

POLYTHERAPY IN BONE REGENERATION: CLINICAL APPLICATIONS AND PRELIMINARY CONSIDERATIONS

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Polytherapy, namely the simultaneous application of three fundamental elements necessary for bone regeneration (growth factors, osteogenic cells and osteoconductive scaffolds) seems to lead to a very high success rate in the treatment of complex non-union (NU) cases and critical bone defects. NU are reported in 5-10% of long bone fractures. The use of autologous bone grafts has been long-considered the gold standard for the treatment of these cases. However the harvesting procedure from the iliac crest increases surgery time and presents some donor site complications which may be elevated. In recent years, surgeons have some alternatives to autologous grafting such as: application of organic or synthetic bone substitute, application of mesenchymal stromal cells (MSC) or growth factors (GF). In the literature there are many studies available about their application in monotherapy, but unfortunately the healing rate doesn't exceed 90%. Polytherapy seems to be a logical option to improve the healing rate, nevertheless, there are not still extensive studies that validate this strategy and moreover, some questions are not resolved.

Polytherapy is a new treatment strategy of biological stimulation for NU and critical bone defects to reach bone healing.

In the literature there are many studies that estimate 5 to 10% of fractures associated with impaired healing or delayed union or NU (1,2). It has been demonstrated that a wide debridement of the pathological tissue, a stable fixation of the NU site and a simultaneous implant of autologous bone graft (ABG), stimulate the enhancement of bone regeneration process and lead to fracture healing. Due to its limited availability, and also to other problems in managing ABG, such as the prolonged surgical time and the additional donor site morbidity, medical scientists started focusing their research on enhancement of bone healing using other active biological substances (3) such as mesenchymal stem cells (MSC) (5,6), synthetic growth factors (GF) such as bone morphogenetic proteins (rhBMP) (4, 7, 8, 9) or autologous growth factors (AGF) contained in Platelet Rich plasma (PRP) (4) and scaffolds (10). The application of these elements in monotherapy (implanting of one single agent) has been adopted as a strategy for a long time. Despite this correct scientific approach, studies has never produced coherent results. The success rates

have not consistently reached levels close to 100% (11,12). The diamond/pentagon concept suggests that using only one component shouldn't be enough to manage aseptic non-unions and underlines that all five elements are necessary (stable fixation, cells, grow factors, scaffolds, vascularization) (13,14). Polytherapy is the strategy that would involve the simultaneous implant of all fundamental components of this concept in order to improve the efficacy of the treatment and reach a higher rate of healing.

MATERIALS AND METHODS

Using PubMed search engine a research of the published paper on polytherapy in long bone non-unions was performed at the 1st of January 2011 with the following keywords: "polytherapy" OR "poly-therapy" AND "ABG" AND/OR "MSC" AND/OR "PRP" AND/OR "BMP" AND/OR "Growth Factors" OR "GF", AND "long bone non-unions" OR "NU". Exclusion criteria were: case reports or referring to children (< 16 y.o.), editorials or letters and articles in other languages than English.

RESULTS

On a considerable number of initially retrieved

Keywords: Polytherapy, long bone non-unions, growth factors, mesenchymal stem cells, autologous bone graft, multi-approach, BMPs

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abstracts on polytherapy (> 900) and based on inclusion / exclusion criteria described, we have included in our research nine studies, which will be summarised here. (9, 12, 15, 16, 17, 18, 19, 20, 21). In a recent study about chondrogenic and osteogenic differentiation of MSC (20) it was demonstrated rhBMP-7 enhances the upregulation of lineage-specific markers, such as type II and type IX collagens (COL2A1, COL9A1) in chondrogenic, and secretes phosphoprotein 1 (SPP1), osteocalcin (BGLAP) and osterix (SP7) in osteogenic differentiation; this is due to the property of increasing alkaline phosphatase activity and to the dose-dependent capacity to accelerate mineralization of osteogenic differentiated MSC. Data even suggest that rhBMP-7 is not a singular lineage determinant, but it promotes both osteogenic and chondrogenic differentiation of MSC by co-ordinating with initial lineage-specific signals to accelerate cell destiny determination. In another recent in-vitro study the application of VEGF with MSC showed to enhance an osteogenesis process. Overall the addition of GF (rhBMP-2) has greatly increased osteocalcin level (OC) released from MSC. This study stated that endogenous IGF-1 and FGF-2 are essential to osteogenesis, but also that excess IGF-1 and FGF-2 are inhibitory to bone formation (18).

