Stem cells and their derivates: an implication for the regeneration of nonunion fractures

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Running head: **Stem cells for nonunion fractures regeneration**

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ABSTRACT

Despite advances in biomedical research, fracture nonunion rates have remained stable throughout the years. Long bone fractures have a high likelihood of nonunion, but the specific biological pathways involved in this severe consequence are unknown. Fractures often heal in an organized sequence, including the production of a hematoma and an early stage of inflammation, the development of a soft callus and hard callus, and eventually the stage of bone remodeling. Deficient healing can result in a persistent bone defect with instability, discomfort, and loss of function. In the treatment of nonunions, mesenchymal stem cells (MSCs) prove to be a promising and safe alternative to the standard therapeutic strategies. Moreover, novel scaffolds are being created in order to use a synergistic biomimetic technique to rapidly generate bone tissue. MSCs respond to acellular biomimetic matrices by regenerating bone. Extracellular vesicles (EVs) derived from MSCs have recently gained interest in the field of musculoskeletal regeneration. Although many of these techniques and technologies are still in the pre-clinical stage and have not yet been approved for use in humans, novel approaches to accelerate bone healing via MSCs and/or MSC derivates have the potential to reduce the physical, economic, and social burdens associated with non-healing fractures and bone defects. In this review, we focus on providing an up-to-date summary of recent scientific studies dealing with the treatment of nonunion fractures in clinical and pre-clinical settings employing MSC-based therapeutic techniques.

KEYWORDS: stem cells, nonunion fracture, pseudoarthrosis, bone healing, exosomes, scaffold

INTRODUCTION

Bone injuries are a major medical concern across the world, resulting in enormous healthcare and social costs. While most bone injuries are capable of healing through bone regeneration by natural callus formation with standard treatments, long bone injuries may result in nonunion fractures also known as pseudarthrosis (Fig. 1). Long-bone fractures, including those of the femur, tibia, and humerus, are among the most common types of non-fatal trauma in the world. The overall probability of nonunion per fracture is 1.9%, however, the probability may increase up to 9% for some fractures in particular age groups.[1] Even with the current treatment techniques for nonunion, atrophic nonunion fracture is still the most challenging to manage. Long bone nonunions carry a heavy socioeconomic burden that is primarily caused by indirect expenses like lost productivity from prolonged treatment.[2]

Nonunions are defined as a disruption of normal healing with the expectation that without targeted and precise treatment, no consolidation will be achieved.[3] Under physiological conditions, fractures and bone defects often heal in an organized sequence that includes the production of a hematoma and an early stage of inflammation, the development of soft callus, the construction of hard callus, and eventually the stage of bone remodeling (Fig. 2). Shear stress and fracture site instability appear to be key biomechanical risk factors for nonunion formation. Furthermore, biological factors, including inadequate blood flow and severe bone and soft tissue damage, are held accountable for fracture healing complications such as infections and significant bone abnormalities.[3] Interestingly, by finding polymorphisms in blood and bone callus samples of nonunion patients, a genetic tendency for nonunion development was revealed. Last but not least, the specific patient variables and comorbidities (i.e. high body mass index, diabetes, osteoarthritis and rheumatism, osteoporosis, etc.) are risk factors for nonunion development.[4]

Long bone nonunions are frequently associated with bone abnormalities. The basic techniques for nonunion care include removing necrotic bone and tissue, filling most of the bone defect, stimulating osteoblast recruitment, raising the concentrations of osteoinductive chemicals, and maintaining a stable mechanical environment. Blood vascular distribution at the site of nonunion has also been proven to be a significant element in fracture healing.[5,6]

There are now a variety of conservative reconstructive therapy methods available for nonunion treatment. The existing available treatments produce insufficient results and have a slew of side effects that complicate bone repair and limb length maintenance.

In this review, we focus on providing an up-to-date summary of recent scientific studies in the field of regenerative medicine for the treatment of nonunion fractures in clinical and pre-

clinical settings employing MSC-based therapeutic techniques (Table 1). A number of the MSC therapies suggested use various scaffolding to potentially aid in the repair of pseudarthrosis. Following that, we discuss many unique MSC-based cell-free approaches and their use in nonunion fracture therapy – two of which employ MSC-derived exosomes, and one reports development of an ultrasound-mediated gene delivery method to induce activity of endogenous MSCs at fracture site.

LITERATURE SEARCH METHODOLOGY

A search was performed (10 November 2022) of the PubMed/ Medline databases. Key-words related to MSCs were combined with synonyms for nonunion fractures, clinical trial, exosome, and scaffold. The search was restricted to the last 5 years and the English language.

OVERVIEW OF CELL THERAPY IN BONE HEALING

The onset of inflammation is a hallmark of the first stage of bone repair. Mesenchymal stem cells (MSCs), endothelial cells, and immune cells travel to location of the broken bone site. In response to signals provided by the hematoma, osteoprogenitor cells from the periosteum, bone marrow, and surrounding tissue move into the fracture location.[7–9] Therefore, it is not unexpected that patients with nonunion showed reduced progenitor cell counts at nonunion sites and in their bone marrow, as well as a systemic mesenchymal and osteogenic cell pool deficit.[10] Due to its osteogenic potential, which was initially noted, the administration of bone marrow (sourced from iliac crest) was suggested to enhance the cellular environment in a disordered bone healing process.[11–13]

