Bone fracture healing: Cell therapy in delayed unions and nonunions

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ABSTRACT

Bone fracture healing impairment related to mechanical problems has been largely corrected by advances in fracture management. Better protocols, more strict controls of time and function, and hardware and surgical technique evolution have contributed to better prognosis, even in complex fractures. However, atrophic nonunion persists in clinical cases where, for different reasons, the osteogenic capability is impaired. When this is the case, a better understanding of the basic mechanisms under bone repair and augmentation techniques may put in perspective the current possibilities and future opportunities. Among those, cell therapy particularly aims to correct this insufficient osteogenesis. However, the launching of safe and efficacious cell therapies still requires substantial amount of research, especially clinical trials. This review will envisage the current clinical trials on bone healing augmentation based on cell therapy, with the experience provided by the REBORNE Project, and the insight from investigator-driven clinical trials on advanced therapies towards the future.

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Introduction

Bone fracture clinical management is oriented to obtain bone healing in the shortest time frame, with the best possible functional recovery, and with less complications. However, an overall rate of 5 to 10% delayed union or nonunion is widely accepted as a perceived proportion...
for bone healing problems, although this figure is not homogenous. Rather, different nonunion rates are found in different types of fracture, somewhat ranging from up to 18.5% in the tibia diaphysis [1] to 1.7% in the femoral shaft after reamed nailing [2].

The definition of delayed union and nonunion or pseudarthrosis certainly deserves more discussion. Those cases that correspond to a different healing rate than expected (slow healing rate) should be clearly separated from those in which the bone healing is no longer expected without treatment. A better understanding of fracture healing biology would help in fostering preclinical studies and clinical proposals in both of these directions: accelerating bone fracture healing in case of slow healing rate, based on biological stimulation, and promoting bone fracture healing in case of no healing expectations, based on redeveloping the bone regeneration capability, whether fully lost or at least under the required threshold to healing.

Major limb injuries related to traffic accidents and multiple trauma are a major health issue in developed countries, resulting in long treatments with substantial socioeconomic effects. But these injuries are also severely impacting less developed countries, where secondary complications frequently generate major disabilities [3]. Long bone fractures are difficult and slow to heal and may require months until consolidation is completed. Long treatments not only associate significant loss of working days with economic effects on the patient and the society, but also carry the risk of nonunion and permanent disabilities related to malunion, joint stiffness, muscular atrophy, or reflex sympathetic dystrophy.

The ability of fractured bone to regenerate and undergo repair may be compromised when insufficient osteogenic reaction is observed in the fracture callus, up to developing an atrophic nonunion. Those cases cannot be solved through a mechanical approach, as occurs with hypertrophic nonunions. Treatment of these atrophic nonunions requires some form of bone healing augmentation, providing that vascularization is sufficient and confirming that infection is absent.

Conventional, standard treatment to augment bone healing is based on bone autograft, today’s most accepted gold standard. The application of autologous cancellous and cortico cancellous grafts, or larger, even vascularized, segmental bone grafts (frequently constructed out of the fibula) when the defect exceeds some centimeters, may permit the most appraised personalized management to this problem. Yet this classical orthopedic approach may be not appropriate. And this happens when the autograft strategy has already failed, when the osteogenic potential of the available donor site is altered (due to cell scarcity, fibrous tissue abundance due to previous harvesting, or other impairments), or when the risk/benefit evaluation of the autologous bone graft obtention is unbalanced or refused by the patient.

Alternatively proposed strategies include those relying on the osteoconductive or osteoinductive capabilities of implanted tissue (such as allograft or demineralized bone matrix) or a synthetic material (such as bio ceramics in different forms and compositions). Also, different strategies have been defined to supplement potential molecular deficiency in the stimulation of local cell differentiation in the osteoprogenitor line (such as BMP or other growth factor local deliveries). These strategies rely on the surrounding or available cells that might eventually produce the required local bone regeneration. The expected fracture healing is seriously constrained in cases where previous efforts to heal the fracture have failed. Particularly in those cases with a supposed cell insufficiency, cell-based alternatives developed over mesenchymal stem cells (MSCs) [4] have been proposed, and are currently under investigation and evaluation.

In this context, this review progresses from clinical concepts of bone healing impairment to advanced therapies under trial [5]. In this journey, cellular and molecular bases of bone regeneration in fracture healing will be considered as the foundations of so-called therapy platforms [6], state of the art and recent contributions to bone induction and augmentation will be appraised, and particular emphasis will be placed on cell therapy proposals and current cell therapy based orthopedic clinical trials.