In 2 animal studies (15,16) the new bone formation in femoral defects of rats, demonstrated by radiographical and histological analysis, was superior in the group treated with the association of human-MSC (hMSC) and rhBMP-7 compared to rats treated with rhBMP-7 or hMSC alone. Similarly, in a dogs study, the simultaneous application of rhBMP-7 and MSC has increased the local population of cells and the connective tissue progenitors in a canine femur defect model (21). In another study conducted on tibial critical size defects of mini-pigs, it has been revealed that area of new bone formation was significantly higher in the group treated with an association of PRP and ABG compared with the group treated with the application of ABG alone. (19)

A multicentric study (22) on 30 cases of femoral NU, was observed the healing in 86,6% (26 on 30) cases operated with rhBMP-7. In a subgroup of this study, 12 cases were treated with ABG and rhBMP-7 association and the union was detected in 83,3% (10 on 12). This can be explained because the two failures had received four previous surgeries, one autologous bone graft and were originally a grade II open fracture.

In Zimmerman (12) et al. study 26 long bone tibial shaft NU were treated with rhBMP-7. The healing was found in 24 on 26 (92%) cases treated, but after deep analysis of subgroups of this series was founded that 8 cases were operated with the simultaneous application of rhBMP-7+ABG and 18 cases with rhBMP-7 alone. The

result in rhBMP-7 alone group was a union rate of 88,8% (16/18 cases), while in ABG-rhBMP-7 association was observed a clinical and radiographic healing in 100% of cases.

Finally, in wider study in literature (Giannoudis's series), the association between ABG and rhBMP-7 in 45 long bone NU led a successful rate of 100%, with a median time of clinical and radiographic healing of almost 6 months. (17).

DISCUSSION

Theoretically ABG possesses all the desirable biological properties necessary to bone regeneration: osteogenicity, osteoinductivity and osteoconductivity. Despite failure rates have been reported as high as 50% (23). A reasonable explanation of this failure rate may be the variations that exist from host to host in terms of the presence and concentration of MSC and GF, linked to the age and general condition of the patient. Moreover the harvesting and handling process before implantation could affect the effectiveness of ABG. This used as a biological stimulant for the treatment of long bone NU shows range of healing between 60% and 100% (8, 12, 23).

Nevertheless it has not few disadvantages such as difficulties connected with harvesting, chronic residual pain in the donor site in about 30% of cases (24), prolongation of surgical and anaesthesiologic times, frequent complications such as infection (24) with low patient compliance and high direct costs. However, the ABG is still regarded as the gold standard treatment (25).

Alternative agents such as scaffolds (allo, xeno, synthetic grafts), autologous MSC and GF (rh-BMPs and PRP) for bone biological stimulation have been recently developed but applied as monotherapy such as fundamental strategy for long time. These are analyzed here:

a) Both organic (allografts and xenografts) or synthetic bone substitutes (HA, TCP, BCP) have exhibited osteoconductive properties but not osteoinductive capacity. They have some disadvantages used alone: long time required for reabsorption and complete substitution with newly-formed bone, inadequacy for filling large bone gaps, especially in the treatment of atrophic long bone NU (10). In literature the healing rates of their application in monotherapy range to 50 to 80%.