The iliac crest has a reservoir of spongious bone that is widely employed as a source of bone autograft, supplying extracellular matrix (for osteoconduction), growth factors (for osteoinduction), and patient's cells (leading to local osteogenesis). However, in 5% of instances, significant problems such as donor defect hernias, vascular injuries, sciatic nerve damage, deep infection, deep hematoma requiring transfusion, and iliac wing fracture have been recorded. Pain at the extraction site occurs in 5% of instances, superficial hematoma or seroma or superficial infection at the extraction site occurs in 13% of cases, and ilioinguinal neuralgia occurs in 2% of cases. In order to decrease these negative effects on patients, researchers moved towards utilizing mesenchymal stem cells instead.[14]

MSCs have been shown to not only have the ability to develop into a variety of cell lines based on the available niche, but also to support their therapeutic potential through paracrine action. This highlights their broad potential for use in biological treatment for a wide range of

disorders. MSCs' potential to mediate immunomodulatory activities made them a promising choice for innovative disease therapy.[13,15,16]

MSCs utilized in treatment are derived from a variety of sources. Umbilical cord, bone marrow, and adipose tissue are the most common sources. The precise mechanism of MSCs' influence on bone fracture regeneration remains unknown. Furthermore, the safety of MSC-based treatments must be considered, as well as an uniform protocol of delivery, and ultimately, eligible patients for this therapy must be identified.

Overall, local cell therapy for nonunion is claimed to have shown positive outcomes, the evidence for the best cell harvesting, processing, and application method is currently lacking.

DISCUSSION

Mesenchymal Stem Cell-based Therapy in Clinical Setting

Recent clinical trials used MSCs in conjunction with well-established and extensively used fixation procedures to promote nonunion healing. Following research teams put a lot of effort to provide osteoinductive/ osteogenic variables in order to establish an optimal environment to assist the healing microenvironment at the fracture site, with the goal of improving clinical results. Using different matrices is also an excellent concept for designing a therapeutical model for MSC administration into fracture sites, and they are commonly employed in these innovative clinical studies.

Research team of prof. Dilogo presented the first case report combining Masquelet technique with umbilical cord-derived MSCs (UC-MSCs), bone morphogenetic protein-2 (BMP-2) and Hydroxyapatite (HA). Each technique represents one of the four pillars of the diamond concept, namely osteogenic, osteoinductive, osteoconductive and stable fixation. This particular female patient suffered with infected nonunion of the right femoral shaft with significant bone loss underwent number of unsuccessful surgeries. Based on the patient’s medical history, a modified Masquelet procedure was planned.

The Masquelet surgical procedure consisted of two steps. The segmental bone defect was bridged using a polymethylmethacrylate (PMMA) cement spacer, and the bone was stabilized using orthopedic hardware in the first stage. Since the wound was grossly infected, 2 months later patient underwent a second procedure consisting of debridement, spacer removal and ORIF using reverse distal femoral locking plate. During the surgery a 12 cm bone defect

was noted after spacer removal, which was filled using bone substitute material containing HA along with BMP-2 and 50 millions of allogeneic UC-MSCs to enhance bone regeneration.

The patient was rehabilitated and followed for up to 12 months, and clinical and radiographic imaging were performed. The patient had clinical union 6 months after surgery and claimed no pain when walking, but a clinical examination revealed a 2 cm leg length difference. At the 8-month follow-up, the patient complained of pain when moving her knee and elected to have the patellar implant removed. Clinical assessment after 11 months revealed that the patient could execute complete weight bearing walks with no discomfort and no change in leg length disparity. The scar was clear and free of infection.

Because the researchers employ UC-MSCs implantation as a replacement for autogenous and synthetic bone graft, they consider this method to be a modified Masquelet approach.[17] According to current research, the induced membrane plays a function in bone transplant osteogenesis and vascularization.[18–20]

The case report of Dilogo et al. suggests that the Masquelet technique may be an applicable approach to overcome large bone defects, with the supplementing of allogeneic UC-MSCs and BMP-2 it provides growth and osteoinductive factors. As presented, this technique may play a crucial part in improved healing of the nonunion fracture, in line with the diamond concept.[17]

The recent paper of Prat et al. presents two cases of persistent atrophic pseudoarthrosis. After demonstrating the feasibility of the treatment and osteogenic potential of the cell-based treatment in an ovine model of critical size segmental tibial defect, 2 patients with atrophic pseudoarthrosis were treated with a tissue-engineering product (TEP) composed of autologous bone marrow-derived MSCs (BM-MSCs) manufactured under good manufacturing practice (GMP).

Firstly, the group compared the osteogenic efficacy of a tissue-engineering technique based on autologous BM-MSCs seeded onto cadaveric spongy bone matrices from a tissue bank to autografts and acellular scaffolds in ovine model. Commercially particulated deantigenized bone matrix from human cadaveric donors was employed as a natural osteoinductive and osteoconductive agent in the scaffold. This method was chosen because cells connected to the scaffold lasted longer at the site of delivery, as proven by prof. Caminal and her team in a sheep model of avascular osteonecrosis.[21]

Animals in this study were divided into 3 groups: group 1, gold standard; group 2, acellular scaffold; group 3, cell-seeded scaffold. Following that, the tibiae were stabilized using two osteosynthesis plates, and the plate fixation remained stable in all animals over the

3-month follow-up period. At the end, tibiae from the three experimental groups were collected for macroscopic and histological analyses. Intense bone remodeling activity and vascularization were found in histological sections collected from animals in groups 1 and 3, but no tumorigenesis or ectopic tissue development were observed, indicating the safety of the cell-based therapy suggested here.

