2021

Current and Future Perspectives in the Treatment of Fracture Nonunions

Zebadiah Nelson

Title: Current and Future Perspectives in the Treatment of Fracture Nonunions

Author: Zebadiah Nelson

Affiliations:

South Dakota State University Department of Biology & Microbiology.

Zebadiah.nelson@jacks.sdstate.edu

Table of Contents

Abstract:

Fracture nonunions comprise one of the serious clinical complications in orthopedics. Fracture nonunions result when the time to union is greater than 6 to 9 months. It is estimated that approximately 5% to 10% of all fractures progress to fracture nonunion due to a host of complicating patient variables and risk factors. New scoring systems have emerged which help in directing the treatment strategies. Fracture nonunion management should be directed toward maintaining the diamond concept which introduces osteogenic cells lines, an osteoconductive scaffold for those cells to grow, as well as the necessary growth factors, and a stable mechanical microenvironment. This paper will focus on some of the promising research that has been done in the field of orthopedics for the treatment of fracture nonunion, particularly the use of mesenchymal stem cells, bone marrow aspirates, and biophysical manipulations such as the use of electromagnetic fields, and their effects on cell cultures, animal models, and clinical studies.

One Sentence Summary:

This review explores some of the latest avenues of research which seek to improve the treatment process for fracture nonunion by enhancing the rate of bone growth.

Introduction

After a fracture, bone tissue has the remarkable ability to repair and remodel itself in an expedient and well-orchestrated manner. This healing process relies on the coordination and dissemination of many growth signals and inflammatory signals throughout the injured bone tissue which work together to form a structurally sound bone to support the weight of the human body. The molecular signaling events that occur during bone healing present a unique case study in systems biology, and as such, there are many molecular targets which can be exploited to speed up or resume the process. When this delicate signaling process becomes interrupted through lack of stability, lack of healing time, or molecular inhibition at the level of growth factors, it can result in a fracture nonunion or delayed union. One of the first studies which examined the genetic basis for nonunion was done in 2011. This study sought to understand whether there were differences in genes encoding growth factors. It was found that there were single nucleotide polymorphisms (SNPs) in bone morphogenetic proteins (BMP-7, BMP-2); as well as Smad6, and Noggin. Smad6 and Noggin are both inhibitors of the BMPs and in this study it was found retrospectively that patients who developed nonunion had SNPs in these genes which interfered with healing (*12*). Although the definition of a fracture nonunion in the clinical setting can be arbitrary and much depends on the specific patient criteria, as noted by Zura et al. such as age, prescribed drugs, tobacco use, mobility, and some genetic factors previously noted (*13*).

formally, a nonunion can be defined as a complete halt or delay to the healing process in bone tissue. The FDA defines fracture non-union as a fracture that is 9 months old which has not made any significant healing progress within 3 months. A subset of this pathology is delayed

union which is defined as a lack of bony union within a 6 month window of time after the initial injury, the distinguishing factor is that a delayed union shows some evidence of the healing process, whereas a nonunion does not. Both pathologies are managed using similar procedures (*14*).

Much of the diagnostic measures in fracture nonunions are dependent upon current epidemiological estimates, which put the annual incidence of fracture nonunions in the United States at 100,000 per year. This number represents only about 5% to 10% of all fractures that occur (*15*). That may even be a low estimate of the incidence which depends entirely on the diagnostic parameters used by physicians and the bones with which it occurs. Observational Cohort studies reveal that the fracture nonunion risk was varied with the bone in question and the severity of the initial fracture. It is also reported that men are more prone to nonunion than women.

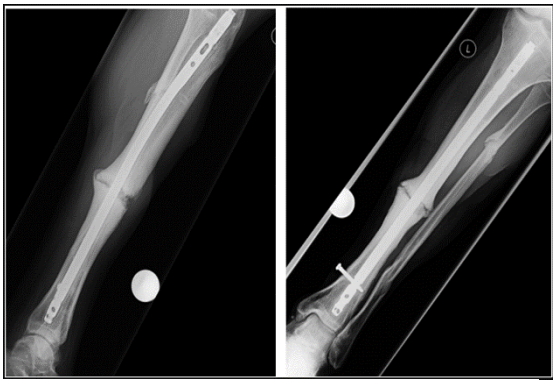

Figure 1. This radiograph shows a hypertrophic nonunion of the tibia with anteroposterior and lateral view. Of note is the characteristic bony callus formation around the fracture gap sometimes referred to as elephant's foot (*5*).

When a nonunion develops, there are structural classifications for the character of the fracture site. One of the first incidences of an effort to classify the structural elements of fracture nonunion site was done by Weber and Chech in 1976 (*16*). This classification is used to describe the structure of the nonunion site through radiographic appearance only. A hypertrophic nonunion indicates that a callus has developed to stabilize the fracture site (figure 1), these calluses develop in the shape known

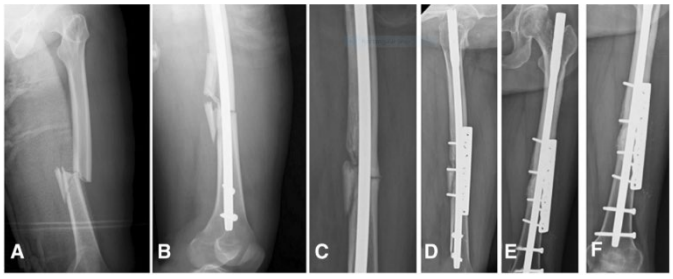

Figure 2. Atrophic nonunion of femoral shaft fracture following a vehicle accident. Patient is a 38-year-old female. Initial treatment was performed with intramedullary nail fixation technique with antirotational plating (D-F). Union achieved after 5 months (F) (*6*).