b) The Demineralized Bone Matrix has an osteogenic potential due to the presence of "BMP" and other factors such as transforming growth factor beta (TGF- β), insulin-like growth factor (IGF) (26). However, it has been demonstrated a less osteoinductive capacity compared to ABG and has a questionable variability of concentration of BMP-2 and BMP-7 in some products (27).

c) Present in bone marrow of patients, MSCs are considered osteogenic progenitor cells with demonstrated ability to repair bone defects (6). They can be used to enrich a scaffold or direct implanted in NU site. Their efficacy is directly linked to their concentration and it has been demonstrated that at least a 3x or 4x concentration is required to have a bone regeneration capacity. For this reason scientists tried to expand them in culture in an undifferentiated line but the isolation and differentiation of MSCs in vitro requires time, work and significant costs. (28)

So, recently, new techniques (29) have become available. MSCs can be harvested from bone marrow of iliac crest and concentrated by: 1) centrifugation processes, that separate the cellular components by their density; or 2) by filtration processes that exploit the selective natural affinity of MSCs to animal or synthetic bone matrix, soaked in negative pressure of MSC. Both these procedures can be performed in the surgery room directly. These new methods show two clear advantages: less costs compared to in vitro expansion and drastic lower donor site morbidity compared to traditional open harvesting from iliac crest.

The human application of MSC obtained by centrifugation has showed to heal 53 patients in a series of 60 long bone NU (5). This study underline the influence of the number and concentration of progenitor cells as key factor to determine the healing. Indeed, in the union group (88,3%) the concentration of progenitor cells observed was $> 1500/\text{cm}^3$, while in the failed group (11,7%) the concentration detected was $< 1000/\text{cm}^3$. Finally, Hernigou's study has showed that percutaneous autologous bone marrow graft is a safe treatment for aseptic atrophic non-unions of the tibial diaphysis, in absence of local or systemic complications. In literature the healing rates of their application in monotherapy range to 50 to 88%.

d) AGFs are contained in PRP. It is an advanced product of blood management, a biologically active concentration of mediators extracted from platelets from patient's plasma that seems to accelerate the normal and physiological bone healing process. Various cytokines (PDGF, TGF β 1- β 2, IGF1-2, VEGF) with a chemotactic, mitogenetic and angiogenetic properties are delivered by degranulation of activated platelets.

In literature there are many studies but few clear evidences of bone healing capacity. A recent randomized study about the use of the PRP in 60 long bone NU cases, has scored their lower healing capacity (68,3%) compared to rh-BMP7 (89,7%) (4).

In conclusion, the AGFs contained in PRP are promoters to cellular division (mitogenesis) but not specific for bone cells and unable to promote the

differentiation of mesenchymal cells and to induce new bone formation. For these reasons they are not very useful when used alone or with scaffolds in the treatment of long bone NU (4).

e) In 1990, Celeste and Wozney were the first to isolate three isoforms of BMP. Nowadays we know 12 isoforms, of which BMP-7 and BMP-2 are fundamental in human biology for the osteoinduction. To date only BMPs, differently from other GFs, can be defined osteoinductive agents. Recombinant human rh-BMPs, obtained with genetic engineering techniques, are identical to the naive human BMP.

In 2001 Friedlaender et al. has scored the same healing ability of rhBMP-7 and ABG in a series of 124 tibial non-unions (8). Rh-BMP-2 showed its higher efficacy even in open tibial fractures in BESTT study (30) and in spinal fusions compared to ABG (31).

In a large review of 2007 by De Long et al. the authors conclude that only rhBMPs has a level 1 evidence to support the clinical use (10). Dinopoulos et al. in 2007 has examined the use of the two available BMPs (rhBMP-2 and rhBMP-7), specifically in long bone NU, reaching the conclusion that rhBMP-7 only has level 1 evidence and can represent a reasonable alternative to autologous bone graft in such diseases (7).

A recent randomized study of 60 long bone non-unions and a recent multicentre study of 68 tibia non-unions, have exhibited that use of rhBMP-7 leads to a union rate higher to autologous bone graft (from 86,6% to 89,7%) (4).