**Clinical bone healing impairment: from hypertrophic to atrophic nonunions**

In a normal biological environment, many skeletal fractures heal uneventfully in the first 6 to 8 weeks. In case of an impaired bone healing process due to a disturbed biological or mechanical environment, or in cases where thick cortices are involved such as in femoral and tibial diaphysis, fractures may take a longer time to heal [7]. Per conventional definition, if a fracture is not healed after 4 months, it can be considered a delayed union. If no bony healing is obtained in 6 months after the fracture, it can be clinically considered as nonunion, although the diagnosis requires specific radiological features showing bone ending changes.

There are two distinct variants of nonunions with opposed underlying pathomechanisms, namely hypertrophic and atrophic nonunions. A hypertrophic nonunion presents with a large, vital callus, although inefficient to regenerate bony union. On conventional radiographs, the hypertrophic nonunion displays a large, broadened callus towards the fracture gap, with a radiolucent area instead of bone bridging. Due to its radiological features (Fig. 1), the hypertrophic nonunion is also called elephant foot nonunion [8]. Its basic problem is the mechanical disturbance of the chosen fixation technique. The most recognized

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**Fig. 1.** Radiological AP and lateral views of a tibial midshaft hypertrophic nonunion.
etiology underlying hypertrophic nonunions is the inefficient and unstable fixation of the fracture allowing for multidirectional motion of fracture fragments.

Whereas limited axial compressive movements can increase callus formation and accelerate fracture healing [9], shear displacement has demonstrated to hinder callus formation [10]. To a critical value, an increasing interfragmentary motion leads to an increase in callus formation. Above a critical threshold, especially in combination with larger gap sizes, interfragmentary motion leads to hypertrophic nonunions [9,11,12]. Most frequently, the treatment of hypertrophic nonunions is surgically oriented. Exchange of the limited amount of compressive forces [13,14]. Secondarily, additional treatment by ultrasound or external shock wave therapy has also been proposed, although definite evidence is still lacking and significant controversy remains about this issue [15,16].

The pathomechanisms leading to atrophic bone nonunions are completely different. Claimed underlying causes usually incorporate biological impairment, sometimes in combination with mechanical factors. In most cases, atrophic nonunions are the expression of impaired biological support for bone healing, as for damaged vascular supply, and destruction of the periosteum and endosteum. This impairment is frequently associated to cofactors such as polytrauma or soft tissue damage, with detraction of surrounding tissues [17]. Consecutively, fracture healing is impaired because of the deficiency of important mediators, blood supply or other indispensable biological parameters.

Mechanical reasons can also be involved in the development of atrophic nonunions. Excessively rigid fixation, insufficient compressive forces, and a fracture gap too wide to allow bony bridging of the fragments can also contribute. In radiological images, the atrophic nonunion demonstrates the absence of callus tissue, the narrowing of bone ends, and a large radiolucent zone in the fracture gap (Figs. 2 and 3). The treatment of atrophic bone nonunion requires a surgical intervention. The aims of this intervention are the elimination of disturbing mechanical factors through modification of osteosynthesis and reduction of the fracture gap, the increase of compressive motion between fracture fragments, and the biological improvement by introduction of new cells, vascular supply and healthy bone tissue into the fracture gap [18].

Clinical and imaging diagnoses

In the regular follow-up of patients after bone fracture, the course of fracture consolidation is reviewed by conventional, two orthogonal projection radiographs. Therefore, the development of a nonunion can be monitored clinically and through imaging. In case of insufficient fracture healing, early modification of osteosynthesis like dynamization of an intramedullary nail can influence the further course of healing and re-orient a delayed union or even some nonunions towards adequate bone consolidation. Patients with a manifest nonunion usually complain about pain in the fracture area with, and sometimes even without, weight bearing. The affected bone is usually sensitive upon pressure and patients are not able to bear full weight [17].

In cases of suspected infectious genesis of nonunion with possible additional symptoms like reddening, hyperthermia and elevated body temperature, laboratory analysis should be obtained for infectious parameters such as white blood cell count and inflammation parameters [19].

After clinical and laboratory evaluation, conventional radiographs in two orthogonal planes represent the basic diagnostic imaging tool, where the radiolucent gap between bone endings is associated to closure of intramedullary canals of diaphyseal bone endings. Besides, the basic characteristics of the nonunion (status of consolidation, hypertrophic/atrophic nonunion, segmental bone defects) can be evaluated for a more precise diagnosis.

If the amount of consolidation or the radiological signs of nonunion do not become obvious in conventional radiographic evaluation, a computer tomography (CT) of the affected region is mandatory. Three-dimensional reconstructions and exact illustration of the fractured region, with the amount and location of possible callus bridges, can be evaluated through CT imaging (Fig. 3).

In some cases, especially with doubtful aseptic pathogenesis of the non-union, additional diagnostic evaluation should be performed. While bone scintigraphy no longer represents the state of the art diagnostic imaging tool, fluorodeoxyglucose positron emission computer tomography (FDG-PET-CT) has become more and more relevant in clinical daily routine. This imaging tool combines the exact imaging from the CT with additional information about the metabolism of the examined area with a high diagnostic sensitivity for a chronic osteitis. FDG-PET-CT has been shown to be of good diagnostic accuracy in bone pathology discrimination [20] and chronic osteomyelitis [21].