as an elephant's foot. this is typically due to a lack of initial stability from the point of injury with sufficient vascularization present which contributes to the elephant's foot formation. Hypertrophic nonunions typically are a result of a mechanical failure from inadequate fixation or premature patient mobility with adequate fixation (*17*). This excess mobility results in a lack of healing despite all the necessary biological components and vascularization being present at the site of tissue injury (*18*). Typically, hypertrophic nonunions maintain adequate vascularization for healing and good clinical outcome is usually achieved with fixation using nail, plate, or intramedullary approaches (*19*). An atrophic nonunion (figure 2) shows no callus development which can indicate that there is likely a pathology at the cellular level which can be explained by the risk factors and at the level of growth factors which is interferes with the mechanisms of normal osteogenesis. Lack of blood supply to the fracture site along with strain forces are also thought to be factors which can lead to atrophic nonunion(*18*). Oligotrophic nonunion shares similar characteristics with both and is often considered an intermediate form. In the case of these 3 types of nonunion, these distinguishing characteristics are based on appearance in radiographs (*20*) (*21*). Fracture nonunions place a considerable burden on healthcare infrastructure and the treatment costs increase substantially. The pain, prolonged hospital stays, potential for infection, all result in a higher cost burden and risk to the patient. Due to the substantial pain during recovery, this can increase the dependency on prescription opioids, which was reported as high as 78% in some patient studies (*22*). Because of the complex nature of fracture nonunions and the diverse variability that is present in a typical clinical presentation, fracture nonunion of any category presents a unique challenge to orthopedic surgeons. The need for novel inexpensive treatment options is necessitated by the diversity of this pathology and burden placed on the patient and the healthcare system. This paper will explore the varying

routes of research that exist to develop novel treatments and some of the future perspectives within the field.

Emerging Classification Systems

As previously noted, there exists a large amount of variability in the way that each fracture nonunion presents, as such, no two are alike, each patient must have a unique treatment approach that is tailored to their medical history and fracture pathology. As with any disease state, it is imperative to be able to measure and classify it so that a proper treatment regimen can be directed against it. This concept of variability within orthopedic medicine makes research of the fracture healing process in nonunions challenging to measure. Sources of variability within orthopedic research exists in the following numerical and qualitative categories: age, sex, developmental state, bone type, drug interactions, fracture incidence, patient weight, and prior fractures (*15*). To date, this has remained one of the greatest challenges to overcome in proving which treatment is the most effective for patients at varying severity levels. Additionally, due to this variability there has been a need to be able to algorithmically classify the character of fracture nonunion so that the correct treatment approach can be tailored to the patient based on their past medical history and current healing progress. Within the past decade, this has ushered in a new scoring system to sort fracture nonunions based on a host of categories. These categories fall under 4 themes: The growth factors present, cellular microenvironment, bone matrix (scaffold), as well as the osteogenic cell lines present. These 4 categories together

Figure 3. The "diamond concept" of fracture healing management proposed by Giannoudis et al. places equal importance on the mechanics, osteogenic cells, osteoconductive scaffold, and the growth factors present at the fracture site.(*8*)

form what is known as the "Diamond Concept" shown in figure 3 (*23*) (*8*). Previous classifications like the Weber and Chech classification system relied solely on radiographs for the determination of the severity of nonunions. Calori et al proposed a new Nonunion Scoring System (NUSS) (shown in figure 4). New scoring systems like NUSS take it a step further by incorporating a wholistic approach (combining risk factors with diamond concept), as well as stability of the fracture, fracture gap, bone alignment, and soft tissue status. In this scoring system, a number is assigned to a nonunion characteristic based on its severity, higher severity traits receive a higher number and lower severity traits receive a lower score. Patients who can be classified with a lower overall score typically require a less complex intervention than those that receive a higher score which may require multiple different treatment approaches based on how their score reflects the overall "Diamond Concept" of management (*23*).

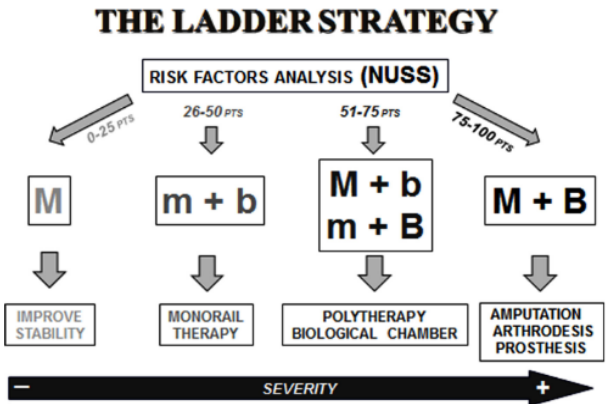

Figure 4. The ladder strategy devised by Calori et al shows the implementation of the NUSS scoring system which indicates the severity of a fracture nonunion. This system helps to direct the treatment approach and eliminates the guess work. M is a major mechanical issue, m is a minor mechanical problem, B is a major biological issue and b is a minor biological issue. (*4*)

A retrospective clinical study by Calori et al sought to validate the NUSS. This study included 300 patients over the age of 18 with nonunion of a long bone fracture. 198 patients were male, 102 patients were female. Tibial shaft nonunion was found in 52% of patients, femoral shaft nonunion in 17%, 11% in the humerus, 6% in the radius, 4% in the ulna. The patients were divided into four total groups based on their NUSS scoring; patients in group 4 (shown in figure 4) (M+B) were not included in the study because their major defects prohibited the study of the classification system. Clinical outcome was assessed both through radiographs and through

absence of severe pain and range of motion testing in comparison to contralateral unaffected limb. This study demonstrated that statistically significant rates of union can be achieved if fracture nonunions are classified in such a way that every patient characteristic and wound trait is considered in order to direct the appropriate treatment course. The authors of this study recommend that NUSS be implemented in every orthopedic center to maximize positive clinical outcome for nonunion patients. The latest work that has been done in classification of fracture nonunions is relevant to the overall discussion because classification of severity largely determines which treatment modality best fits the patient (*4*).

Gold Standard for Treatment of Fracture Nonunion

In order to get a better picture of the frontiers of research in any treatment or therapeutic, it is important to first understand the standard of treatment currently in use as a baseline comparison. As such, the use of autologous bone grafts is the gold standard in orthopedic medicine for the treatment of atrophic long bone fracture nonunions. This treatment most commonly employs the use of host iliac crest cancellous bone as an osteogenic scaffold to facilitate the process of osteogenesis. A careful examination of the patient outcome for autologous bone grafting is warranted as well as the reasons for approaching the treatment of nonunions from a different approach apart from the gold standard of care. Some would argue that the search for alternative treatment options is largely driven by an industry reliant on producing new forms of bioimplants and adjuvants. Conversely, as both general and orthopedic surgery moves toward ever increasingly minimally invasive techniques, particularly in orthopedics, this can be a benefit as it improves patient outcomes and postoperative pain. Additionally, the limiting factor for autologous bone grafts is the quantity of cancellous bone that can be harvested from the donor site. Iliac crest autograft comes with its own risks as well, there is a significant

level of donor site morbidity and complication rates (*24*) (*25*). Despite the increasing development of alternatives to the autograft, many advantages persist. The primary advantage to autologous bone grafting is that the tissue maintains histocompatibility since it is collected from the patient's own body. Secondly, the autologous graft is osteogenic, osteoinductive, and osteoconductive. The most frequently used autologous graft is from cancellous bone found in the iliac crest. Cancellous bone has trabecular bone structure which is a porous material lined with osteoblasts attached to their porous scaffold which is made of mineral hydroxy appetite and collagen fibers. Cortical bone grafts are less advantageous because there is less porous surface area for osteoblasts to reside on and are often less osteoconductive (*26*).