Based on the pre-clinical results, the researcher group moved with this approach forward to the clinical setting. They provided treatment description for the two patients, which consists of MSCs collection from BM aspirates obtained from patients, followed by a validation from GMP-compliant process. The scale-up in vitro generated a sufficient number of viable cells for seeding 15 cm^3 and 25 cm^3 of deantigenized bone particles. The final dose of 6.83 × 105 and 6.55 × 105 MSCs/cm3 for treatment of patients 1 and 2, respectively. Human BM-MSCs showed fibroblastic morphology and the characteristic MSC immunophenotype and differentiation potential confirmed the MSC nature of the cells.

The patient 1 showed no intraoperative or postoperative problems, and the clinical development of case 1 was satisfactory in the follow-up period, with a clear tendency toward healing that finally culminated in complete bone healing at 6 months, as determined by both Computed Tomography (CT) scan and X-ray evaluation. The patient resumed regular life and employment after this point.

Unfortunately, the case 2 patient had extensive medical history regarding the tibial fracture. This patient underwent several surgical procedures, presented with acute infections prior to enrollment in this study. Upon enrollment, treatment with TEP composed of expanded autologous BM-MSCs following the Masquelet technique was performed. The cement was removed three weeks after the resection, and with help of external fixation, the creation of a pseudomembrane demonstrated adequate environmental signals for bone regeneration. Unfortunately, the patient developed a neuropathic pain syndrome that could not be addressed pharmacologically, and she repeatedly requested amputation. The supracondylar amputation was performed after a psychological examination.

This study supports the safety, reproducibility, and feasibility of treating recalcitrant pseudoarthrosis with a combined procedure involving MSCs (as a biological agent in a TEP) and a standard surgical technique, but clinical trials are ultimately required to define both safety and efficacy profiles, as well as to help understand the mechanisms underlying their potential osteogenic effect in nonunions.[22]

Zhang et al. published their current research on the therapeutic benefits of combining the Ilizarov technique with intraosseous injection of autologous bone marrow derived

mesenchymal stem cells.[23] The Ilizarov external fixation technique, developed by Gavriil Abramovich Ilizarov in 1951, is a common surgery for the treatment of bone nonunion, bone lengthening, and bone deformity repair. To provide tension and support to the bone, metallic wires are introduced percutaneously and joined to a metallic ring. This technique addresses considerable number of complications and drastically improves quality of the union, it also allows unrestricted access to the site of damage to monitor healing and infection rates. [19,24] Zhang team's open-label trial included 25 patients with infected tibial non-union, who were randomly assigned to either the Ilizarov technique alone or in combination with an intraosseous injection of BM-MSCs. All patients were monitored for 12-34 months (mean 16 months), and both groups met the main goal of stable tibial fracture union. The data presented in this study suggest that utilization of BM-MSCs as an add-on therapy significantly reduced the duration of Ilizarov, which is much welcomed by patients. The trial patients suffered bone defect of size within 3–12 cm and were able to achieve bone union by 2.5 months, which is a statistically significant conclusion.[23]

Since the researchers used a commercially available method to isolate MSCs, additional characterization of the stem cell population is required in the future. A broader clinical research in other types of bone unions is also needed to emphasize the positive effect of this combined treatment.[23]

Study published by team of prof. Gomez-Barrena aimed to evaluate the efficacy of clinical and radiological bone consolidation (at 3, 6, and 12 months follow-up) of long bone delayed unions and nonunions, with subgroup analysis of affected bone, patient's gender, tobacco use, and time since the original fracture. Gomez-Barrena et al. had responded to lack of available evidence from trials in difficult clinical settings with cell therapy, by proposing a phase I/IIa open, prospective, multicentric, non-comparative interventional clinical trial. The primary endpoints of this trial evaluated safety and feasibility of Advanced Therapy Medicinal Product (ATMP) given to patients. ATMP, comprised of the autologous expanded BM-MSCs and CE-marked bioceramic, were produced under GMP. [25]

Twenty-eight individuals were recruited, mean age was 39 ± 13 years, 57% were males, and mean Body Mass Index 27 ± 7. Thirteen (46%) were active smokers. There were 11 femoral, 4 humeral, and 13 tibial nonunions. Initial fracture occurred at a mean ± SD of 27.9 ± 31.2 months before recruitment. Patients surgically received GMP-expanded BM-MSCs coupled to MBCP+^{TM} (100% synthetic CE-marked class III implant), a bone replacement produced in 1-2 mm granules constituted of 20% HA and 80% beta tricalcium phosphate (ß-TCP).

Clinical and radiological consolidation was reported in all 25 patients who completed the one-year follow-up. This study demonstrated success in femoral, humeral, and tibial nonunions surgically treated with ATMP by clinical and radiographic consolidation. Bone biopsies indicated bone regeneration, and there was no difference in the time it took to attain consolidation among the afflicted bones, however it was slower in tibial nonunions. At 6 and 12 months, smokers had worse consolidation scores; gender and time since the initial fracture had no impact.[25]

This study has several limitations, the first consideration is the study's design, where a comparative, randomized, controlled trial would provide more data. This randomized comparison research is already underway, as the team published a trial protocol in 2018, providing a comparison of ATMP to the standard treatment via the gold standard; iliac crest autologous graft.[26] Also, more cases are needed to determine efficacy, particularly in some categories.[25]

Successfully treated cases, provided by recent research, demonstrate new possibilities for nonunion treatment by moving away from traditional ineffective procedures. MSC use is a fast growing emphasis in many medical domains, particularly orthopedics, and warrants additional exploration. To assess the effectiveness of MSCs in conjunction with fixation techniques and/or scaffolds for the treatment of long-bone nonunion, future randomized clinical studies with bigger sample numbers are required.