The combination of clinical examination, laboratory analysis and radiological imaging by conventional radiographs, CT and possibly PET-CT should be sufficient for a clear diagnosis. Underlying cellular and molecular mechanisms are key to develop specific treatments and its review is mandatory to further progress in clinical research strategies.

Cells and molecules in bone healing after fractures and pharmacological new therapies

Bone healing of fractures and small bone defects is a unique and very effective process involving complex and well-orchestrated interactions between cells, cytokines, osteo-conductive matrix and a mechanically
motactic activity towards endothelial cells, bone remodeling.

sis and chondrogenesis, to interleukin-1 (IL-1), IL-6 or tumor necrosis factor-

stable environment with a good blood supply, according to the "diamond concept" [22] to generate new bone instead of a fibrous scar, as occurs in other connective tissues.

This complex dynamic process requires the precise orchestration of various events during overlapping stages [23] with distinctive histological characteristics, from the initial inflammatory response, the formation of a cartilaginous soft callus, the formation of a bone hard callus, and finally the bone union followed by remodeling. As is widely accepted, this bone repair in adults recapitulates the normal development of the skeleton during embryogenesis [24]. Moreover, the current paradigm of bone tissue engineering also relies on biomimetics to reproduce bone formation from development biology [25,26]. Prenatal bone formation starts with mesenchymal cell condensation and subsequent differentiation to chondrocytes (through endochondral ossification) or, in precise cases, straight forward to osteoblasts (through intramembranous ossification) [27]. Both processes are implicated in the callus formation after fracture [24]. However, callus formation in adult bone is highly influenced by factors such as inflammation, presence of pluripotent and osteoprogenitor cells, gap distance between bone fracture endings, and mechanical stabilization and loading. The endochondral ossification mechanism predominates in the majority of fracture healing cases, advancing through several phases that involve multiple cellular and molecular events [28] in the so-called “bone healing cascade” [29] from hematoma and inflammation to angiogenesis and chondrogenesis, to finally complete osteogenesis followed by bone remodeling.

The interruption of vascular endothelium integrity is the first step following trauma, accompanied by a disruption of the blood supply and hematoma formation, associating the presence of necrotic material. This facilitates a potent inflammatory response related to the production of pro-inflammatory cytokines from aggregated platelets, as interleukin-1 (IL-1), IL-6 or tumor necrosis factor-α, which have chemotactic activity towards endothelial cells, fibroblasts, lymphocytes and monocytes–macrophages [30]. Specifically, transforming growth factor b1 (TGFb1) is a potent chemotactic stimulator of mesenchymal stem cells that enhances osteoblast precursors and chondrocyte proliferation, and may participate in recruitment of bone cells in the trauma area [31]. In addition, TGFb1 induces the production of extracellular bone matrix proteins such as collagen, osteopontin, and alkaline phosphatase [7] and regulates different cell types implicated in bone turnover and fracture healing [31]. Sox9, Runx2, and Osterix are three essential transcription factors that have important roles in the cell-fate decision process by which mesenchymal cells become chondrocytes and osteoblasts, through activation of cell type-specific genes [32]. Macrophages can also produce and secrete these factors including bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF), which induce angiogenesis and bone formation [33,34]. Neovascularization of damaged tissue is crucial to successful bone healing, providing oxygen and delivering progenitor cells [35]. The vascular endothelium lost integrity produces hypoxic conditions that induce chondrogenesis, as occurs in the central avascular area of the callus [36]. In this regard, VEGF is a key osteogenic and angiogenic factor that is expressed under the control of hypoxia-inducible factor (HIF)-1α in low oxygen tension [35]. Overexpression of HIF1α in mature osteoblasts, in mice with distraction ostegenesis, stimulates bone regeneration indicating an angiogenic response related to new bone formation. BMPs, parathyroid hormone (PTH)-related protein (PTHrP) and other osteogenic factors stimulate the expression of VEGF in osteoblastic cells [37,38]. In this reparative phase, neoangiogenesis and chondrogenesis predominate to bridge the gap in the fracture and complete bone healing, but this soft callus is then replaced with a hard callus connecting bone fragments with new bone. Osteoblasts can form woven bone rapidly, but it is randomly arranged and mechanically weak [28], requiring bone remodeling by which newly formed woven bone is replaced by lamellae through the activity of osteoclasts and osteoblasts [39].

