demonstrating the inflammatory, and consequently, immunomodulatory properties of fracture hematoma. Additionally, MSCs cultured with fracture hematoma supplemented medium displayed upregulation of osteogenic and angiogenic genes such as EGF, FGF2, and VEGF, contributing to the induction of the osteogenic phenotype.[57] In a recent review of 89 manuscripts, the same authors observed a significant increase in bone morphogenetic proteins and growth factors, mainly BMP-2, PDGF and TGF in fracture hematomas.[51]

All these molecular mediators contribute to the attraction of BM cells to the fracture hematoma. In animal models, there is some evidence indicating that cells from BM migrate to the fracture site and help in the healing process, differentiating into osteoblasts to make up the early calluses.[58] In humans, cells extracted from fracture hematoma exhibit characteristics of multilineage mesenchymal progenitor cells, the population which is derived from BM and holds osteogenic, chondrogenic and adipogenic differentiation potential.[59] These results may be considered as a clear proof of the interconnection between the early hematoma and bone marrow cells in the healing process.

In this scenario, the use of BMA clot as an early hematoma might not only replace local tissue with new cells but also trigger an immunomodulatory cascade that will ultimately attract peripheral mesenchymal stem cells to the injury site (endothelial progenitors, muscle-derived cells, circulating progenitors).

6. The role of fibrinolysis on regenerative process

The subsequent events after the clotting and inflammatory response are mainly dictated by fibrinolysis. The fibrinolytic activity of human MSCs is known to be critically involved in the invasion into fibrin clot and in the extracellular matrix remodeling.[60] In other words, degradation of extracellular matrix is required for cells to colonize the collagenous layers of the damaged tissue. Supporting this theory, a laboratory study described for the first time the expression of key components of the fibrinolytic cascade by human MSCs.[60] uPA/tPA (urokinase plasminogen activator and tissue plasminogen activator – major drivers of fibrinolysis) dependent plasminogen activation expressed by human mesenchymal stem cells strongly evidenced the critical participation of MSCs in tissue regeneration, in an autocrine and paracrine fashion.

It has also been demonstrated that human monocytes/macrophages produce tPA and uPA.[61] Growth factors and cytokines regulate their expression on macrophage surfaces. Fibrin degradation facilitates MSCs and macrophages invasion and subsequent proliferation and differentiation within the injured area. The tPA secretion is enhanced by tissue bFGF and PDGF during the proliferative phase and uPA secretion by TGF-b during the regenerative phase of the healing cascade, respectively.[62]

7. Enhancement of the BMA clot

In vitro preparation of MSCs with photobiomodulation (PBMT) prior to cell transplantation has been suggested as a preliminary stage to enhance regenerative potential. Khandra et al. showed that there was increased cellular attachment, proliferation, differentiation, osteocalcin synthesis, and transforming growth factor β1 production in human osteoblast-like cells after low level laser irradiation (photobiomodulation).[63] Nurkovic et al. demonstrated that PBMT accelerates MSC proliferation.[64] The low intensity laser irradiation varies around the red and infrared levels (660 nm−1100 nm) and an energy density of 0.5−5J/cm^3 appears to be effective in enhancing tissue regeneration.[65] A recent study demonstrated that PBMT can act on migration, differentiation and production of extracellular matrix molecules by progenitor cells.[66]

Based on the results of *in vitro* studies, significant evidence of an upregulation of the regenerative capacity of MSCs has been accumulated. The combined use of BMA plus PBMT seems to be a good strategy in the clinical scenario. An *in vivo* study recently demonstrated that this combined therapy significantly yielded greater bone formation than controls or either treatment alone in murine bone defects.[65] Bone formation markers such as osteocalcin-positive cells, proliferating cell nuclear antigen and newly formed bone area were significantly more evident in the group treated with BMA plus PBMT. From the orthobiologic point of view and the authors' anecdotal experience, photobiomodulation seems to be an enhancer for the BMA clot in clinical application.

8. Author's note

It is the author's preference the use of hyaluronic acid (HA) in the making of the "BMA clot matrix" in order to obtain a more fluid product to inject. The mixing of the bone marrow aspirate with HA

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is achieved with the use of a three way stop cock to connect syringes. It is advantageous to break down some clots as anticoagulants are not used in this product. Also the formation of this matrix has a potential enhancement in its biological effects. HA has been shown to be an anti-inflammatory molecule able to increase the viscosity of synovial fluid and promote endogenous production of HA.[67]

In this sense, the development of a new mixed product may be valuable due to their dissimilar biological mechanisms, combining the regenerative potential of the BMA with the rheological properties of HA. It has been shown that the proliferation rate is higher in chondrocytes cultured in the media containing PRP + HA compared to the cultures with PRP or HA alone. Glycosaminoglycan content is significantly higher in chondrocytes cultured in PRP and HA blend[68] and extracellular matrix production is higher with the use of high AH concentration. Additionally, hyaluronic acid can also increase the release of growth factors from PRP, which may promote an enhanced healing effect on certain tissues.[69]

9. Conclusion

The rising importance of osteoimmunological aspects in bone healing supports the essential role of the initial hematoma as a source of inflammatory cells. These cells release the cytokine pattern that directs cell recruitment towards the injured tissue. The application of BMA clot as a source of early hematoma to ameliorate damaged tissues seems to be quite logical from an orthopedic standpoint. BMA clot carries progenitors, plenty of cytokines and a natural scaffold that can replenish damaged areas. Mostly, the paracrine and immunomodulatory effects provided by the BMA matrix attract new cells, encouraging the cross-talk between coagulation and the inflammatory response. This event renders the healing process more reliable. Although the mechanism by which BMA clot acts has not yet been fully elucidated, clinical application seems to be safe but further studies are still desirable.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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