A retrospective cohort study by Flierl et al. sought to understand which bone grafting technique provided the best option for reducing the time to union in long bone fractures. Patient inclusion criteria included patients with ages ranging from 18 to 85 years old, admitted to the hospital from January 1st, 1998 to December 31st, 2010. This study found that autologous or autograft was the most effective treatment for reducing time to union when compared to allograft and injection of growth factors like rhBMP-2 (*25*).

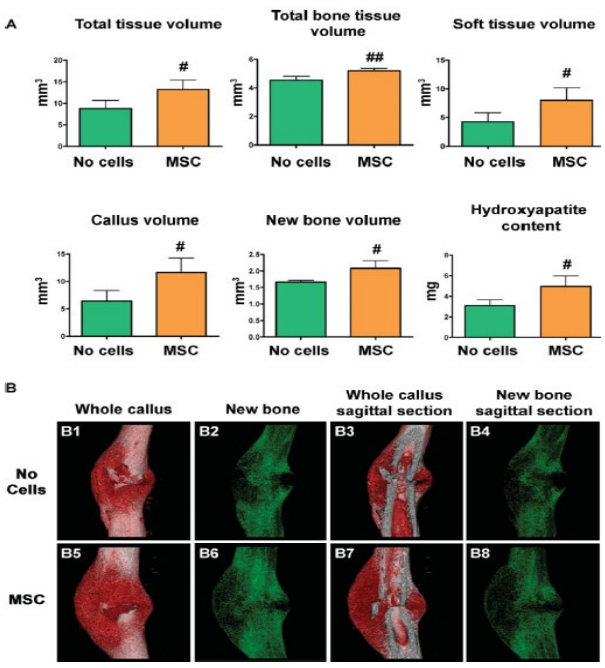

Figure 5. Micro-CT scans that were done 14 days after initial fracture reveal that fracture callus size, density, volume increase with the presence of MSCs. Also shown is the increase of hydroxy appetite HA (bone mineral) content (mg) with the presence of MSCs. (*2*)

Emerging Cell-Therapies, Mesenchymal Stem Cells, and Growth Factors

Mesenchymal stem cells are the progenitors to mature osteoblasts and are

crucial during the bone healing process. They are found in many tissues, of note, bone marrow. Also crucial to the fracture healing process is the cytokine and growth factor environment surrounding the fracture. The two bone morphogenetic proteins mentioned earlier, which are members of the TGF-β superfamily, BMP-2 and BMP-7 are important in the process of bone repair/growth and MSCs are defined as being cells which are plastic-adherent when cultured, they express the following surface molecules: CD105, CD73, and CD90; they have multipotent potential to differentiate into chondrocytes, adipocytes, and osteocytes.(*27*) By harnessing their regenerative power in the case of a fracture nonunion, the healing process can be sped up by as much as half the time. placing them directly into the wound site. MSCs can be induced to differentiate into osteoblasts in vitro when exposed to the necessary nutrient mediums and growth factors that would be found in the wound site of a fracture nonunion(*27*).

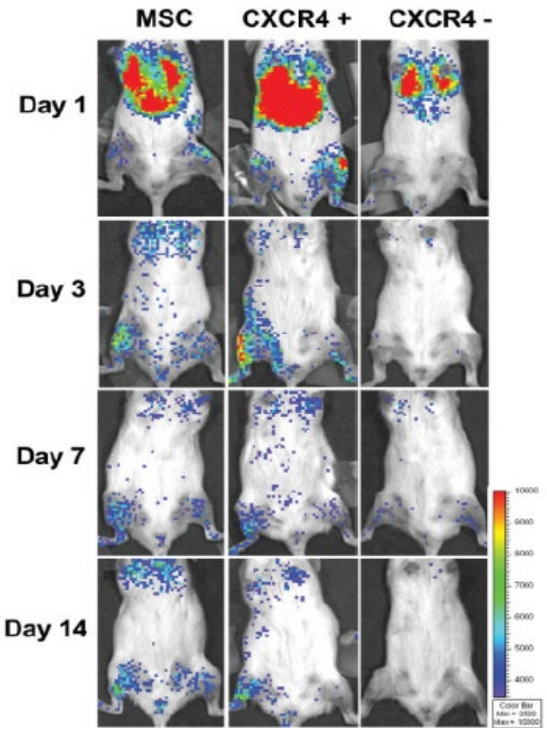

Figure 6. BLI showing the CXCR4 dependent migration of MSCs to the fracture site in rats, BLI shown is done on a time course of days 1, 3, 7, and 14. Initial localization is in the lung fields of the rats, unsorted MSC are used as a control (MSC), little to no migration is seen in rats with CXCR4(-) (*2*).

Transplanted MSCs have shown success in animal models, particularly with rats. One such study sought to understand the way in which MSCs were able to migrate to the fracture site and to determine their effect on the callus formation during healing. It was shown through MSC expressing luciferase, that MSC migration to the callus site is largely dependent on the chemokine receptor CXCR4, and that MSCs improved the mechanical properties of the fracture site by enhancing the thickness of the

cartilage and eventual bone callus around the fracture site (Figure 5). The authors found that approximately 30% of the MSCs cultured from 4 mice were found to express CXCR4 using immunoselection. The MSC cultures were grouped as MSC-CXCR4(+) and MSC-CXCR4(-). Based on adenoviral vector *Firefly luciferase* under transcriptional control of a cytomegalovirus promoter, bioluminescence imaging (BLI) was performed on a time course from 1 to 14 days following a tibial fracture in the rats shown in figure 6. This data validates the migratory behavior of MSCs toward the site of tissue injury. The authors also note findings of increased fracture callus volume, density of hydroxy appetite, and total tissue volume which increases the stability of the fracture site and aids in the healing process, as shown in figure 3. Further findings of this study reveal that MSCs will lodge themselves into the endosteum that surrounds the fracture callus. BMP-2 expression was found to be a prominent feature of the MSCs once they migrated to the fracture site through *BMP-2-Lac Z* which is an essential growth factor for controlling the fracture repair process, additionally no BMP expression was noted in specific regions of the fracture callus which the authors hypothesize indicates an underlying regulatory mechanism that would require further investigation. Taken together, these results indicate the strong potential for MSCs to be utilized as a therapy for patients with difficult fracture nonunions. This study provides foundational evidence for the use of cell-based therapies in humans to modulate the molecular and inflammatory microenvironment to promote osteogenesis (*2*).