This cellular and molecular background justifies different strategies to promote bone regeneration based on molecular osteoinduction. The use of agents that increase vascularization and osteoblastic maturation could contribute to early callus formation. In this context, PTH exerts anabolic actions throughout cAMP–PKA pathway activation, implicating on bone formation in vitro and in vivo [40] and interacting with important bone local factors such as PTHrP, BMPs, Wnt-β-catenin, EGF, and FGF [40]. The possibility of using Wnt pathway molecules as anabolic agents in bone repair is complex because their effects depend on the cell differentiation state [41]. In addition, this pathway is implicated in tumoral processes. Studies with Wnt pathway antagonists such as DKK-1, SFRP and sclerostin are in progress. Several studies demonstrated that the use of these factors can promote bone formation in rodent models associated with a decreased BMD and higher bone turnover [42,43]. Sost (sclerostin-encoding gene) is a key modulator of bone remodeling and its expression was rapidly reduced in the callus, indicating that this would allow osteoblasts to escape from its inhibitory effect to promote bone repair [44].

However, translation into clinical trials is limited at this point. PTH trials in fractures [45,46] aimed at accelerating fracture repair, particularly in fractures that are seldom prone to nonunion. BMPs have been frequently used in clinical trials with significant heterogeneity. A systematic review and metaanalysis on 11 trials observed comparable times to fracture healing between BMP and controls and confirmed some evidence of increased healing rates with BMP without a secondary procedure compared with usual care control in acute tibial fractures [47], but observed that achieving union for nonunited fractures was similar to bone graft substitutes. Other strategies such as local application of FGF2 were found to accelerate tibial shaft fractures [48], although no data are available in nonunions.

Bone healing augmentation: grafts and biomaterials

Bone grafting is widely used in hospitals to repair injured, aged or diseased skeletal tissue. In Europe, about one million patients encounter
surgical bone reconstruction annually and the numbers are increasing due to our aging population. Bone grafting intends to facilitate bone healing through osteogenesis (i.e. bone generation) at the site of damage, but this is only attained when augmentation includes cells capable of forming bone. Other options to augment this bone repair include osteoinductive (i.e. bone inducers) and osteoconductive (i.e. bone guides) capabilities of the supplied coadjuvants to the surgical treatment.

Bone autograft is the safest and most effective grafting procedure, since it contains a patient’s own bone growing cells (to enhance osteogenesis) and proteins (to enhance osteoinduction), while providing a framework for the new bone to grow into (osteoaduction). However, bone autograft is limited in quantity (about 20 cm³) and its harvesting (e.g. from the iliac crest) represents an additional surgical intervention, with frequent consequences of pain and complications [49].

The next solution is allograft bone directly coming from tissue banks (fresh-frozen) or prepared to be conserved (dried or lyophilized). This solution does not contain living cells and some matrix proteins are destroyed by virus-inactivation treatments and the freezing process, thus it only guarantees osteoconductive properties. Moreover, allograft bone may transfer disease or lead to immunological reactions [50]. An interesting alternative is to combine allograft with MSCs from concentrated bone marrow, as has been proposed in bone defects after revision hip surgery [51] but also preliminarily explored in long bone pseudarthrosis [52]. Yet the number of cells may be an issue, as the available evidence in preclinical models recommends a high number of cells [53] and many ongoing clinical trials are thus based on high number of MSCs that require cell expansion, as will be discussed later.

Since both autograft and allograft have drawbacks, scientists have long searched for biocompatible materials that could be used in place of the transplanted bone [50,54]. Several biomaterials can be chosen depending on the goal (mechanical strength or filling) and approach (percutaneous or surgical). The most widely used biomaterials are calcium-phosphate ceramics, which usually combine hydroxyapatite and tricalcium phosphate as granules or, more rarely, sticks, and exhibit interconnected pores each measuring 100–400 μm. These biomaterials promote the adhesion, proliferation, and osteoblastic differentiation of MSCs, as well as the production of the collagen matrix that subsequently undergoes mineralization. Collagen sponges and biodegradable polymers can also be used. The biomaterials must be absorbable, at a variable rate depending on their anticipated biomechanical role, and must allow the ingrowth of newly formed blood vessels from the neighboring tissues. Good quality vascularization of the tissue in contact with the implant is crucial.

Although most of the available synthetic bone substitutes possess some of the positive properties of autograft (particularly, osteoconductive capabilities and occasionally, osteoinductive properties), none has all the benefits of one’s own bone yet (osteogenic properties). Basically and besides bone autografting, which is the only truly osteogenic material, orthobiological solutions today available to surgeons include osteoconductive and osteoinductive products, such as different preparations of bone allograft (fresh-frozen or dried by lyophilization, warranting osteoaduction), different synthetic substitutes (with variable properties but particularly osteoconductive), and synthetic pharmaceuticals with osteoinductive properties (such as bone morphogenetic proteins, BMPs). Available evidence confirms the outcome of fractures and non-unions treated by surgical techniques augmented by autograft [55] and by BMPs [47]; thus this information may be compared to efficacy studies about other solutions.