Mesenchymal Stem Cell-based therapy in pre-clinical setting

The emphasis in the next section is on preclinical in-vivo data. This allows researchers to better grasp the potential and indications for innovative methods to MSC treatment in animal models. Data from MSC therapy of long-bone nonunion fractures in animal models was supplied by the researchers. This is a key element to consider when transferring data to the clinical field. In reality, while various models are suitable for evaluating bone regeneration, not all of them accurately mimic human tissue properties. To facilitate findings transferability, chosen models should exhibit physiological and pathological parallels; hence, bigger models more closely mimic the human state.

The use of autologous BM-MSCs to treat a rabbit with a radial nonunion segmental defect was studied by Zamani Mazdeh et al., in relation to the effects of 17-estradiol. Twenty rabbits were randomly assigned to one of four experimental groups: 1. autologous fibrin clot -

control; 2. autologous fibrin clot with MSCs; 3. 17-estradiol (E2) treatment; and 4. E2+MSC treatment. [27]

Healing was evaluated by radiology (weeks 0, 2, 4, 6, 8, and 10) and histopathology (week 10). Radiological assessment results revealed that the E2+MSC group performed the best compared to the other groups from week 4 to week 10 inclusive. The analysis of the radiological evaluation data indicated that the statistical significance between the healing levels of the E2+MSC group and that of the other groups began at week 4, while and the control group at week 6 for the first time.

Furthermore, histopathological analysis revealed that the E2+MSC group reached the highest score, which was substantially higher than the E2 and control groups. Hence, the collected data confirms that the in-vivo administration of in situ 17-estradiol improves the osteogenic ability of planted BM-MSCs while also accelerating bone repair.

What the researchers discovered, was that BM-MSCs did not mend a critical-sized defect. Furthermore, except for week 2, the scores of the E2+MSC group were considerably larger than the MSC group in all time points. This lends credence to the notion that estradiol enhances MSC differentiation potential in order to repair critical-sized bone defects. [27]

The study suggests that estradiol not only boosts the differentiation capacity of BM-MSCs into osteoblasts, bone regeneration, and the amount of future bone healing; but it also speeds up the rate of bone healing, resulting in a shorter healing period. As a result of delivering more efficient and less time-consuming healing in combination with a prospective treatment for patients, in-vivo application of in situ estradiol looks to be a viable technique for future cell-based therapeutic therapies of nonunion bone fractures. [27]

In 2021, Osagie-Clouard and her team published a pre-clinical study, utilizing locally delivered MSC-treatment in fibrin glue; in combination with different doses of teriparatide (parathyroid hormone (PTH) 1- 34) in nonunion rat model with mechanical support of fixator (Fig. 3). PTH is the N-terminal fragment of the whole 84- amino acid polypeptide hormone. It is licensed in both the United States and Europe for the treatment of osteoporosis.[28] Despite a large body of clinical research demonstrating PTH's utility in increasing bone density, its use as a pharmacological adjunct to fracture healing has been primarily in preclinical studies. Such studies have shown beneficial effects on callus formation, with increased trabeculae and an accelerated remodeling phase.[29,30] In clinical studies, PTH has been demonstrated to reduce time of radiological healing of distal radii fractures in the osteoporotic population, enhance pelvic ring fracture healing, and accelerate healing in both sternal and humeral nonunions and delayed unions.[31–33]

When compared to PTH or MSCs treatment groups alone, combination treatments (differing in dosage) resulted in more mineralized tissue and improved mechanical integrity at the fracture site. In contrast to the control groups, combination therapy groups increased trabecular number (TbN), whereas high-dose combination therapy also raised trabecular thickness (TbTh). The increased callus integrity/maturity in the high-dose combination group explains the difference in TbTh and TbN.

The findings reported in this study showed, that PTH usage on its own has a dosage dependency in connection to callus maturity and mechanical characteristics. PTH was prepared to a concentration of 25 μg/kg (low dose) and 100 μg/kg (high dose). Nonetheless, because rats have an uncertain receptor binding profile, the provided high dosage may not be completely absorbed or active, and hence the acting dose may be significantly lower than the concentration administered.

Essentially, the consistency of our PTH findings, as well as the extent of the gains found with combination groups, justify the choice to utilize a five-day, Monday-Friday dosing schedule. This study report had only one end point at 35 days. Hence, the endochondral ossification increases may have been overlooked due to the lack of a 14-day marker. As a result, an earlier timepoint would have provided an answer to the topic of enhancement vs acceleration.

Osagie Clouard's team was the first to use locally injected MSCs to explore this combined treatment in a weightbearing limb. The published data emphasizes local administration of MSCs in conjunction with systemic PTH as a therapy deserving of further investigation; the data given supports the efficacy of combining both techniques. Futhermore, additional research on the underlying mechanistic mechanisms is required.[34]

The research papers reviewed above demonstrate that the introduction of MSCs as a therapeutic is worthy of future examination. The evidence, provided by these pre-clinical studies, supports the usefulness of using stem cell-based approaches in treatment of pseudarthrosis. Furthermore, greater investigation into the underlying molecular pathways is needed.

