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Distraction Osteogenesis and Its Challenges in Bone Regeneration

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1. Introduction

1.1 Distraction osteogenesis

Bone is amongst the very few tissues in the human body that possess intrinsic capacity to heal spontaneously following injury. However, beyond a certain critical size defect, bone cannot heal by itself and outside intervention is required. Numerous techniques are available for the management of these defects, including the gold standard autogenous bone grafts, allografts, bone graft substitutes, vascularized fibular bone grafts and systemic administration of anabolic agents. All these techniques, however, do have limitations (Dimitriou et al., 2011; Nauth et al., 2011). Such instances of severe bone loss, whether due to congenital bony deficiencies or acquired causes, pose an immense challenge to the treating physicians, and it is in these cases that distraction osteogenesis could offer a viable and successful alternative to these techniques. Distraction osteogenesis (DO) is a surgical technique in which the intrinsic capacity of bone to regenerate is being harnessed to lengthen bones or to replace large segments of bone. It consists of the application of an external fixator to the affected bone (Figure 1), followed by an osteotomy of the bone and then gradual and controlled distraction is applied to the two bone segments. This controlled distraction, usually by an external fixator, generates new bone within the distracted gap. When the desired lengthening is obtained, distraction is stopped and the external fixator is kept on until the newly formed in the distracted gap is mechanically strong enough to allow removal of the fixator. DO is considered a type of in vivo bone tissue engineering and is superior to other methods of bone regeneration in the management of cases of bone loss, because this technique allows the spontaneous formation of de novo native bone without the need for bone grafts. DO also has the unique ability to regenerate bone and soft tissues simultaneously.

2. Historical aspect of distraction osteogenesis

Codivilla from Italy is credited for having performed the first lengthening procedure by applying skeletal traction through a calcaneal pin, following osteotomy of the femur (Codivilla, 1905). However, it was a Russian surgeon, Gavriil Ilizarov, who pioneered the biological principles of bone and soft tissue regeneration and popularized the technique of distraction osteogenesis, when he discovered that under slow and gradual distraction, new

bone will regenerate in the distracted gap (Ilizarov, 1989a; Ilizarov, 1989b). Starting in the early 1950s, he worked in a village in Siberia, Kurgan, unknown to the rest of the world. Then, in 1982, he successfully treated a famous Italian explorer "Carlo Mauri" for a resistant non-union of his tibia and it was only then when his principles were made known to the Western World.

3. Ilizarov principles or the law of tension stress (Green, 2011)

Ilizarov developed the *law of tension-stress,* which describes the process of new bone and soft tissue regeneration under the effect of tension-stress caused by slow and gradual distraction. His biological principles can be summarized as follows:

3.1 Minimal disturbance of bone and soft tissues

Ilizarov showed that formation of new bone at the osteotomy site is definitely influenced by the amount of damage to the bone, medullary cavity, and periosteum. He described the new concept of corticotomy, where only the cortex of the bone is cut, preserving the periosteum and medullary cavity. The value of corticotomy has recently been questioned, because the medullary blood supply regenerates in 7 to 10 days following a complete osteotomy. The integrity of the periosteum is the only important factor for new bone formation at the site of the osteotomy.

3.2 Delay before distraction

(Latency phase) Duration of delay varies from 5 days in a child to about 10 days in a skeletally-mature patient. This allows the formation and organization of a hematoma.

3.3 Rate and rhythm of distraction

The optimum rate was found by Ilizarov to be 1mm/day and the optimum rhythm of distraction was 0.25mm every 6 hours. Elongation of more than 2mm/day may lead to slowing of osteogenesis, while elongation of 0.5mm/day or less may lead to premature consolidation. An autodistractor causing a continuous gradual and slow distraction of 1.0 mm/day was found to be superior to a rhythm of four times a day.

3.4 Site of lengthening

Metaphyseal lengthening leads to better osteogenesis than diaphyseal lengthening. The metaphyseal region contains much more cancellous bone than the diaphyseal region and this type of bone has a much higher potential for osteogenesis.

3.5 Stable fixation of the external fixator

Similar to fracture healing, this has been shown to be of paramount importance**.** Some axial micromotion is, however, beneficial to the consolidation of the regenerate bone.

3.6 Functional use of the limb and intense physiotherapy

During the whole lengthening procedure, this is of foremost importance in order to obtain a satisfactory outcome.

4. Phases of distraction osteogenesis

As shown in Figure 1, DO consists of the following phases:

- Latency phase: This is the phase immediately following the osteotomy, where there is no distraction. It lasts 5 to 10 days
- Distraction phase: the two bone segments are gradually distracted at a rate of 1.0 mm day in several increments, until the desired amount of lengthening is obtained.
- Consolidation phase: distraction is ceased and the two bone segments are held in place until the newly formed bone in the distracted gap consolidates (about one month per cm lengthened)
- Removal of the fixator.