The importance of BMPs in fracture healing and bone growth as a whole cannot be overstated. BMPs are members of the TGF-β superfamily, they bind to two separate classes of receptors which form heterodimers, ultimately leading to the phosphorylation of Smad proteins, Smad then behaves as a transcription factor for proosteogenic target genes. Runx2 being of

importance, which regulates the osteogenesis process (*28*). It has been shown in murine models that deletion of the BMP genes leads to loss of proper skeletal development. When BMP2 and BMP4 were both knocked out osteogenesis was halted completely (*29*). This data is consistent with the findings of Dimitriou with regard to reduced function of BMP proteins through SNPs in the human genome (*12*).

A promising type of cell-based therapy which harnesses the power of MSCs takes bone marrow aspirate concentrate (BMAC) or isolated and expanded MSCs from bone marrow and injects them directly into the fracture site. This utilizes the patient's own bone marrow which is centrifuged down to yield a concentrate (Buffy Coat) of stem cells which are then injected directly into the fracture site. This technique is typically used in conjunction with an osteoconductive scaffold for cells to adhere because although MSCs are osteogenic they require a conducive environment to adhere and proliferate (osteoconductive scaffolds) (*10*).

A level III clinical study of note was carried out by Hernigou et al, this study had 60 patients with aseptic atrophic nonunion of the tibia. Bone marrow was aspirated from the iliac crest and centrifuged down to a concentrate; each patient received 20 cc of concentrate which was injected directly into the fracture site. This research team observed that there was a positive correlation between the concentration in CFUs of fibroblasts and the total volume of the bone callus (*10*). Of the sixty patients, 7 of these patients did not achieve bony union. The 6 patients who failed to achieve union had an average cell count of 634±187 progenitor cells/cm^3 (p=0.001 significantly lower), than the 53 that achieved which union had an average cell count of 2835±1160 progenitor cells/cm^3. One important aspect of this study is that

Figure 7. Comparison between successful unions and failures of union to the concentration in cells/cm^3 at the graft site (*10*).

it demonstrates that there is a positive correlation between the concentration of cells per cubic centimeter at the fracture site and the rate of union. The authors note that there is no associated negative inflammatory response which could interfere with healing. A limitation to this study is that the MSCs were not isolated as they were in a mix of other progenitor cells in the aspirate, including mononuclear cells, which contributed to the milieu of cytokines. additionally, there was no placebo control group included (*10*).

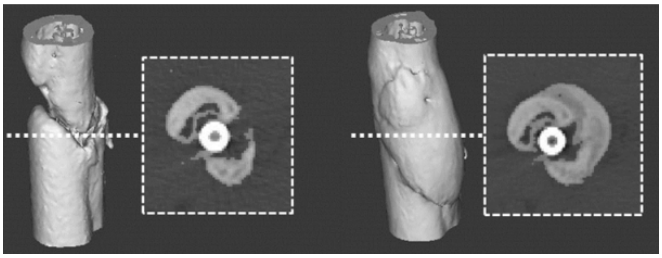

Figure 8. Computed tomography images performed on a 45 year old patient prior to BMAC grafting and 6 months after (right) show significant callus formation on the femoral shaft around the fracture site (*7*).

A study done by Hernigou et al. sought to examine the clinical outcome of injecting 20 cm^3 of bone marrow aspirate which was concentrated and harvested from the patient's iliac crest. There were 60 patients who suffered from atrophic nonunion of the tibia. Union was achieved in 53 out of 60 patients. In the fractures that failed to unite, the concentration ($p=0.001$) and number of cells ($p<0.01$) were significantly less than the fractures that did achieve union. The authors note a positive correlation in their findings between the amount of bony callus formation and the number and concentration of fibroblast CFUs injected into the graft. Also noted in this study is that directly injecting the BMAC in vitro as opposed to

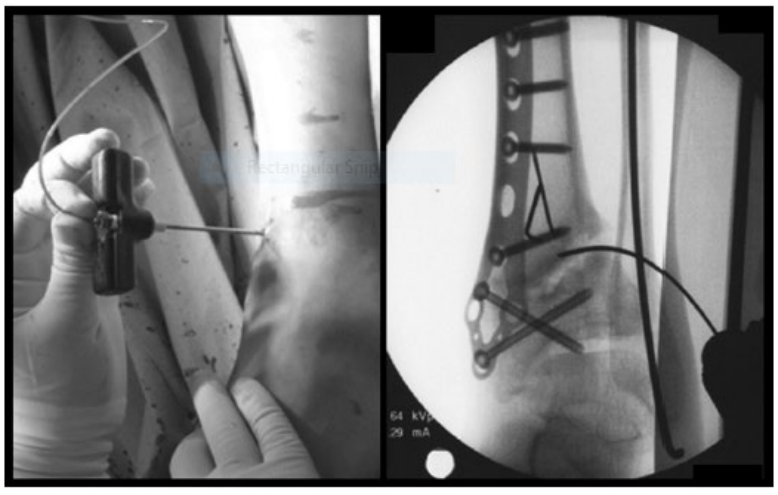

Figure 9. Procedures performed by Sugaya et al injected the bone marrow aspirate concentrate percutaneously directly into the fracture gap with the bone marrow harvest needle over the course of 1 minute (*7*).

expansion ex vitro minimized cellular aging, and reduced cell viability which are some of the complicating factors with expansion of stem cells prior to injection (*30*).

Sugaya and colleagues performed a retrospective cohort study which analyzed patient outcomes with atrophic fracture nonunion treated with autologous BMAC injections (Figure 9) on 17 patients. This study is unique in that it also measured the pain in patients using visual analog scale (VAS) on patients following BMAC grafting. Statistically significant reduction in VAS was noted ($P<0.001$) in patients who achieved union with BMAC. Persistent and severe fracture site pain is one of the symptoms of nonunion and the authors of this study believe that achieving stability in the fracture site sooner was responsible for the alleviation of the pain. Of the 17 patients who underwent treatment with BMAC 13 were observed to have radiographic union after 12 months with no patients progressing to complications (*7*). The limitations to this study were that the patient sample size was small, and it was retrospective. RCT trials need to be conducted in multiple fracture nonunion sites to validate the results (*7*).