An alternative strategy to accelerate bone healing includes the use of degradable biomaterials in combination with osteogenic factors. Besides the already mentioned growth factors, emerging anabolic osteogenic factors are under scrutiny. This applies not only to PTH but also to PTHrP whose C-terminal 107–111 domain (also known as osteostatin) exhibits osteogenic features in vitro, and stimulates bone formation in vivo [56–60]. PTHrP also conferred both osteogenic and angiogenic preclinical features when coating Si-based ceramics both in vitro and in vivo [61,62].

But besides bone grafts, substitutes and their augmentation with growth factors and anabolic strategies, cell therapies have been proposed to evolve towards new osteoinductive and osteogenic solutions that could safely and efficaciously compete with currently available standards.

**Advanced therapy (AMTP) proposed solutions**

In view of these limitations and the increasing number of bone grafting procedures, surgeons are looking for alternatives with added value compared to osteoconductive substitutes, such as cell therapy and tissue engineering [63].

According to the above mentioned diamond concept [22], MSCs play a crucial role in bone repair, and thus cell therapy can serve as an alternative to autologous bone grafting. A large number of osteoprogenitor cells may be implanted at the injury site, either alone or combined with a matrix. Autologous bone marrow (BM) is rich in growth factors and osteoprogenitors as MSCs are present in the mononuclear cellular fraction of the bone marrow. Bone marrow MSCs are currently the most appropriate cells for inducing bone repair, as they have a strong osteogenic potential and are easily obtained by culturing iliac crest aspirates. Several MSC-based cell therapy modalities have been developed, i.e., with and without cell culturing, and with or without a matrix. The mononuclear cell fraction of the bone marrow, which contains the MSCs, can be used directly by percutaneous injection of aspirated BM into the injury site. To increase the number of injected mononuclear cells and consequently of MSCs, it is possible to separate the mononuclear cells by centrifugation and concentrate them 3-fold to 6-fold [64] with good results in pseudarthrosis [65]. The healing rate increased in proportion to the injected MSC concentration. Patients whose fractures did not heal received fewer than 1000 MSCs per mL and fewer than 30,000 MSCs in total, whereas those whose fractures healed received significantly higher MSC concentrations and counts, with a mean of 1500 MSCs per mL and 54,000 MSCs in total, in a volume of 20 mL.

Concentrated or unconcentrated mononuclear cells can be mixed in the operating room with a synthetic or natural osteo-conducting matrix (e.g., allogeneic bone graft or coral) before implantation. Few published studies assessed the combined use of concentrated or unconcentrated BM with a biomaterial [66–68]. This method is a valid option for everyday practice, provided that CE-marked (that is, approved for clinical use in Europe) biomaterials are used and concentration (if used) is achieved via an approved procedure.

Mononuclear cells may also be cultured in vitro to allow selection and expansion of an adherent fraction corresponding to MSC. This increases the number of MSC to millions of cells. Expanded MSCs can be extemporaneously mixed with scaffolds during surgery (Fig. 4) so that this composite material is used in the same way as bone grafts. Quarto et al. [69] first reported the use of cultured BM MSCs combined intra-operatively with hydroxyapatite blocks to fill large bone defects (4–7 cm). They successfully treated 3 patients, with defects in the tibia, humerus, and ulna, respectively. A subsequent study confirmed healing of the defects after 6–7 years [70]. The expanded MSCs may also be injected alone percutaneously in the site of fracture or osteotomy with interesting results in two studies [71,72].

Tissue engineering combines bone marrow cells or mesenchymal stem cells, synthetic scaffolds and molecular signals (growth or differentiating factors) in order to form hybrid constructs. In a classical approach, bone tissue engineering consists of harvesting bone marrow from a patient, isolating MSCs by their adherence to tissue culture plastic, expanding and differentiating those cells in culture to a sufficient number, and then seeding them onto a suitable synthetic scaffold to be expanded in vitro on this scaffold during several days/weeks. This allows for scaffold colonization and for cell differentiation, before grafting
of this processed composite material at the affected site, prior to implantation into the same patient [73].

For bone reconstruction purposes, human MSCs have been seeded and cultured on porous calcium phosphate ceramics in osteogenic media (dexamethasone, ascorbic acid, l-glycerophosphate). Early proposals lead to clinical studies with low numbers of patients using this approach, but the outcomes were inconsistent showing low efficacy in bone regeneration. From these, it is clear that the strategy requires significant tuning [74,75].