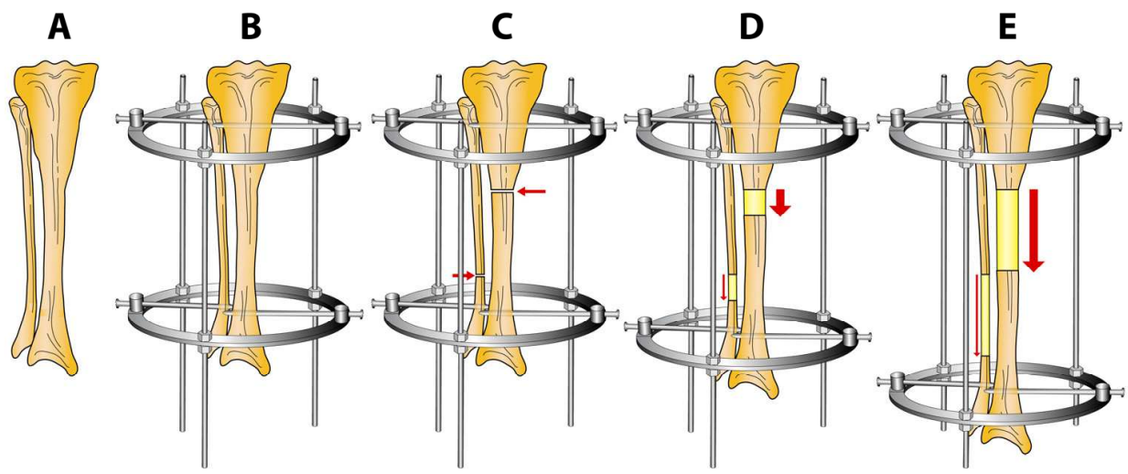


Fig. 1. Steps of the technique of distraction osteogenesis used for limb lengthening;
A. Shows the bone to be lengthened; B. Application of the external fixator; C. Osteotomy of the proximal tibia; D. Start of distraction and E. End of distraction when desired amount of lengthening is obtained.

5. Clinical significance of distraction osteogenesis

DO is a very successful technique of bone regeneration, widely used for lengthening bones and in the management of bone loss secondary to congenital or acquired causes, both in long and tubular bones of the axial skeleton as well as flat bones of the craniofacial skeleton. It has many indications, including:

5.1 Distraction osteogenesis as a bone lengthening technique

DO could be used in restoring the length of bones in numerous conditions including congenital causes of bone defects specifically congenital limb deficiencies or acquired causes due to growth plate injuries leading to growth arrest and subsequent limb length discrepancy (Birch and Samchukov, 2004; Murray and Fitch, 1996), (Figure 2).

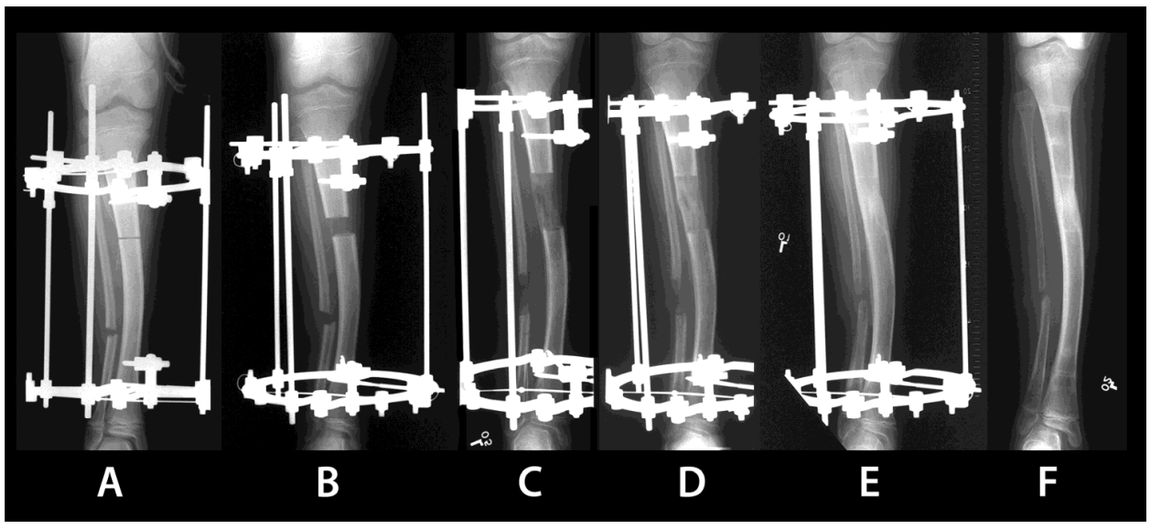


Fig. 2. Lengthening of short tibia showing various phases of the distraction process. A. Application of the fixator and osteotomy of the tibia; B. Start of distraction; C. End of distraction; D. and E. Consolidation phase, without any distraction, until bone in the distracted gap consolidates; F. Removal of the fixator.

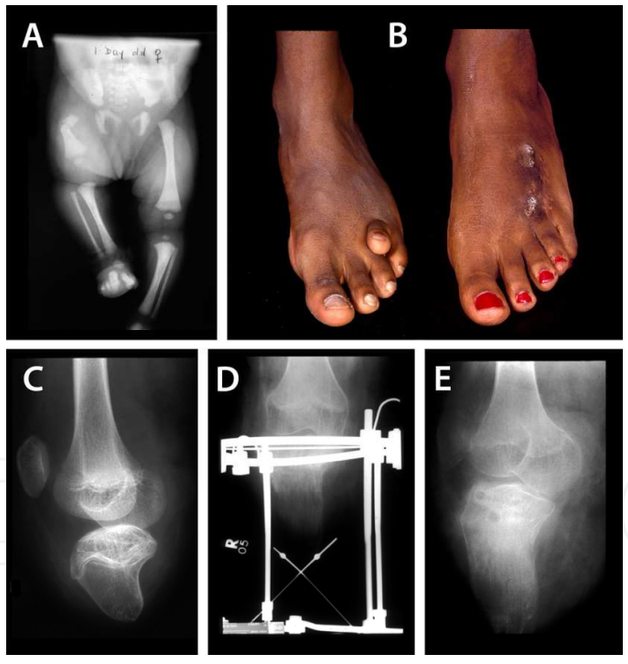


Fig. 3. Examples of short bones lengthened by distraction osteogenesis. A. A very short femur; B. Short 4th metatarsal bone pre and post lengthening; C. Amputation stump pre-lengthening, D. During distraction phase, and E. Post-lengthening.