Along with injecting bone marrow aspirate concentrate directly into the fracture site, studies have examined the patient outcomes when BMAC is combined with adjuvants such as recombinant human bone morphogenic protein 2 (rhBMP-2) and demineralized bone matrix (DBM) as an osteoconductive scaffold. One clinical study sought to demonstrate the use of (rhBMP-2) combined with demineralized bone matrix (DBM) to treat fracture nonunions with a fracture gap > 5 cm (*31*). Prior to this study, there was little evidence demonstrating that a BMAC treatment course could be used to treat fracture gaps greater than 5 cm. The authors of this study call the mixture of BMAC with DBM or rhBMP-2 the modified Hernigou technique. In 49 patients with tibial fracture nonunion, they examined the radiologic healing time for fracture gaps greater or less than 5 cm. They concluded that there was no significant difference

in the time to union (p=0.81) between patients with varying fracture gap size. 79.4% of the patients in this study achieved radiographic union in an average 4.7 months. This study provides a good basis for the application of BMAC with rhBMP-2 or DBM as an osteoinductive scaffold (*31*).

Noninvasive Treatments (biophysical stimulation)

It has been known for more than a century that bone responds to physical stimuli with growth. Julius Wolff, a German surgeon, first proposed in 1892 that bone will rearrange its architecture to support the loads applied to it (*32*). Among other foundational works in the understanding of bone was the discovery of the piezoelectric nature of bone. The findings of Yasuda, et al in 1957 reveal that dried bone crystal exhibits piezoelectric effect one tenth that of quartz crystal when a strain tension was applied (*33*). Piezoelectric effect exists when flexion or torsion is applied to a crystal structure. This torsion causes electrons to move from one end of the crystal to the other. These concepts were further expanded by Bassett et al. in the 1960s whose group described that wet bone was also able to produce an electrical potential when mechanical stress was applied to it (*34*). This provided a conceptual framework for Wolff's law. Over the past 40 years, these foundational ideas have been explored in the context of inducing a growth response with the application of an electric field or through ultrasonic waves in the form of low-intensity pulsed ultrasound. Biophysical stimulation involves applying direct energy therapeutics to an injured tissue in order to enhance some cellular function to bring about a therapeutic, notably, enhanced mineralization and proliferation of osteoblasts (*35*). Numerous studies have shown that pulsed electromagnetic fields seem to enhance the proliferation rate of osteogenic cell lines, and also to induce differentiation to osteogenic cell line, and osteogenic mineralization (*36*). Despite promising results in animal and tissue culture models, evidence

pointing to a mechanism remains elusive to researchers in this field. In the clinical setting, many experiments have shown results of decreased healing time, however, there is a lack of consistency in the measurement parameters for clinical experimentation, such as frequency of EMF and intensity (*37*).

Proposed Mechanisms of PEMF stimulation

In a broad view, these studies can be categorized by their objectives and their methods. Research has been focused on three primary areas of experimentation: induction of cell differentiation, proliferation, and mineralization all to form a better understanding of how PEMF can be used for fracture repair. Fibroblast stem cells are used to determine if that cell line can be induced into the osteoblast fate by electromagnetic stimulation, if bone cell precursors can be induced into osteoblasts more efficiently, or if preexisting osteoblasts can be induced to create more bone matrix than normal to increase healing as shown in figure 10 (*3*).

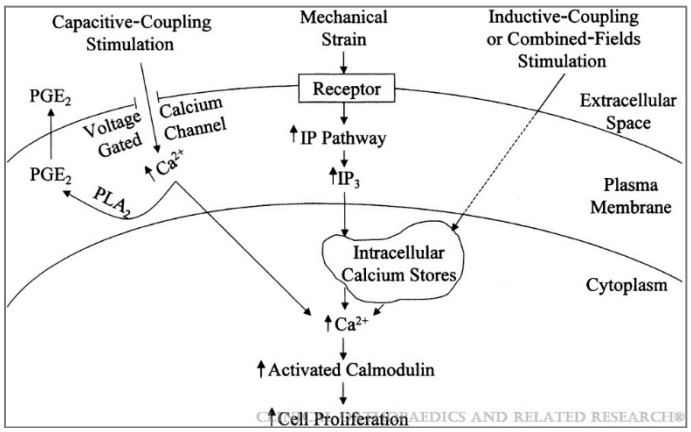

Figure 10. This schematic shows the various proposed signal transduction pathways that can be induced with treatment of electromagnetic fields or a mechanical strain. All pathways end with increased activation of calmodulin which leads to increased cell proliferation (*3*)

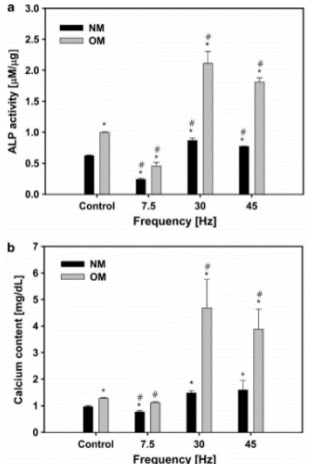

Figure 11. Alkaline phosphatase production and calcium content both measured and compared to frequency of PEMF exposure at varying days. Increased production of both ALP and the concentration of Calcium can be observed for day 10 and 20 at 40 and 45 Hz. NM=normal growth medium, OM=osteogenic growth medium (*11*).