The reasons of the limited clinical success may be due to several bottlenecks in the multidisciplinary field of bone tissue engineering, particularly about biomaterials and cell limitations. Biomaterials used as bone void fillers are inspired by the bone extracellular matrix (hydroxyapatite, collagen I) but need to be colonized by cells and vascularized in order to promote bone tissue formation and healing. The regenerative capabilities of current biomaterials are still limited to small bone defects. Regarding cell limitations, barriers are found in the autologous approach, the cell selection, the association of cells and materials, and the osteogenic differentiation of implanted cells. The autologous approach for isolation and osteogenic differentiation of MSCs is highly demanding in terms of logistics, production and safety of culture conditions leading to a costly therapeutic procedure. The selection of a restricted population of cells from different donors with age and genetic diversities remains a challenge for regenerative medicine at this early stage of research due to patient variability. The association of biomaterials and osteogeniprotot cells raises technical challenges (i.e. cell sources, types, doses, timing) and regulatory issues (devices with medicinal drugs) to implement clinical trials. Moreover, bone formation requires different cell populations that cooperate to set up complex 3D tissue under the guidance of biomechanical cues while vascularization plays a major role in tissue healing. Finally, osteogenic differentiation induced in vitro is not fully supported by the in vivo release of osteogenic factors from the graft itself.

An alternative to the previous strategies is to implant the composite material (cell + scaffold) into a heterotopic site, e.g. in a richly vascularized muscle, to promote angiogenesis and blood vessel growth into the construct for some weeks and then to transfer it to the affected site with vascular anastomoses for the transferred muscle flap containing the implant [76], but only anecdotal experience is available.

Most crucial in the lack of evidence on satisfactory AMTF solutions that could be acceptable in current clinical practice is the insufficient number of clinical trials with reasonable, standardized, and preclinically well-supported cell products. Scientific preclinical proofs of efficacy are frequently weak, and the proposed cell products are also difficult to reproduce in a standardized manner, based on the provided information in many publications, which compounds the difficulties to confirm these products in well-designed clinical trials.

Safety and preclinical rationale to launch clinical trials on cell therapy

Not only are complex design and management of clinical trial regulation and subsequent approval applicable to cell therapy, but specific ethical and regulatory issues are also present. Therefore, a substantial amount of efforts are required to support clinical trial proposals on preclinical strong arguments and data. In this context, cell therapy is considered an advanced therapy (AT) by the European legislation [77], where cells or tissues are considered "engineered" if they have been subjected to substantial manipulation and are not intended to be used for the same essential function or functions in the recipient as in the donor. Principles applying to advanced therapies include marketing authorization (pre-market approval), demonstration of quality, safety and efficacy, and post-authorization vigilance. Manufacturing of these products requires authorization by the competent authority of the member state ensuring national traceability and pharmacovigilance requirements as well as specific quality standards. The regulatory requirements, currently derived from the field of pharmaceutical medications, will have to evolve in accordance with the specific characteristics of cell therapy trials in surgery.

At present, only autologous MSCs are used for bone repair cell therapy. Intra-operative BM concentration in the operating room using small centrifuges and CE-marked kits does not require authorization and is performed under the responsibility of the surgeon. Safety of this procedure has been confirmed by Hernigou on 1873 patients [78]. For the cultured MSCs, Tarte et al. [79] found no evidence of deleterious changes or malignant transformation of cultured MSCs used in two national multicentric immune-hematology trials. However, the immunomodulating effects of MSCs and their stromal properties (ability to maintain the survival and growth of associated cells) warrant caution in patients treated for neoplastic diseases, most notably bone malignancies.

Preclinical rationale requires solid indices of feasibility and efficacy. In this field, preclinical studies only orient towards the real feasibility and efficacy, whose definite proof requires clinical trials. Bone research scientists scaling their hypothesis to clinical trials on bone cell therapy should be aware of the underlying strategy that should orient their preclinical research to maximize the probabilities of satisfying regulators and obtaining the required approvals to launch trials. Understandably, this strategy will be modified as upcoming evidence may make some requirements unnecessary, while other new data may recommend different preclinical approaches prior to clinical trials.

In this context, the REBORNE European Union FP7th large integrating project (www.reborne.org) has fostered our consortium to organize the current preclinical requirements to request approval from multinational European competent authorities. Both in vitro and animal studies have been launched to preclinically support the derived clinical trials. Particularly, a clinical multicentric phase I/IIa trial (EudraCT 2011-005441-13, NCT01842477) aiming at safety and efficacy of cellular therapy was started in May 2013 to assess the use of cultured, expanded autologous BM cells intra-operatively loaded onto biphasic calcium-phosphate granules as an alternative to autologous cancellous bone grafting in patients with long bone nonunion or delayed union.

On-going cell therapy clinical trials to treat long bone non unions

The review of international clinical trial databases is the only updated source of on-going clinical trials. Search can be performed initially through the WHO International Clinical Trials Registry Platform — ICTRP [80]. This platform incorporates weekly updates of the European Clinical Trials Database — EudraCT [81], the ClinicalTrials.gov database [82], the International Standard Randomised Controlled Trial Number
### Table 1
Clinical trials related with long bone fracture and mesenchymal cell therapy, cited in trial registries and completed or recruiting patients, with sufficient information about intervention.