5.2 Distraction osteogenesis as a bone transport technique

DO is also widely used for the reconstruction of large segmental skeletal defects by a special technique called bone transport (Figure 4). The magnitude of this problem is enormous.

Approximately 150,000 segmental skeletal defects are sustained in the United States each year as a result of trauma (Cierny and Zorn, 1994). To this must be added a significant number of bone defects following debridement after severe cases of osteomyelitis (Cierny and DiPasquale, 2011) and resections for malignant bone tumours (Tsuchiya, 2011).

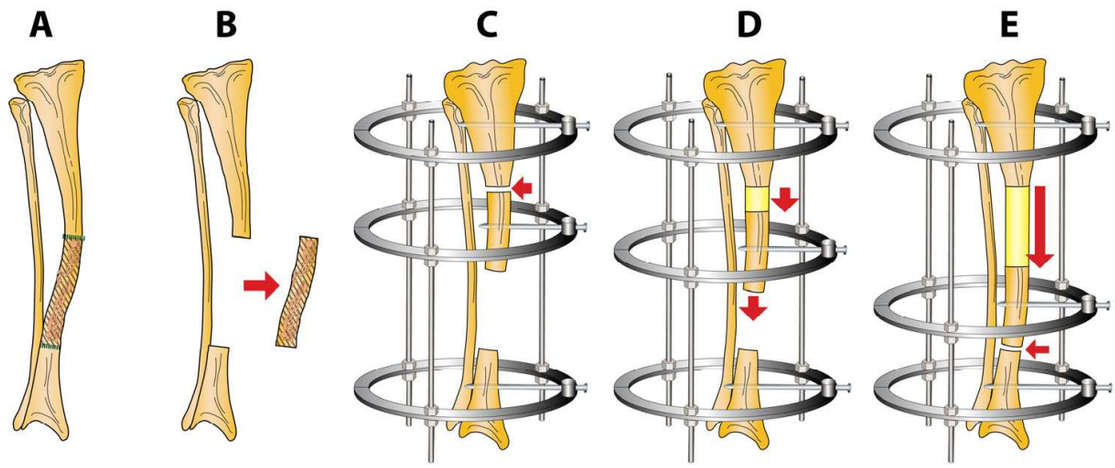


Fig. 4. A and B. Showing the segmental bone defect; C. Application of the external fixator, the bone defect and osteotomy of the proximal tibia; D. Start of distraction and transport of the healthy bone segment distally to fill the bone defect, while at the same time, new regenerate bone is formed in the distracted gap proximally; E. Completion of the bone transport.

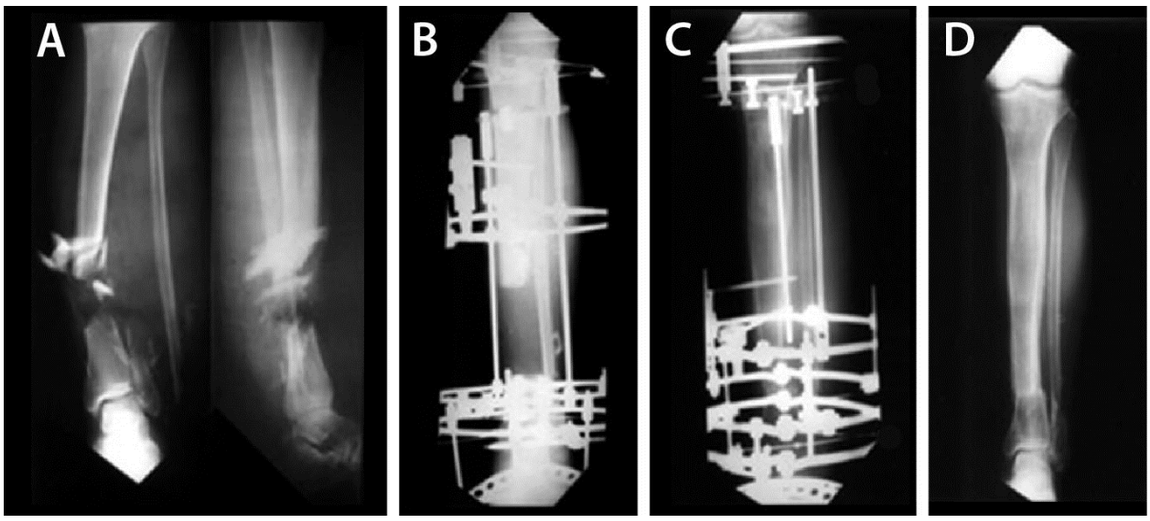


Fig. 5. A. Showing large segmental bone loss of the tibia due to severe trauma; B. Application of the external fixator; C. Completion of the bone transport and successful and successful bone formation in the distracted zone; D. Complete bone regeneration of the affected bone.