Differentiation

A goal of this research has been to determine if differentiation of mesenchymal stem cells into osteogenic

cell fate has can be reliably achieved. Significant work has been done to determine the molecular events associated with application of PEMF through a pulse generator and the resultant cellular differentiation that occurs with the application. Recently, Martini et al. found that bone BMP-2 can be upregulated with the application of PEMF at 1.5 mT intensity in human MSCs. It was further found in this study that p38 (mitogen activated protein kinase, helps with signaling differentiation and apoptosis) activity was upregulated in the presence of PEMF which is important for production of osteoblasts (*38*). Typically, though, alkaline phosphatase or ALPase is used a marker for differentiation from osteoblast to osteocyte by the deposition of bone matrix around the developing cells. Similarly, work done by Kang et al also suggests using gene expression markers such as ALP, and RunX2 as important osteogenic markers for quantifying the differentiation to osteogenic cell line. The ALP concentration and Calcium concentration were synergistic when compared with the production of RunX2 (*11*). A unique aspect of this study is that they suggest that there appears to be an effective EMF frequency threshold for the effective stimulation of ALP and Calcium concentration. 7.5 Hz seems to inhibit the osteogenic proliferation effects in the adipose derived stem cells being studied. This result was occurring regardless of the growth medium present. They propose in their work that osteogenic differentiation can be controlled by manipulating the electromagnetic frequency value. This was a significant finding because the methods employed by this research team showed that under a unified set of experimental parameters and environmental variables, they were able to modulate the growth rate of these adipose derived stem cells into osteogenic cell lines. The authors of this work speculate that there appears to be some underlying mechanism for induction of osteogenesis that is involved in the resonance frequency for ions such as calcium. This

movement would be in the form of efflux from the cell. Triggering a strong efflux of calcium ion from the cell may aid in the production of extracellular matrix and ultimately bone tissue (*11*).

Proliferation

Many studies discuss the cell proliferation activity that can be measured in fibroblast murine cells under the stimulation of PEMF. One such study sought to understand the role of the mTOR pathway in light of the PEMF exposures. The mTOR pathway has been implicated as an important growth pathway in the differentiation and proliferation of osteoblasts. Rapamycin was used in this study as a method of suppression of differentiation and proliferation (*1*). When administered in vivo, it can be seen that rapamycin inhibits the mTOR expression levels despite PEMF exposure. This indicates that activation of the mTOR pathway is essential for proliferation and differentiation of osteoblast cells. Inhibition of the mTOR pathway resulted in minimal osteoblast differentiation and proliferation. It was also found that an intensity of 0.4 mT yielded strong results with mTOR phosphorylation as opposed to the 0.1 mT intensity which was also evaluated. The effects of the PEMF on the growth pathway seemed to be transient, only lasting ten minutes. This led the research team to investigate whether intermittent phases of PEMF was effective as a transient application. The results of this testing showed that the intermittent PEMF application sustained the prolonged phosphorylation of the mTOR pathway. BrdU was used to measure the effectiveness of continuous intermittent PEMF exposure. It was found that BrdU uptake was consistently higher than the continuous PEMF exposure indicating more cell divisions. ALPase activity in cells was minimal with PEMF exposure in this

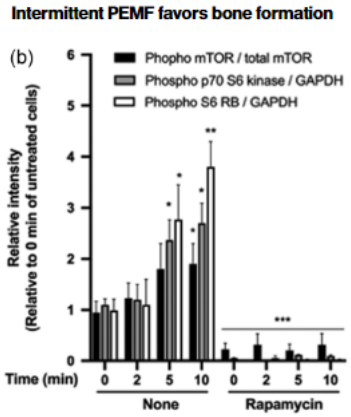

Figure 12. The effects of rapamycin on the activation of the mTOR pathway. Activation of the following pathways are depicted with varying time course for PEMF exposure. The effects 10 minutes (*1*).

experiment when compared to the control indicating that PEMF has more effect on osteoblast proliferation and not the differentiation to osteocyte fate (*1*). This study can serve as a model for how clinical studies should be approached. Instead of application windows lasting days and weeks, emphasis should be placed on an intermittent stimulation window for greater effect if it is to be used a treatment option for fracture nonunion (*1*).

One study analyzed varying age demographics for human bone-marrow stem cell samples from female patients aged 21-30 years of age, and 31-65 years of age. It was found that PEMF exposure using a commercially available therapeutic device increased the cell count in the 21-30 age demographic. It was shown in this study that PEMF exposure activated extracellular regulated kinases (ERKs) through phosphorylation with stimulation at just 15 min per day. This study also measured the ALP levels, type I collagen (COL1A1) through qRT-PCR. These proteins are osteoblastic and mineralization markers that play an important role in the development of an osteoblast. They found that Alp and COL1A1 expression were significantly increased but there was no increase in the osteocalcin (OC) expression with PEMF. A key finding from this study is that PEMF increased mineralization of the bone-marrow stem cells during the mineralization but not during differentiation. Of note, this study finds that there appears to be a factor related to the age of the individual when using hBMSC samples from female participants. The PEMF exposure was able to induce proliferation of pre-osteoblast cells up to the age of 30 but the effects on cell cycle regulators and matrix proteins were minimal as age of donors increased (*9*). These findings together, suggest that PEMF can stimulate differentiation, mineralization, and activation of TGF-β signaling in the bone marrow stem cells of females. It is thought that Eddy currents (loops of electrical current) are generated around the cell initially which can enhance the phosphorylation reaction of ERK which enhances BMSC

proliferation. After the BMSCs reach confluence (all surface area of plate has been covered by a monolayer of cells) and the growth medium is changed to that of differentiation, it is proposed that these Eddy currents cause decrease in fibrillin 2, which then increases TGF-β2 availability. As depicted by the researchers in figure 13 (*9*).

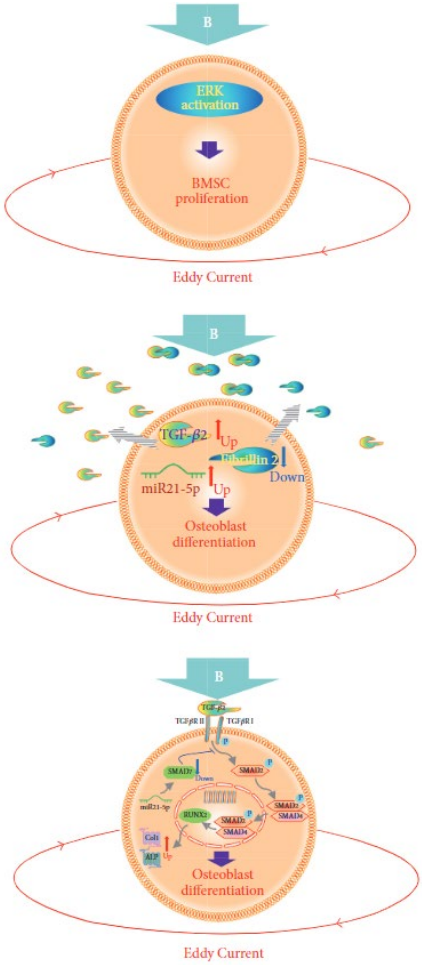

Figure 13. Proposed mechanism of action by Selvamurugan et al showing that (B) magnetic field acts on the hBMSC to induce eddy currents which surround it and increase the effects of ERK which leads to increased proliferation of hBMSC. TGF-β2, miR21-5p, are upregulated, while fibrillin 2 is down regulated. All of which leads to the autocrine signaling of TGF-β2 and increased activity of SMAD2/4 with an end result of increased alkaline phosphatase activity and COL1(*9*)