Novel matrices for MSC delivery systems

It is generally believed, that combining MSCs with a good delivery mechanism will allow for improved regeneration, particularly with the assistance of bio-functional scaffolds, which would provide the benefit of structure and qualities similar to native tissue. The construction of a scaffold is a critical step in bone tissue creation. A appropriate scaffold has a three-dimensional structure that resembles the extracellular matrix, allowing cells to connect,

migrate, and proliferate.[35] As a result, numerous research labs have concentrated their efforts on developing an unique scaffold that is best suited to facilitate nonunion fracture healing.

In latest study of Xue et al., the research team concentrated their efforts on investigating MSC osteogenesis and migration stimulation potential of High mobility group box 1 (HMGB1)-gelatin sponge scaffolds combined with MSC sheets, for the local treatment of nonunion fractures of rat tibial osteotomy model.[36]

HMGB1, one of the main local stressors in the danger-associated molecular patterns (DAMPs) family, has been demonstrated to be an efficient osteogenic growth factor.
As previously disclosed by team of prof. Xue, HMGB1 stimulates MSC osteogenesis and migration. As a result, the potential of HMGB1 as an osteogenic cytokine that promotes local bone repair warranted additional exploration.[37,38]

Gelatin sponges, utilized in this study, are a natural substance with a porous structure and great biocompatibility, and their hydrophilicity makes them suitable carriers for HMGB1 local distribution.

The low surface-to-volume ratio of the scaffolds is known to be limiting the quantity of implanted stem cells. The use of MSC sheets as a source of high-density cells might be one answer to this reoccurring challenge of tissue engineering research.[39–41] The findings indicate that adding HMGB1 to gelatin sponge scaffolds increased their biocompatibility in terms of in vitro MSC proliferation and osteogenic differentiation of MSCs and MSC sheets. The outcomes also revealed that the STAT3 signaling pathway was involved in the increased osteogenic differentiation of MSCs. Lentiviral vector-mediated HMGB1 overexpression in MSCs resulted in considerable elevation of p-STAT3 during the osteogenic induction of lenti-HMGB1 MSCs. The suppression of STAT3 expression in MSCs during osteogenic induction, whether by siRNA knock down or the addition of the SH-4-54 inhibitor, prevented these cells from undergoing osteogenic differentiation. Hence, the data suggest that the STAT3 signaling pathway is involved in the HMGB1-induced osteogenic induction of MSCs.

In an in vivo study, HMGB1-gelatin sponge scaffolds combined with MSC sheets supported new bone formation in surgically treated fractures. In this study, the porous gelatin sponges were selected as the scaffold because of their commercial availability and excellent biocompatibility. Confocal laser scanning microscopy (CLSM) and DNA assays showed that the gelatin sponge scaffolds supported cell attachment and proliferation. In scaffolds loaded with HMGB1, enabling its controlled release, both cell attachment and proliferation increased significantly.

In in vivo experiments, HMGB1-gelatin sponge scaffolds together with MSC sheets encouraged new bone growth in surgically repaired fractures. The cell sheets wrapped around the gelatin scaffolds, functioned as artificial periosteum, while HMGB1 released in controlled manner from the gelatin sponge enhanced the cell sheets' microenvironment and encouraged host cell invasion and osteogenic differentiation. The cell sheet's osteogenic differentiation experiment also discovered that it preserved a high level of osteogenic differentiation potential. In the future, the team plans on creating cell sheets utilizing ontogenetically produced cells.[36]

Carvalho et al. published most recent study utilizing a synergistic biomimetic technique to create matrices that rapidly generate bone tissue, which is an important part of weight bearing bone fracture recovery. They utilized collagen matrices, enhanced with two selected key matrix proteins, the most prevalent non-collagenous proteins, osteocalcin (OC) and osteopontin (OPN), in rabbit nonunion fracture models.[42] The data presented indicate that combination of collagen with osteocalcin (OC) and/or osteopontin (OPN), increased the rate and quantity of synthesized bone matrix by increasing MSC proliferation, accelerating osteogenic differentiation, enhancing angiogenesis, and demonstrating a sustained bone formation response from MSCs derived from a variety of human tissue sources (marrow, fat and umbilical cord). Moreover, the researchers favored OC/OPN to BMP due to limitations such as high manufacturing costs and the large dosage requirements, raising concerns regarding their cost effectiveness. The goal of this work was to establish a novel technique for rapidly generating and maintaining functional bone creation using OC and OPN, as well as to determine the mechanism underlying their synergistic effect on bone regeneration. OC-enhanced collagen matrices and OPN-enhanced collagen matrices were also studied in comparison to OC/OPN mineralized scaffolds, to validate the synergistic impact of these two bone ECM proteins. [42]

Carvalho and colleagues were the first to investigate the synergistic effect of different concentrations of bone matrix non-collagenous proteins, OC and OPN, incorporated into type I collagen gels, while demonstrating enhanced proliferation and accelerated osteogenic differentiation of human MSCs and angiogenesis, ultimately resulting in increased mineralization and bone regeneration of the nonunion fractures. Furthermore, in vivo evaluation of OC/OPN mineralized scaffolds in a critical sized-defect rabbit long-bone model revealed no foreign body reaction during bone tissue formation. This recently established biomimetic technique has proven to its ability for rapidly forming mineralized bone tissue and securing a sustained bone formation response by MSC from numerous sources, allowing for speedier patient recovery and treatment of nonunion fractures in an elderly and sick population.[42]