5.3 Distraction osteogenesis in the craniofacial skeleton

The clinical use of DO is not only limited to orthopaedic problems, but extends to the field of cranio-facial surgery. Mandibular distraction was first performed in 1973 in a canine model (Snyder et al., 1973). However, it was only in 1992 when it was first reported in humans (Mccarthy et al., 1992). Currently, both extraoral and intraoral devices may be used depending on the condition to be treated (Goldwaser et al., 2011). Commonly treated conditions include craniofacial deficiencies, syndromic craniosynostosis, Pierre Robin Sequence, posttraumatic deformities, and sleep-related breathing disorders (Vander Kolk et al., 2001).

6. Types of external fixators used in distraction osteogenesis for long bones

There are two types of external fixators used in DO: circular and monolateral. Circular fixators, also called ring fixators, include the standard circular frames (Figure 6A) described by Ilizarov and the more recent Taylor Spatial Frames (Feldman and Chaudhry, 2011). Monolateral fixators consist of a single bar transfixing bone with screws and pins (Figure 6B). Hybrid fixators also have been developed, consisting of a combination of circular and monolateral constructs.

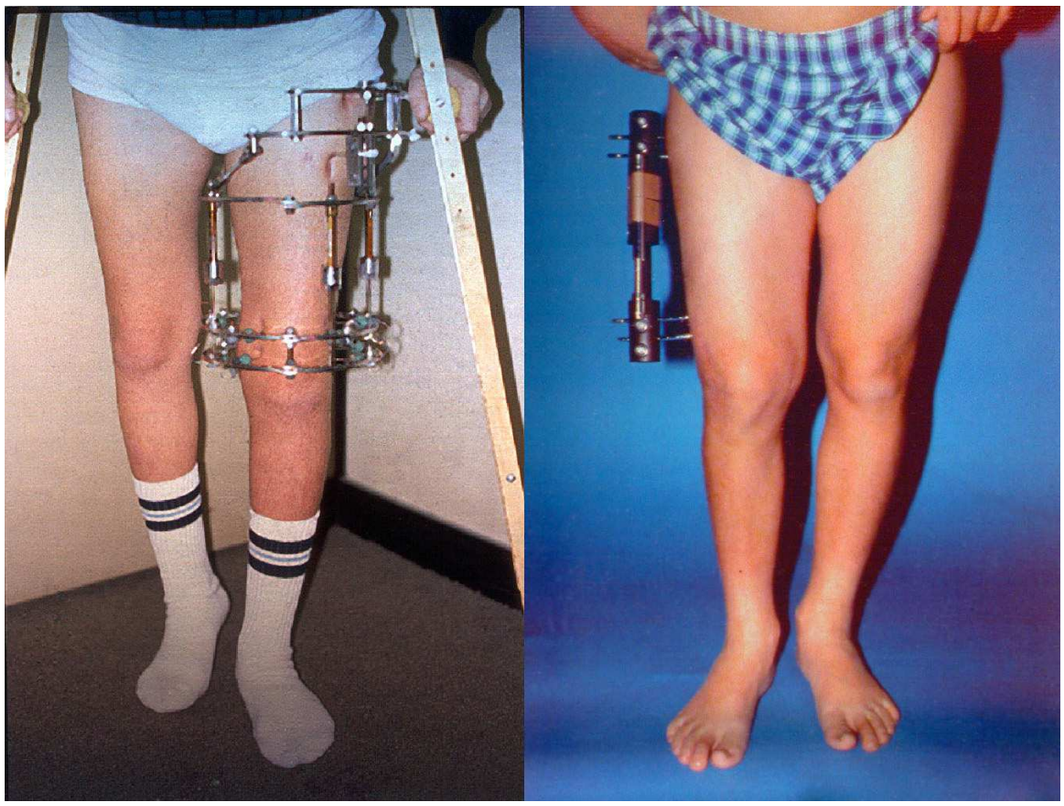


Fig. 6. A. (Circular); B. (Monolateral) External fixators.

7. Cellular events in distraction osteogenesis

The histological features of DO closely resemble those of fracture healing, as shown in Figure 7. Immediately after the osteotomy, a hematoma is formed. As distraction progresses, this hematoma is organized into fibrous and fibro-cartilaginous tissue in a longitudinal pattern along the direction of distraction and gives a striated appearance. New bone starts to be formed as early as two weeks after distraction. This new bone is formed from the periosteum, from the cortex at the site of the osteotomy and from the spongiosa and proceeds from the osteotomy cuts towards the center, always forming a fibrous, radiolucent interzone between the two advancing edges of the mineralization front (Aronson et al., 1990; Aronson et al., 1989; Aronson et al., 1997; Hamdy et al., 1997). Besides the formation of new bone in the distracted gap, all the surrounding soft tissues are also stimulated and lengthened, including skin, muscles, connective tissue, nerves and vessels (Makarov et al., 2009).