<table>
<thead>
<tr>
<th>Title</th>
<th>Identifier</th>
<th>Sponsor</th>
<th>Principal investigator</th>
<th>Study phase</th>
<th>Indication</th>
<th>Intervention</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of non-union of long bone fractures by autologous mesenchymal stem cell</td>
<td>NCT01206179</td>
<td>Royan Institute, Tehran, Iran</td>
<td>Mohssen Emadedin</td>
<td>1</td>
<td>Long bone nonunion</td>
<td>BMAC injected percutaneously</td>
<td>Completed 2011</td>
</tr>
<tr>
<td>Percutaneous autologous bone-marrow grafting for open tibial shaft fracture (IMOA)</td>
<td>NCT00512434</td>
<td>University Hospital, Tours, France</td>
<td>Philippe Rosset</td>
<td>1</td>
<td>Open tibial fractures</td>
<td>BMAC injected percutaneously</td>
<td>Completed 2013</td>
</tr>
<tr>
<td>The efficacy of mesenchymal stem cells for stimulate the union in treatment of non-united tibial and femoral fractures</td>
<td>NCT01788059</td>
<td>Emadi Kamyab Hospital, Khorasan, Iran</td>
<td>Mohammad Taghi Peivandi</td>
<td>2</td>
<td>Nonunion femur and tibia</td>
<td>BMAC injected percutaneously</td>
<td>Completed 2013</td>
</tr>
<tr>
<td>BMAC with bone substitute or DBM</td>
<td>NCT00250302</td>
<td>Hadassah Medical Organization, Jerusalem, Israel</td>
<td>Meir Liebergall</td>
<td>Distal tibia fracture</td>
<td>BMAC + carrier</td>
<td>Completed 2011</td>
<td></td>
</tr>
<tr>
<td>Clinical trial based on the use of mononuclear cells from autologous bone marrow in patients with pseudoarthrosis</td>
<td>NCT01813188</td>
<td>IFUSRM, Murcia, Spain</td>
<td>Luis Meseguer</td>
<td>2 randomized vs autologous bone graft</td>
<td>Tibial pseudoarthrosis</td>
<td>BMAC + TCP + DBM</td>
<td>Recruiting participants</td>
</tr>
<tr>
<td>Evaluation the treatment of nonunion of long bone fracture of lower extremities (femur and tibia) using mononuclear stem cells from the iliac wing within a 3-D tissue engineered scaffold</td>
<td>NCT01958502</td>
<td>Emadi Kamyab Hospital, Khorasan, Iran</td>
<td>Mohammad Taghi Peivandi</td>
<td>2</td>
<td>Nonunion femur and tibia</td>
<td>BMAC + collagenic 3-D scaffold with BMP-2</td>
<td>Recruiting participants</td>
</tr>
<tr>
<td>Use of adult bone marrow mononuclear cells in patients with long bone nonunion</td>
<td>NCT01581892</td>
<td>HUCA, Oviedo, Spain</td>
<td>Jesus Otero</td>
<td>1–2</td>
<td>Nonunion</td>
<td>BMAC + osteogenic matrix</td>
<td>Recruiting participants</td>
</tr>
<tr>
<td>Expanded MSC alone</td>
<td>NCT00916981</td>
<td>University Hospital of Liege, Belgium</td>
<td>Jean Philippe Hauzeur</td>
<td>1–2</td>
<td>Long bone nonunion</td>
<td>Expanded MSC injected percutaneously</td>
<td>Completed 2012</td>
</tr>
<tr>
<td>Pivotal phase 2b/3 study on autologous osteoblastic cells implantation in hypotrophic non-union fractures</td>
<td>EUCR2011-005584-24-NL</td>
<td>Bone Therapeutics S.A., Belgium</td>
<td>Stefan Desmyter</td>
<td>2b-3 randomized vs autologous bone graft</td>
<td>Long bone nonunion</td>
<td>Expanded MSC injected percutaneously (PREOB®)</td>
<td>Recruiting participants</td>
</tr>
<tr>
<td>Phase 1/2a study on allogeneic osteoblastic cells implantation in delayed-union fractures</td>
<td>NCT02020590*</td>
<td>Bone Therapeutics S.A., Belgium</td>
<td>Enrico Bastianelli</td>
<td>1–2</td>
<td>Long bone nonunion</td>
<td>Allogeneic osteoblastic cells injected percutaneously (ALLOB®)</td>
<td>Recruiting participants</td>
</tr>
<tr>
<td>Expanded MSC + bone substitute</td>
<td>NCT00424567</td>
<td>Aastrom Biosciences, USA</td>
<td>Matthew Jiménez</td>
<td>1–2</td>
<td>Long bone nonunion</td>
<td>Cultured bone marrow tissue + bone matrix</td>
<td>Completed 2007</td>
</tr>
<tr>
<td>Feasibility study of Aastrom tissue repair cells to treat non-union fractures.</td>
<td>NCT01626625</td>
<td>Indonesia University</td>
<td>Phedy Phe</td>
<td>1 compared to iliac bone autograft</td>
<td>Nonunion</td>
<td>Expanded MSC + HA</td>
<td>Recruiting participants</td>
</tr>
<tr>
<td>Mesenchymal stem cells; donor and role in management and reconstruction of nonunion fracture</td>
<td>EUCR2011-005441-13</td>
<td>INSERM, France, (FP7 European Project REBORNE)</td>
<td>Enrique Gómez Barrena</td>
<td>1–2a</td>
<td>Long bone nonunion</td>
<td>Expanded MSC + HA + TCP</td>
<td>Recruiting participants</td>
</tr>
</tbody>
</table>