In conclusion, rhBMP-7 is a drug with clear and constant dose able to induce new bone formation and the only growth factors with a recognized ability to induce the differentiation of osteoprogenitor cells in vivo in a pre-chondroblastic and pre-osteoblastic lineage. However, rhBMP-7 alone or with scaffolds doesn't reaches a success rate over 92% (range 75% - 92%) in 778 consecutive long bone non-unions (4, 7, 8, 9, 11). Despite monotherapy is a correct scientific approach, the implant of one single agent has showed a success rates not consistently reached levels close to 100% (4, 5, 7, 8, 9, 10, 11, 12, 22, 26, 27). The idea of polytherapy is to realize a strategy that involve the simultaneous implant of all fundamental components of the diamond/pentagon concept. The adoption of this strategy seems to be a logical pathway to lead a consistently higher success rate (14), but, can all these components, adopted as a concept of monotherapy, be managed together?

While the combination of rhBMP-7 and bone allograft has established a synergistic effect in NU healing, however to date, few studies have evaluated the synergistic action between MSC and rhBMP-7. Nevertheless, in some animal studies the combination of them was scored to have a higher osteogenic and osteoinductive power than

the application of rhBMP-7 or MSC alone (15, 16, 21). Instead, the association of PRP and ABG has exhibited controversial results in animal bone defects studies. (19). Given the frequent poor vascularity that often accompanies an atrophic non-union, we wonder if the PRP, as an angiogenetic agent, can be useful in combination with other bone growth factors (rhBMPs). VEGF has shown to be an important component for the neoangiogenesis process at the fracture site and given the encouraging results of synthetic VEGF in bone defects (32), we wonder if the synthetic VEGF can be useful in combination with other synthetic osteoinductive agents (rhBMP), osteoconductive scaffolds and MSC. Unfortunately, we do not have studies on these applications.

In the last year human studies on association between ABG and rhBMP-7 in recalcitrant non-unions were produced. The results obtained seem to give a higher healing rate to their monotherapy application (healing rate 83-100%) (12, 22, 33). However, more extensive and controlled studies to validate their efficacy are necessary.

CONCLUSION

A large variety of treatment strategies, implants, and approaches have been utilised over the last years in order to manage long bone NU with a wide range of results. However, using such a polytherapy approach requires some considerations:

Which is the better timing? Implanting all the desirable components at same operative setting or grafting each individual component separately at different time point? There are such a wide number of variables that need to be investigated: the type of cells (differentiated or undifferentiated), the dose and carrier medium, the combination of scaffolds with a growth factors, the optimal scaffold and the type of carrier of the osteoinductive molecules. (34). Unfortunately, there are still no human studies based on association of these elements that provide an answer to all these variables.

Our experience comes from a long clinical and surgical pathway at the Operative Unit of Reporative Orthopaedic Surgery at

Orthopedic Institute Gaetano Pini of Milan where we take care of NU, critical bone defects and episeal bone necrosis with particular attention and interest in the application of new biotechnology in bone regeneration. Our experience started from the analysis of risk factors that lead to a NU (1), through the creation of a new classification system of the disease (N.U.S.S.) (35), arriving to the drafting of guidelines for treatment of NU and bone defects for the Italian Society of Orthopaedics and Traumatology (S.I.O.T.) (36). The pathway has not ended, however, our effort is to find and validate new

applications of biotechnology and new materials, to better understand exactly the dose of these therapies related to dimension, type and localization of the bone defect and to quantify the dose that remain in the application site. We want to focus on the importance and on the effects of the association of these elements because many of them may interact as inhibitor or as co-activators and this strictly influences the healing process. In conclusion, our opinion is that future studies should take into consideration all these parameters that, to date, are unanswered, so that surgeons will apply the physiological principles and molecular mechanisms of polytherapy to minimize the potential failure risk in difficult fracture cases and atrophic long bone NU.

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