8. Molecular events and mechanism of bone formation in distraction osteogenesis

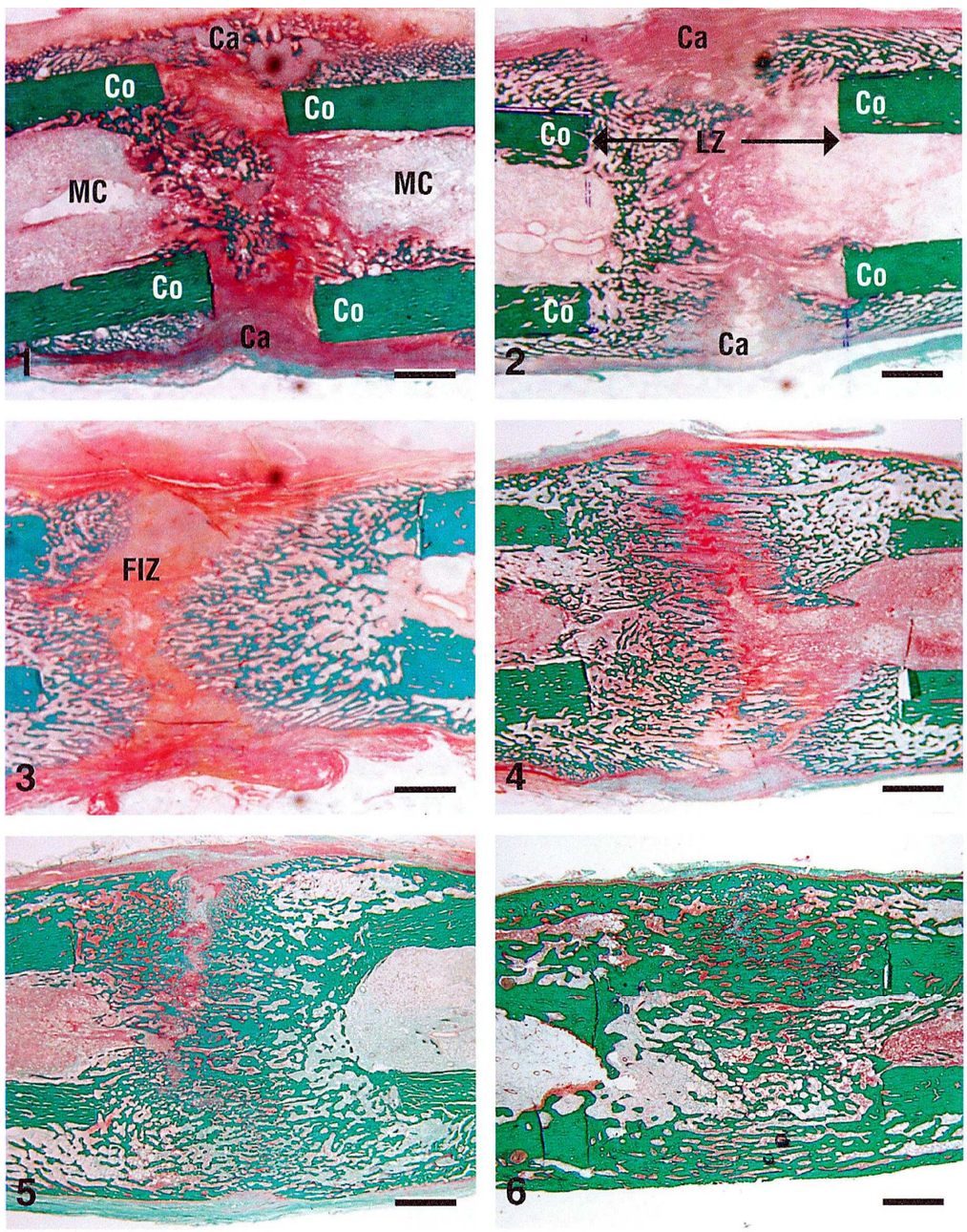
New bone formation in DO occurs through mechanotransduction, the process by which the mechanical tension-stress forces induced by distracting the bony segments at the site of the osteotomy, are converted into a cascade of molecular signals, which in turn activate numerous cellular events (differentiation, proliferation and secretory functions), that ultimately lead to new bone formation (Huang and Ogawa, 2010). We and others (Ai-Aql et al., 2008; Haque et al., 2007; Haque et al., 2008; Haque et al., 2006; Rauch et al., 2000) have shown that the mechanical forces applied during DO lead to the temporal and spatial expression of numerous cytokines, growth factors including BMP (Bone Morphogenetic Proteins), FGF (Fibroblast Growth Factor), IGF (Insulin Growth Factor), TGFB (Transforming Growth Factor B), PDGF (Platelet Derived Growth Factor), vascular factors VEGF (Vascular Endothelial Growth Factor), HIF (Hypoxia Induced Factor), as well as extracellular matrix proteins (Sato et al., 1998) and matrix metalloproteinases (Marucci et al., 2002). During the distraction phase, while the distraction forces are still being applied, many of these molecules are up-regulated and then, once the distraction forces cease at the end of the distraction phase, they become down-regulated.

9. The role of angiogenesis in distraction osteogenesis

DO is a vascular dependent process and new bone formation in DO is associated with robust neo-angiogenesis and neo-vascularity (Choi et al., 2000; Choi et al., 2002). There is an increased expression of numerous vascular growth factors in the distracted zone, including VEGF, HIF, basic FGF and Angiopoietin (Pacicca et al., 2003). Mobilization of endothelial progenitor cells have been described to play a major role in new bone formation in DO (Lee et al., 2008).

1. Haematoma formation after osteotomy and start of distraction
2. Mid-distraction showing start of bone formation
3. End of distraction (2.0 cms) showing fibrous interzone in the middle of the distracted zone. New bone formation is laid down longitudinally along the direction of distraction stress

4-5. Fibrous interzone decreases as new bone formation advances from the osteotomy ends towards the centre of the distracted zone
6. New bone completely bridges the distraction gap.