The team tested 5 different OC/OPN concentrations, above and below bone matrix physiological values. They discovered biomimetic OC/OPN-enhanced collagen matrices that promote early osteogenic differentiation of MSC and maintain bone formation response. Additionally, the amount of OC and OPN released from the collagen gels was measured, in order to indirectly prove the link between these proteins and collagen. The measurement took place after 24 hours and 21 days and the records show that OC and OPN were not released from the gels even after 21 days. The presented study shows the benefits of OC/OPN synergy on several aspects of bone regeneration, including MSC proliferation, osteogenic differentiation, mineralization, and angiogenic characteristics. However, more research is needed to determine the usefulness of these matrices as biomimetic scaffolds that promote bone healing in bone tissue engineering and regenerative medicine.[42]

MSC cell-free therapy employing MSC-derived extracellular vesicles

Despite the initial premise that MSC-based treatment techniques aid in tissue regeneration by replenishing tissue through their capacity to differentiate, it has now become widely accepted that the true therapeutic strategy is found in their secretory activities. These cellular techniques have several technical difficulties, including labor-intensive and time-consuming cell expansion, dedifferentiation during cell expansion, inconsistency in large-scale manufacturing, and loss of intrinsic activity upon administration coupled with MSC-based cell treatment.[43,44] MSC-exosomes, which are typically seen in MSC secretomes, are extracellular microvesicles (30-150 nm in diameter) consisting of lipid bilayer capable of impacting cells and tissues via numerous signaling pathways without eliciting an immune response (Fig. 4) Exosomes are significant paracrine factors that can be employed as therapeutic methods for tissue healing, particularly in the field of bone regeneration, because they are the primary mediators of intercellular communication with cells (Fig. 5).[45–47]

Exosomes produced from BM-MSCs (BM-MSC-Exos) were implanted into the fracture site in a rat model of femoral fracture in research reported by Zhang et al.[48] The data showed that increased osteogenesis and angiogenesis accelerated bone healing processes. The researchers revealed for the first time that BM-MSC-Exos may stimulate angiogenesis and osteogenesis in a rat nonunion model. The reported results suggest that BM-MSC-Exos can significantly boost bone regeneration. Immunohistochemistry (IHC) and western blotting used to examine the expression levels of osteogenesis-related genes in vitro and in vivo. The study indicates that the levels of OPN and osteoglycin (OGN) expression had dramatically risen. IHC examination confirmed the enhanced expression of OCN, OPN, and OGN in BM-MSC-Exos-

stimulated target bone tissue. Hence, the team first time proven that BM-MSC-Exos could promote angiogenesis and osteogenesis in an experimental model of nonunion rats. [48]

Using western blotting, IHC, and immunocytochemistry (ICC), the authors found that the expression levels of BMP-2, Smad1, and RUNX2 were significantly enhanced in callus tissue and MC3T3-E1Cs induced by BM-MSC-Exos. Consequently, the authors stated the possibility that BM-MSC-Exos may promote osteogenic development by activating the BMP-2/Smad1/RUNX2 signaling pathway, which may play a primary role in BM-MSC-Exos-mediated boost of osteogenesis.

The ability of HUVECs to proliferate, move, and form tubes was greatly induced by BM-MSC-Exos in vitro. Hence, the in vivo findings revealed that BM-MSC-Exos might significantly boost angiogenesis. RT-PCR and western blotting were used to examine the expression levels of angiogenesis-related genes in target tissues induced by BM-MSC-Exos, and HIF-1 and VEGF were shown to be considerably enhanced. [48]

The outcomes presented by Zhang et al. provide the first evidence for the potential of BM-MSC-Exos in the treatment of nonunion, implying that BM-MSC-Exos might be a promising therapeutic method in the treatment of nonunion. The current study, however, has numerous drawbacks. The therapeutic approach via exosomes for nonunion has not yet been developed; this technique cannot temporarily substitute surgery. In clinical nonunion, surgical debridement and fixation adjustments are frequently required, which differs from the animal nonunion model.[48]

Research team of prof. Hu, claims in their most recently published work, they firstly explored the dysregulated microRNAs (miRs) in BM-MSCs-derived extracellular vesicles (B-EVs), and then investigated the effects of B-EVs and the molecular mechanisms in fracture repair in mouse model.[49] In this study, C57BL/6 wild-type (WT) mice, and CD9−/− mice, on C57BL/6 genetic background, were utilized and allocated into following groups: WT+negative control (NC) group (WT mice injected with EV-NC after GW4869 (-inhibitor of EV secretion) intervention), WT+ EVs group (WT mice injected with B-EVs), WT+ EVs-Mock group (WT mice injected with B-EVs prior transfected with miR-335 Inhibitor NC), WT+ EVs-Inhibitor group (WT mice injected with B-EVs prior transfected with miR-335 Inhibitor), CD9−/−+NC group (CD9−/− mice injected with EV-NC after GW4869 intervention), CD9−/−+ B-EVs group (CD9−/− mice injected with B-EVs), CD9−/−+ EVs-Mock group (CD9−/− mice were injected with B-EVs prior transfected with miR-335 Inhibitor NC), CD9−/−+ EVs-Inhibitor group (CD9−/− mice injected with B-EVs prior transfected with miR-335 inhibitor). On the

first and eighth days after fracture, all groups of mice were injected with 100 μL EVs isolated from BM-MSCs or 100 μL EV-NC following GW4869 therapy at the fracture site. .[49]

The data confirmed that B-EVs deliver miR-335 to osteoblast-like cells, where it upregulates miR-335 expression while inhibiting VapB expression. Reduced VapB expression boosted Wnt/β-catenin pathway activation, increased α-SMA, OCN, GDF-10, and FGF-2 content, and facilitated osteogenic differentiation and fracture repair. B-EV administration improved the production of bone and cartilage tissues, osteoblast differentiation, and fracture repair, according to hematoxylin and eosin staining, toluidine blue staining, and IHC of BMP2. Consequently, the B-EV therapy improved fracture healing in a rat model and increased osteoblast development in cell cultures.