The possible activity of electromagnetic fields causing Eddy currents is disputed. Evidence put forth by Kavand et al suggests that a more probable mechanism of activation of these pathways lies in tuning the PEMF parameters such that they match the ion cyclotron resonance frequencies of calcium ion (*39*). This is an important step in understanding how exactly electromagnetic fields may interact with a biological system. Because the extracellular fluid surrounding cells has little medium with which to carry electrons in an induced current, the mechanism must be through the action on specific solute ions in that fluid. This current is then carried by sodium, potassium, and calcium ions. Greater research emphasis should be placed on elucidating the effects of PEMF which matches the cyclotron resonance frequency of the principal ion, being calcium, on the cell membranes (*39*).

Growth Factor Synthesis

As reported by Chang et al. PEMF effect on bone formation could depend on the developmental phase of the cell line used which introduces a complicating variable in experimentation and one that must be considered (*40*). The current in vitro studies have shown that biophysical stimulation causes stem cells to differentiate into an osteoblast, secrete matrix glycoproteins such as osteopontin and osteocalcin. It should be noted that the biophysical manipulation techniques used in vitro are comparable to the results obtained from TGF-β1, BMPs, and IGF-1. Additionally, increased mineralization process, and reduction in the formation of osteoclast cells which resorb bone matrix. Wang et al. found that in osteoblastic murine cell line, MC3T3-E1, the growth factors, BMP-2 through 7 were considerably upregulated compared to normal agonist mRNA expression. Additionally, alkaline phosphatase activity was increased above normal levels within the cells. This indicates that controlled exposure of osteoblastic cell lines to PEMF can induce osteoinductive growth factors eventually leading to terminal differentiation of those cells (*41*).

Clinical Evidence for PEMF

The use of PEMF extends beyond both animal models and tissue culture experiments, it has been attempted in the clinical setting in the treatment of fracture nonunions with highly mixed results. The underlying reason for this is that the clinical experiments need to be tailored to an understanding of the underlying mechanisms of action. Due to this fact, there is no widely agreed upon standard set of experimental parameters (*42, 43*). Many studies have had favorable results to date, however, due to the broad diversity in the experimental design, i.e., low sample sizes, non-randomized, there has been no conclusive evidence (*42*). Indeed, there is even variation in the signal intensity, waveform, and frequency of the applied PEMF

on both animal models and human patients. All of these factors contribute to the lack of consensus in the clinical evidence (*44*).

Discussion

The treatment of fracture nonunions remains a great challenge in orthopedic medicine. Autologous bone graft remains the gold standard for the treatment of atrophic nonunions however the risks associated with the procedure warrant new avenues of research that explore the use of bone marrow aspirates, MSC injection, and demineralized bone matrix to enhance the fracture healing process. The use of biophysical manipulation and electromagnetic fields to induce fracture healing still require further evidence and narrowing of the experimental parameters in tissue culture experiments. Clinically, the use of biophysical manipulation as a noninvasive means to enhance fracture healing still remain in level III evidence, more randomized controlled trials and elucidation of a unified mechanism need to be done.

Acknowledgements

I would like to thank my academic advisor, Dr. Greg Heiberger for providing the guidance and thoughtful advice in navigating through the scientific writing process, and my external reviewer, Dr. Jaime Lopez for providing valuable feedback which guided my thought process.

References

1. H. Miyamoto *et al.*, Intermittent pulsed electromagnetic field stimulation activates the mTOR pathway and stimulates the proliferation of osteoblast-like cells. *Bioelectromagnetics* **40**, 412-421 (2019).
2. F. Granero-Moltó *et al.*, Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells* **27**, 1887-1898 (2009).
3. R. K. Aaron, B. D. Boyan, D. M. Ciombor, Z. Schwartz, B. J. Simon, Stimulation of Growth Factor Synthesis by Electric and Electromagnetic Fields. *Clinical Orthopaedics and Related Research®* **419**, 30-37 (2004).
4. G. M. Calori *et al.*, Validation of the Non-Union Scoring System in 300 long bone non-unions. *Injury* **45**, S93-S97 (2014).
5. E. Gómez-Barrena *et al.*, Bone fracture healing: Cell therapy in delayed unions and nonunions. *Bone* **70**, 93-101 (2015).
6. P.-J. Lai *et al.*, Augmentative antirotational plating provided a significantly higher union rate than exchanging reamed nailing in treatment for femoral shaft aseptic atrophic nonunion - retrospective cohort study. *BMC Musculoskeletal Disorders* **20**, 127 (2019).
7. H. Sugaya *et al.*, Percutaneous autologous concentrated bone marrow grafting in the treatment for nonunion. *European Journal of Orthopaedic Surgery & Traumatology* **24**, 671-678 (2014).
8. P. V. Giannoudis, T. A. Einhorn, D. Marsh, Fracture healing: the diamond concept. *Injury* **38 Suppl 4**, S3-6 (2007).
9. N. Selvamurugan, Z. He, D. Rifkin, B. Dabovic, N. C. Partridge, Pulsed Electromagnetic Field Regulates MicroRNA 21 Expression to Activate TGF-beta Signaling in Human Bone Marrow Stromal Cells to Enhance Osteoblast Differentiation. *Stem Cells Int* **2017**, 2450327 (2017).
10. P. Hernigou, A. Poignard, F. Beaujean, H. Rouard, Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am* **87**, 1430-1437 (2005).
11. K. S. Kang *et al.*, Regulation of osteogenic differentiation of human adipose-derived stem cells by controlling electromagnetic field conditions. *Exp Mol Med* **45**, e6 (2013).
12. R. Dimitriou, I. M. Carr, R. M. West, A. F. Markham, P. V. Giannoudis, Genetic predisposition to fracture non-union: a case control study of a preliminary single nucleotide polymorphisms analysis of the BMP pathway. *BMC Musculoskelet Disord* **12**, 44 (2011).
13. R. Zura, S. Mehta, G. J. Della Rocca, R. G. Steen, Biological Risk Factors for Nonunion of Bone Fracture. *JBJS reviews* **4**, (2016).
14. G. M. Calori *et al.*, Non-unions. *Clin Cases Miner Bone Metab* **14**, 186-188 (2017).
15. R. Zura *et al.*, Epidemiology of Fracture Nonunion in 18 Human Bones. *JAMA Surgery* **151**, e162775 (2016).
16. B. G. Weber, O. Cech, in *Pseudarthrosis: pathophysiology, biomechanics, therapy, results*. (1976), pp. 323-323.
17. C. Andersen, N. M. Wragg, M. Shariatzadeh, S. L. Wilson, The Use of Platelet-Rich Plasma (PRP) for the Management of Non-union Fractures. *Current Osteoporosis Reports*, (2021).
18. D. S. Elliott *et al.*, A unified theory of bone healing and nonunion: BHN theory. *Bone Joint J* **98-b**, 884-891 (2016).
19. M. Uzun, M. Çakar, A. M. Bülbül, A. Kara, Treatment of Aseptic Hypertrophic Nonunion of the Lower Extremity with Less Invasive Stabilization System (New Approach to Hypertrophic Nonunion Treatment). *Advances in Orthopedic Surgery* **2015**, 631254 (2015).
20. C. Schlundt *et al.*, Clinical and Research Approaches to Treat Non-union Fracture. *Current Osteoporosis Reports* **16**, 155-168 (2018).