Co: Cortex, Mc: Medullary cavity, Ca: callus, FIZ: fibrous interzone.

Fig. 7. Cellular changes during distraction osteogenesis of the tibia of 2.0 cms in a rabbit model of DO using modified uniplanar fixator (trichrome staining), *Reprint with permission from Bone. 2000 Jun;26(6):611-7*:

10. Types of bone formation in distraction osteogenesis

While many aspects of DO, both at the cellular and molecular level have been elucidated, the exact mechanism and type of bone formation in DO is still being debated. Numerous authors have reported that regenerate bone formation in DO is mostly intramembranous (Aronson et al., 1990; Fink et al., 2003). However, others reported the presence of predominantly endochondral bone (Delloye et al., 1990; Kojimoto et al., 1988). Ilizarov believed that new bone formation in a canine model of DO, was mostly intramembranous, although he also described the presence of islands of cartilage-like cells (Ilizarov, 1989a; Ilizarov, 1989b). Our own results in several animal models of DO (dogs, rabbits and mice), revealed a combination of both types of ossification (Hamdy et al., 2003; Hamdy et al., 1997). Yasui et al (Yasui et al., 1997) in a rat model of DO, described a third type of ossification called transchondroid. It is also possible that the type of intra-membranous formation in DO is different from that of bone development, as reported by Isefuku et al (Isefuku et al., 2004), who showed that DO in Cbfa1 heterozygous knockout mice, new bone formation was the same as that of the controls. While the debate regarding the type of bone formation in DO will likely continue, many factors have been identified as playing a major role in determining which type of bone formation will predominate. These include stability of the fixator, vascularity of the surrounding tissues, rate and rhythm of distraction and the animal species in which DO was tested.

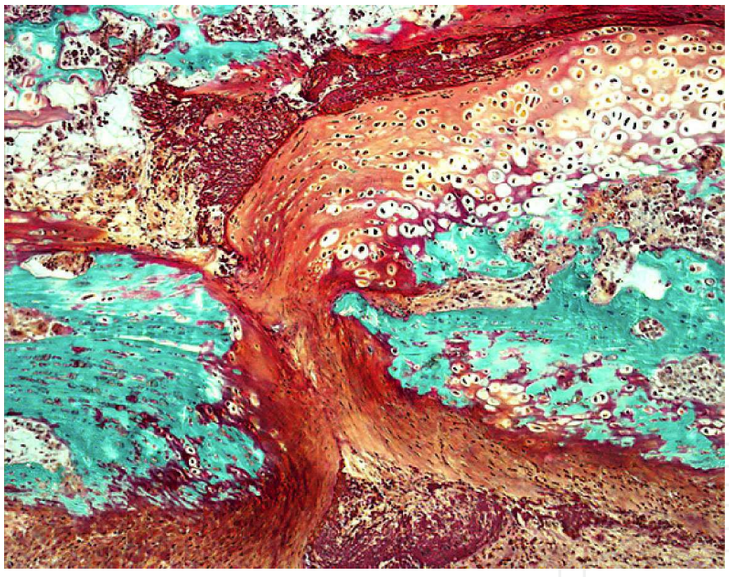


Fig. 8. Histological sections at the middle of distracted zone of rabbits sacrificed at 5 weeks post surgery. Trichrome Goldner staining showing mineralized tissue (bone and cartilage) in green, unmineralized cartilage in orange red. Chondrocyte-like cartilage is seen in large amounts.

11. Problems and issues associated with the technique of distraction osteogenesis and current research aimed at addressing these issues

Although very popular and very successful worldwide as a method of bone regeneration, this technique has several problems, specifically the long period of time that the external

fixator needs to be kept on until the newly formed bone in the distracted zone consolidates. It has been shown by Ilizarov that the distraction phase cannot be increased, as this may lead to poor regenerate bone formation and soft tissue problems, such as contractures and neurovascular problems. The consolidation phase is long, requiring the fixator to be kept on about one month for every cm lengthened. For example, a lengthening of 6.0 cms, would require the fixator to be kept for about 6 months. This, in turn, can lead to or exacerbate many medical, psychological, social and financial problems for the patient and his family (Paley, 1990). These include pin site infections; pain that may require narcotic medications; edema of the lengthened limb causing discomfort and pain; psychological complications; missing school days for children or working days for adults; long and continuous follow-up in the form of regular outpatient visits, frequent radiological examinations and re-adjustment of the frame. The question then arises: how to accelerate the consolidation of the regenerate bone, so that the external fixator could be removed at an earlier time?

12. Attempts at accelerating distraction osteogenesis

Numerous attempts at enhancing newly formed bone have been described and include the application of external biophysical stimuli (i.e. mechanical loading), and administration of biological agents, systemically or locally. Most of these studies have been performed in animal models of DO (Sabharwal, 2011). These include;

12.1 Mechanical loading, axial compression and dynamization

The effect of mechanical loading on the maintenance of bone mass has long been recognized. The mechanical stresses induced in the soft tissues during distraction are converted into a cascade of signals (mechanotransduction) that lead to the activation of numerous osteogenic pathways. At the cellular level, there is cellular differentiation, angiogenesis formation, mineralization of bone matrix as well of bone remodeling. There are no strict guidelines as to the amount of compression or shortening necessary to enhance bone regeneration, however many authors recommend compression of three to five days after completing the lengthening. Some authors also recommended over lengthening by about 5mm followed by compression of 5mm (Hwang et al., 2009; Mori et al., 2006).