Differentially expressed miRs in B-EVs-treated C57BL/6 WT mice were screened using microarray analysis and validated via RT-qPCR to further investigate the unique mechanism of EV therapy in fracture repair. miR-335 was discovered to be prevalent in MSCs and B-EVs. In the case of bone formation, miR-335-5p is prevalent in undifferentiated MSCs and osteoblasts, and its neutralization in bone marrow mononuclear cells implanted into a massive femur defect of the rat promoted bone repair. Furthermore, Hu et al. discovered that B-EV-mediated miR-335 intervention partially counteracted the encouraging effects of B-EVs on fracture repair in mice and osteoblast differentiation of MC3T3 and MG63 cells. The study also confirmed that miR-335 could target VapB, and that VapB overexpression reversed the pro-osteoblast differentiation impact of B-EVs. VapB expression increased during osteoclast development, while VapB knock-down inhibited bone resorption. Furthermore, after B-EV therapy, the activity of the Wnt/β-catenin pathway was dramatically improved. Wnts also reduced MSCs adipogenesis and enhanced osteogenesis through a β-catenin-dependent mechanism. Meanwhile, VapB overexpression hindered osteogenic differentiation by inactivating the Wnt/β-catenin pathway.[49]

Although this research has numerous great discoveries, it also contains several limitations. Firstly, due to experimental constraints, only miR-335 was chosen as having the largest differential expression among the eight differentially expressed miRs. The other seven miRs also play important roles in MSC osteogenic differentiation, indicating that several miRs may be involved in the synergistic miR-miR interaction network during fracture healing.[50–54] Secondly, although the particular mechanism of miR-335 and VapB in apoptosis is not explained in this paper, it is an intriguing research topic. Further in-depth research of the mechanism is planned for the subsequent study. Furthermore, MSCs from other sources (such

as adipose or umbilical cord) may stimulate bone repair, although this has to be validated in future studies.[49]

Recombinant protein delivery system targeting endogenous MSCs

Bez et al. hypothesized that targeted ultrasound-mediated, microbubble-enhanced therapeutic gene delivery to endogenous stem cells would result in effective bone regeneration and fracture healing. To put this theory to the test, the researchers surgically generated a critical-sized bone fracture in the tibiae of Yucatán mini-pigs, which are a therapeutically relevant big animal model. In order to stimulate the recruitment of endogenous MSCs into the fracture site, a collagen scaffold was implanted in the fracture. Transcutaneous ultrasound-mediated reporter gene delivery transfected 40% of cells at the fracture site two weeks later, and flow cytometry revealed that 80% of the transfected cells expressed MSC markers.[55]

Recombinant human bone morphogenetic proteins (BMPs) have been introduced into the clinic for the treatment of long-bone fractures. In individuals with tibial nonunions, local treatment of BMP-2 and BMP-7 resulted in enhanced healing rates. BMPs, on the other hand, are expensive and have been linked to a high frequency of adverse effects such as infection, heterotopic bone growth, and immunogenic responses, probably due to their usage in megadoses.[56,57] Therefore in effort to deliver the BMPs via nonviral vectors, group of prof. Bez decided to investigate ultrasound as possible delivery system for BMP-6 plasmid DNA, achieving transient local gene expression, mainly targeting endogenous MSCs. [55]

In this study the following groups were established: (i) no treatment (only collagen scaffold); (ii) collagen scaffold with BMP-6 plasmid premixed with micro-bubbles injection (BMP-6); or (iii) collagen scaffold with empty plasmid vector premixed with microbubbles injection. Treatment of all groups was followed by ultrasonic application. This ultrasound-mediated gene therapy led to transient expression and secretion of BMP-6 localized to the fracture site. Ultrasound-mediated BMP-6 gene delivery resulted in full radiographic and functional fracture healing in all animals 6 weeks after treatment, whereas nonunion remained in control animals. To establish the practicality of this treatment technique for human usage, many structural, biomechanical, and safety criteria were assessed. The current research examined the short-term effects of ultrasound-based BMP gene delivery. A long-term research is needed to adequately examine the method's efficiency in bone healing as compared to controls. All of the animals in this study received collagen scaffold implants, which have previously been proven to exhibit osteoconductive qualities. Despite the scaffold, we were able to demonstrate considerably greater union rate, bone growth, and biomechanical qualities in

BMP-6 and ultrasound-treated animals in a short period of time. Additionally, the presented data suggests that the combination therapy of a collagen scaffold, BMP-6 plasmid, microbubbles and ultrasound is superior to the control groups and comparable to autograft-treated animals.[55]

Bez et al. claim, this method could be used for a range of orthopedic purposes in the setting of bone fractures. It is minimally invasive, does not involve ex vivo stem cell manipulation or bone harvesting from the patient, and eliminates the use of pricey growth hormones. Since no other effective means of stimulating bone regeneration in locations of severe bone loss has yet been discovered, ultrasound-mediated gene therapy may be a viable approach that might provide a good answer to this unmet clinical need.[55]