21. T. T. Roberts, A. J. Rosenbaum, Bone grafts, bone substitutes and orthobiologics. *Organogenesis* **8**, 114-124 (2012).
22. E. Antonova, T. K. Le, R. Burge, J. Mershon, Tibia shaft fractures: costly burden of nonunions. *BMC Musculoskeletal Disorders* **14**, 42 (2013).
23. G. M. Calori, M. Phillips, S. Jeetle, L. Tagliabue, P. V. Giannoudis, Classification of non-union: Need for a new scoring system? *Injury* **39**, S59-S63 (2008).
24. J. A. Goulet, L. E. Senunas, G. L. DeSilva, M. L. Greenfield, Autogenous iliac crest bone graft. Complications and functional assessment. *Clin Orthop Relat Res*, 76-81 (1997).
25. M. A. Flierl *et al.*, Outcomes and complication rates of different bone grafting modalities in long bone fracture nonunions: a retrospective cohort study in 182 patients. *Journal of Orthopaedic Surgery and Research* **8**, 33 (2013).
26. M. K. Sen, T. Miclau, Autologous iliac crest bone graft: Should it still be the gold standard for treating nonunions? *Injury* **38**, S75-S80 (2007).
27. M. Dominici *et al.*, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315-317 (2006).
28. C. da Silva Madaleno, J. Jatzlau, P. Knaus, BMP signalling in a mechanical context – Implications for bone biology. *Bone* **137**, 115416 (2020).
29. A. Bandyopadhyay *et al.*, Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet* **2**, e216 (2006).
30. P. Hernigou *et al.*, Percutaneous autologous bone-marrow grafting for nonunions. Surgical technique. *The Journal of bone and joint surgery. American volume* **88 Suppl 1 Pt 2**, 322-327 (2006).
31. P. Desai *et al.*, Bone Mesenchymal Stem Cells with Growth Factors Successfully Treat Nonunions and Delayed Unions. *Hss j* **11**, 104-111 (2015).
32. P. Maquet, J. Wolff, R. Furlong, *The Law of Bone Remodelling*. (Springer Berlin Heidelberg, 2012).
33. E. Fukada, I. Yasuda, On the Piezoelectric Effect of Bone. *Journal of the Physical Society of Japan* **12**, 1158-1162 (1957).
34. Z. B. Friedenberg, C. T. Brighton, Bioelectric potentials in bone. *J Bone Joint Surg Am* **48**, 915-923 (1966).
35. R. K. Aaron, D. M. Ciombor, S. Wang, B. Simon, Clinical biophysics: the promotion of skeletal repair by physical forces. *Ann N Y Acad Sci* **1068**, 513-531 (2006).
36. L. Suryani *et al.*, Effects of Electromagnetic Field on Proliferation, Differentiation, and Mineralization of MC3T3 Cells. *Tissue Eng Part C Methods* **25**, 114-125 (2019).
37. C. L. Ross *et al.*, The effect of low-frequency electromagnetic field on human bone marrow stem/progenitor cell differentiation. *Stem Cell Res* **15**, 96-108 (2015).
38. F. Martini *et al.*, Bone Morphogenetic Protein-2 Signaling in the Osteogenic Differentiation of Human Bone Marrow Mesenchymal Stem Cells Induced by Pulsed Electromagnetic Fields. *Int. J. Mol. Sci.* **21**, 15 (2020).
39. H. Kavand, H. van Lintel, P. Renaud, Efficacy of pulsed electromagnetic fields and electromagnetic fields tuned to the ion cyclotron resonance frequency of Ca(2+) on chondrogenic differentiation. *J Tissue Eng Regen Med* **13**, 799-811 (2019).
40. W. H. Chang, L. T. Chen, J. S. Sun, F. H. Lin, Effect of pulse-burst electromagnetic field stimulation on osteoblast cell activities. *Bioelectromagnetics* **25**, 457-465 (2004).
41. Z. Wang, C. C. Clark, C. T. Brighton, Up-regulation of bone morphogenetic proteins in cultured murine bone cells with use of specific electric fields. *J Bone Joint Surg Am* **88**, 1053-1065 (2006).
42. P. F. Hannemann, E. H. Mommers, J. P. Schots, P. R. Brink, M. Poeze, The effects of low-intensity pulsed ultrasound and pulsed electromagnetic fields bone growth stimulation in acute fractures:

a systematic review and meta-analysis of randomized controlled trials. *Arch Orthop Trauma Surg* **134**, 1093-1106 (2014).
43. P. F. W. Hannemann *et al.*, CT scan-evaluated outcome of pulsed electromagnetic fields in the treatment of acute scaphoid fractures: a randomised, multicentre, double-blind, placebo-controlled trial. *The bone & joint journal* **96-B**, 1070-1076 (2014).
44. T. Lei *et al.*, Effects of four kinds of electromagnetic fields (EMF) with different frequency spectrum bands on ovariectomized osteoporosis in mice. *Sci Rep* **7**, 553 (2017).