12.2 Accordion maneuver

This technique consists of alternating cycles of compression and distraction. Numerous variations of this technique have been described: alternated cycles of compression and distraction the same day, and compression for a period of 1 or more weeks followed by distraction for the same duration (Claes et al., 2008).

12.3 Vibrations

Numerous studies have shown that application of low magnitude and high frequency mechanical stimuli may have a beneficial effect on bone formation. Vibration plates have been and are being used to enhance bone formation. However, there had been no studies in cases of distraction osteogenesis in humans (Hou et al., 2011).

12.4 Ultrasound

Numerous studies have shown the efficiency of LIPUS (Low Intensity Pulsed Ultrasound). The device is applied to the skin previously covered by gel corresponding to the point of fracture or distraction osteogenesis for 20 minutes a day. The treatment is usually self administered by the patient at home and the period of treatment ranges from 2 to 6 months or more, until healing is completed. Ultrasound may also enhance angiogenesis and increase the blood flow around the site of new bone formation (Busse et al., 2009; Claes and Willie, 2007; Romano et al., 2009; Watanabe et al., 2010).

12.5 Extra corporal Shock Wave (ESW)

Positive effects of ESW on regenerate bone formation have been reported in rat mandibular and rabbit tibia models of DO (Lai et al., 2010; Narasaki et al., 2003).

12.6 Electrical stimulation

Electrical stimulation could be applied using capacitively coupled electric field (CCEF) method, direct current (DC) stimulation, electromagnetic stimulation or alternating current (AC) stimulation and has been shown to give positive results in animal studies (Hagiwara and Bell, 2000; Kawamoto et al., 2005; Pepper et al., 1996).

12.7 Bisphosphonates

As both, bone formation and bone resorption occur in distraction osteogenesis, it is reasonable to assume that blocking bone resorption by anti-resorptive agents, such as bisphosphonates, may lead to increased bone formation. Numerous animal studies have documented the positive effect of bisphosphonates in distraction process (Abbaspour et al., 2009). In a case series of 7 patients, bisphosphonates were used for the treatment of poor regenerate bone (Kiely et al., 2007); six patients responded well and completely healed. However, despite the reported good results, there has been no other published series on the positive effect of bisphosphonates on enhancement of poor bone regeneration.

12.8 Systemic drugs

Other systemically administered drugs that have been investigated including, *Calcitonin* (Sen et al., 2006), *Prostaglandin E2* (Yamane et al., 1999).

12.9 Locally applied agents

These include *alpha-tocopherol* (Kurklu et al., 2011); *Adiponectin* (Jiang et al., 2011); *Inhibin A* (Perrien et al., 2011); *Nerve growth factor via a hydrogel* (Cao et al., 2011); *Thrombin peptide 508* (Cakarer et al., 2010); *Bone marrow progenitor cell mobilizing agent* (Davidson et al., 2011); *Calcium sulphate injection* (Song et al., 2004); *Osteoblast-like cells* (Shao et al., 2007); *Stromal cell derived factor-1* (Fujio et al., 2011); *NEL-like molecule-1* (Xue et al., 2011).

12.10 Cell therapy and platelet-rich plasma

The use of bone marrow cells to enhance bone healing has been in use from many years. The problem with direct bone marrow injections is that the number of active osteogenic cells is

very low, and therefore special techniques have been developed to aspirate bone marrow, culture the cells in vitro so as to increase and expand their number and then inject them in the desired area of poor bone formation (fracture site, non-union, distracted zone). The use of platelet rich plasma alone was reported to give positive results in a human study (Latalski et al., 2011). The positive osteogenic effect of culture expanded bone marrow cells has also been reported when combined with platelet-rich plasma in humans and with bFGF in rabbits (Jiang et al., 2010; Kitoh et al., 2007).

12.11 Combination of methods

These include rhBMP-2 combined with HA-TCP biomaterial (Ni et al., 2011); BMPs and NEL-1 (Zhu et al., 2011); Autografts and demineralized bone matrix (Canter et al., 2007); autologous bone marrow demineralized bone matrix (Hatzokos et al., 2011). Another experimental study reported also on the positive effects of an internal drug releasing distractor where small doses of BMP-2 are released from chitosan gel with every distraction (Konas et al., 2009).

12.12 Peptide growth factors

These include TGF-β (Transforming Growth Factor Beta), IGF (Insulin Growth Factors) (Bernstein et al., 2010), FGFs (Fibroblast Growth Factors) (Okazaki et al., 1999), PDGF (Platelet Derived Growth Factor) (Moore et al., 2009), Hypoxia Induced Factor (Wan et al., 2008).

13. Bone Morphogenetic Proteins (BMPs)

Of all the osteogenic growth factors, BMPs seem to be the most promising in stimulating bone formation in the context of DO. BMPs are members of the TGF-β superfamily acting on many systems including the kidney, heart and bone. These molecules are amongst the most powerful osteogenic growth factors and the only osteo-inductive ones that act on undifferentiated mesenchymal cells very early in the differentiation process (Gazzerro and Canalis, 2006; Miyazono et al., 2010; Rosen, 2006) (Figure 9).