NCT: ClinicalTrials.gov; EUCTR: EudraCT; BMAC = bone marrow aspirate concentrate; DBM = demineralized bone matrix; HA = HYDROXYAPatite; TCP = tricalcium phosphate; MSC = mesenchymal stem cell.

All mentioned studies are on autologous cells except if indicated by an *.
Register — ISRCTN, and the Australian New Zealand Clinical Trials Registry, as well as monthly updates of national clinical trial registries.

A particular distinction of European clinical trials on advanced therapies is the large proportion of sponsors from academic and charitable organizations, as seen in a recent review of 318 trials from 2004 to 2010 on 250 therapies [83]. This aspect is reinforced by the fostering of investigator-driven clinical trials from institutions and organizations across Europe [84], spreading the opportunities for more available clinical information about the myriad possibilities that can be considered in the cell therapy field.

Yet, many declared clinical trials in any of the available international and national trial registries, both from academic and industrial sponsors, do not offer results or just provide initial information about the research effort, and then the development of the trial and the final outcomes are difficult to trace. This is equally confirmed in the long bone nonunion cell therapy trials. To further illustrate the current situation, the available on-going trials on the topic of this review are summarized in Table 1.

Excluding trials with unknown status or not yet recruiting, 13 trials related with long bone fracture or nonunion and mesenchymal cell therapy were identified as they have been cited in clinical trial registries as completed (6 of them) or recruiting patients. They may be classified into four groups to allow for comparative analysis. The first group, with 3 trials all completed, includes the simplest technique, percutaneous injection of bone marrow aspirate concentrate (BMAC); none of these trials have been published yet. In the second group (4 trials), BMAC is associated with bone substitutes or demineralized bone matrix (DBM); results have been published about one single trial only [85], observing a shorter time to bone union with cells than in the controls. In the third group, 3 trials intend to test percutaneous injection of expanded MSCs, but the only completed trial is not yet published. In the fourth group, 3 trials address the association of expanded MSC and bone matrix or substitute, but the only completed trial has not been published yet. Needless to say that follow-up of these and other trials on the topic will enlighten the future of the field.

A major criticism on the available trials are the underreported results, which may reflect lack of protocol adherence, patient heterogeneity in small unincentric trials, confounding efficacy results in part due to patient or to protocol variability, or others. Many of these trials do not offer sufficient information about the cell product to correlate with the results in other trials and many are also impossible to reproduce in other centers due to lack of transparency. However, reliability is particularly challenged by the size and design of the currently available trials. Unless large, comparative trials with well-defined cell products are published, evidence on this therapy will remain controversial or even negative.

Future directions

A strong need of clinical results is required to further progress in cell therapy. Launched trials will hopefully provide this information in the near future. If clinical results are positive, far greater challenges may be raised by the development of more complex tissue engineering techniques, and this may allow the treatment of large bone defects and unsolved situations [86] after appropriate in vivo models confirm the specific solution to submit to trials. A multidisciplinary approach will be required to improve implanted cell survival and to ensure prompt vessel ingrowth into the biomaterial via careful selection of structure and shape, together with addition of cytokines and growth factors. The development of new materials and cell combinations (hydrogel-based, bioceramic-based, or other) that could eventually craft solutions for supplying cells and biomaterials percutaneously is expected in the near future. The immunosuppressive properties of MSCs may allow the transplantation of allogeneic MSCS in various orthopedic conditions, with the establishment of cell banks for regenerative medicine. Early trials evaluating allogeneic MSCS in delayed unions are already under way. And last but not least, a future step that may help to further define and spread these therapies is a careful cost–benefit assessment and a broad economic evaluation to clarify the best indications of bone repair cell therapy as a standard procedure, if confirmation of safety and efficacy is clearly derived from current trials.

Conflict of interest disclosure

The authors do not have anything to disclose.

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