Exosomes have many of the outstanding characteristics of an ideal delivery system, such as their structure of a bilipid membrane wrapping an aqueous core, the intrinsic ability to target tissues, biocompatibility, and minimal or no inherent toxicity issues, and their ability to pass biological barriers such as the blood-brain barrier.[58,59] Several strategies were explored by the researcher to overcome the limited production of exosomes and increase their therapeutic potential. The first strategy is to optimize their cargo profile by altering isolated exosomes by overexpressing molecules associated to exosome therapeutic actions (miRNA, protein, etc.). Optimizing the cargo profile of exosomes through parent cell therapy is a promising strategy for improving exosome therapeutic benefits; nevertheless, additional improvements are required.[60,61] The second technique for improving exosome therapeutic use is to optimize their tissue targeting and biodistribution. Exosome ligand alteration increases their accumulation in target organs, as demonstrated by multiple investigations; yet, the amount of exosomes in the target organ remains restricted.[62]

Prior to the widespread introduction and application of novel technologies to promote bone healing employing modified MSCs and their byproducts, important effectiveness and regulatory problems, as well as cost difficulties, must be addressed. Indeed, some of the difficulties with the usage of unmodified MSCs and their byproducts have just lately been raised and examined.[63,64] Extensive in vitro and in vivo preclinical research in small and big animals is required. There are significant difficulties about which cell types or lineages are most suited for selection, as well as the techniques by which these cells will be validated, collected, separated, and increased, as well as their purity, potency, stability, and sterility. Storage methods for simple end-user access, as well as user-friendly delivery mechanisms and technologies, must be developed. Importantly, the cells must be demonstrated to be functional, safe, and cost effective. For unambiguous indications in specific populations, this should be

performed through well-designed prospective, randomized trials with rigorous recording and oversight. Finally, cost-effective analyses and value-based health care decision making will be critical factors of whether these new technologies make it to the clinic or stay a scholarly curiosity.

The focus of future research should be on obtaining more solid evidence confirming MSCs' therapeutical potential in aiding quicker and more successful long bone nonunion fracture healing. To understand the mechanics of MSC-mediated bone repair, the immunomodulatory potential of MSCs must be thoroughly explored. It would be beneficial to explore the unique immunomodulatory profile of MSCs derived from diverse sources in order to determine which tissue-derived MSCs are most suited for nonunion therapy.

It is also necessary to be able to tailor patient-specific treatment based on their individual needs and demographics; and to determine the appropriate combination of scaffold material, growth factor(s), cell-based/cell-free approach, and mechanical environment supported by surgical intervention in order to achieve successful intervention of such complex injury as nonunion fractures.

CONCLUSION

Nonunion is caused mostly by unresolved inflammation and inadequate osteogenesis. Despite breakthroughs in surgical intervention, strong anti-inflammation therapy and osteogenic differentiation activation remain essential treatments for nonunion. To summarize, bone restoration is a mix of biological and biomechanical treatment techniques. While there are several biomechanical therapy options for nonunion and bone healing problems, biological resources appear to be few. Evidence suggests that a combination of variables that promote neo-osteogenesis and neovascularization can heal hard and soft tissue deficiencies. The ideal solution indicates to be a combination of a biomaterial scaffold, cell biology approaches, a growth factor, an optimized mechanical environment (diamond concept), and appropriate surgical repair.

Research ethics and patient consent

Not applicable.

Availability of data and material

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Conceptualization—L.D., and R.Z.; literature search and data curation—V.S., and M.C.; writing – original draft preparation—V.S.; writing – review and editing—V.S, and L.D.; visualization—R.Z.; supervision—L.D. and R.Z.; funding acquisition—L.D. All authors have read and agreed to the published version of the manuscript.

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Table 1. Overview of novel cell-based and cell-free therapeutic strategies for nonunion fractures discussed in this review.

Trial Type	Human/Animal Model	Type of Cells/Product	Administration	Reference
clinical trial	human	UC-MSCs	combined w/HA and BMP-2	Dilogo IH et al.[17]
clinical trial	human	BM-MSCs	combined w/cadaveric spongy bone matrices	Prat S et al.[22]
clinical trial	human	BM-MSCs	intraosseous injection	Zhang H et al.[23]
clinical trial	human	BM-MSCs	combined w/CE-marked bioceramic	Gomez-Barrena E et al.[25]
pre-clinical trial	rabbit	BM-MSCs	combined w/17-estradiol	Zamani Mazdeh D et al.[27]
pre-clinical trial	rat	BM-MSCs	combined w/fibrin glue and PTH	Osagie-Clouard L et al.[34]
pre-clinical trial	rat	BM-MSCs	combined w/HMGB1-gelatin sponge scaffolds	Xue D et al.[36]
pre-clinical trial	rabbit	BM-MSCs	combined w/collagen with OC and/or OPN	Carvalho MS et al.[42]
pre-clinical trial	rat	BM-MSC-Exos	injection	Zhang L et al.[48]
pre-clinical trial	mouse	B-EVs	injection	Hu H et al.[49]
pre-clinical trial	mini-pig	BMP-6 plasmid targeting endogenous MSCs	combined w/collagen scaffold	Bez M et al.[55]

Figure 1. Cells involved in process of bone fracture healing.

Figure 2. A spacer ensured a fixed off-set of fracture, creating a gap. The fixator was attached and the osteotomy gap for all the rats.

Figure 3. Exosomes comprised of a lipid bilayer containing various proteins, RNAs, DNAs, and bioactive lipids.

Figure 4. Principles of extracellular vesicle isolation, techniques of isolation, methods of preservation, lyophilization, and potential applications. The fundamental mechanism of ultracentrifugation. The basic method for combining ultracentrifugation and ultrafiltration to boost exosome therapeutic applications.