BMPs are produced by many cells, including osteoblasts, chondrocytes and platelets. BMP signaling is shown in Figure 14 and involves binding to specific membrane receptors, phosphorylation of Smads 1, 5 and 8 and binding with Co-Smad 4, translocation into the nucleus and activation of transcription factors. In bone, BMPs trigger a cascade of events leading to osteogenesis, chondrogenesis, angiogenesis and up-regulation of numerous growth factors and cytokines. Cross-talk between BMP, FGF and Wnt pathways has been reported. Numerous studies have shown that recombinant BMP2 and BMP7 stimulate new bone formation in critical size defects, long bone non-unions, fracture healing, spine fusion, and augmentation of autografts and allografts (Einhorn, 2003).

13.1 Importance of BMPs in distraction osteogenesis

There is enough evidence, today, to suggest that BMPs, specifically BMP2 and BMP7, do play a major role in regenerate bone formation in DO. First, the expression of various members of the BMP signaling pathway was extensively analyzed, at the protein level using

immunohistochemistry and at the mRNA level using RT-PCR, and it was shown by us (Haque et al., 2007; Haque et al., 2008; Haque et al., 2006; Rauch et al., 2000) and others (Ai-Aql et al., 2008) that BMP ligands, receptors, transcription factors and downstream targets are up-regulated during the distraction phase and then down-regulated once the mechanical forces of distraction cease.

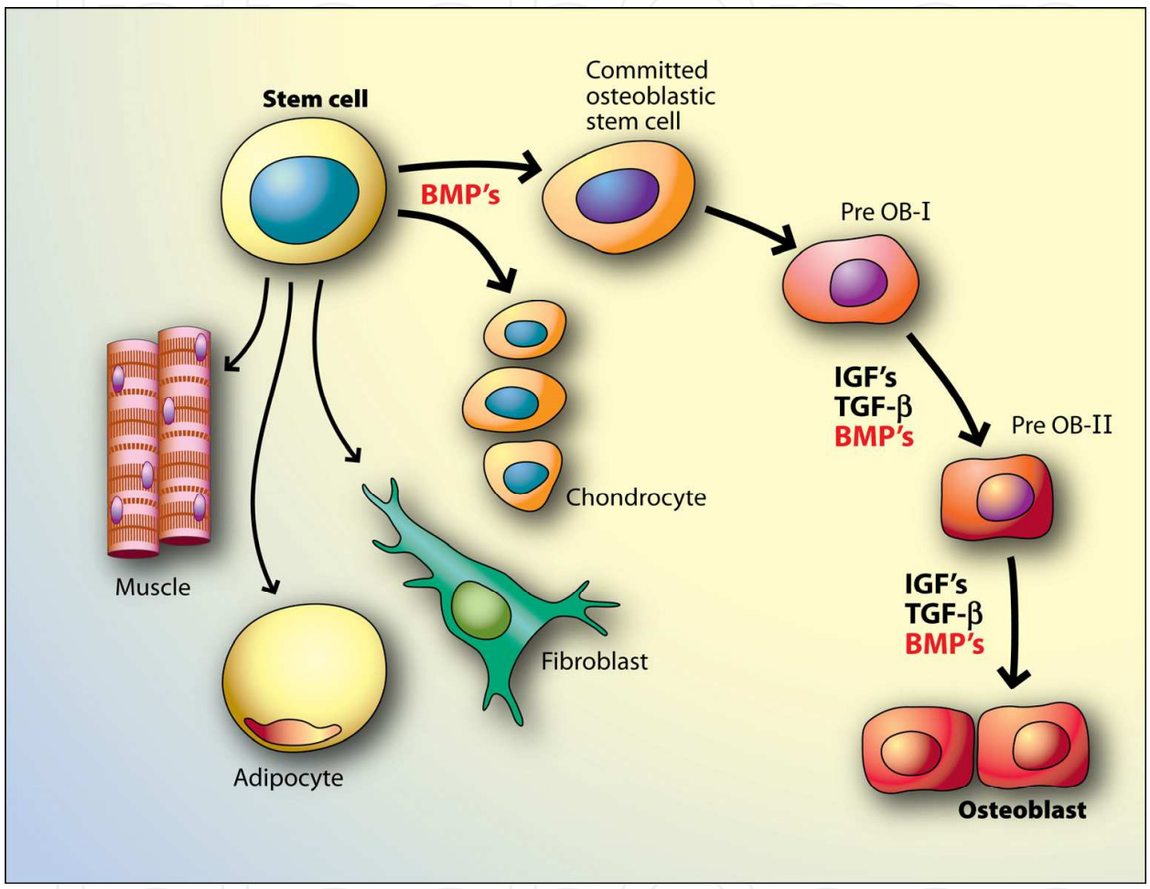


Fig. 9. BMPs acting on undifferentiated cells.

Second, to confirm the important role of BMPs in DO in a mechanistic way, we studied the process of DO in conditional BMP2 knockout mice (supplied by Dr. V. Rosen, Boston) and found that there was a delay in bone formation in the distracted gap of the heterozygous mice (Figure 10), when compared to the control littermates, thus showing that BMP2 is essential for bone formation in DO (Haque et al., 2008) and also supporting the results of (Tsuji et al., 2006) who reported that BMP2 is required for the initiation of fracture healing.

Third, we (Mandu-Hrit et al., 2006) and others (Lesaichot et al., 2011; Li et al., 2002; Mizumoto et al., 2003) have also reported that application of rhBMP7 and rhBMP2 in various animal models of DO, had a significant effect on the acceleration of bone formation in DO.

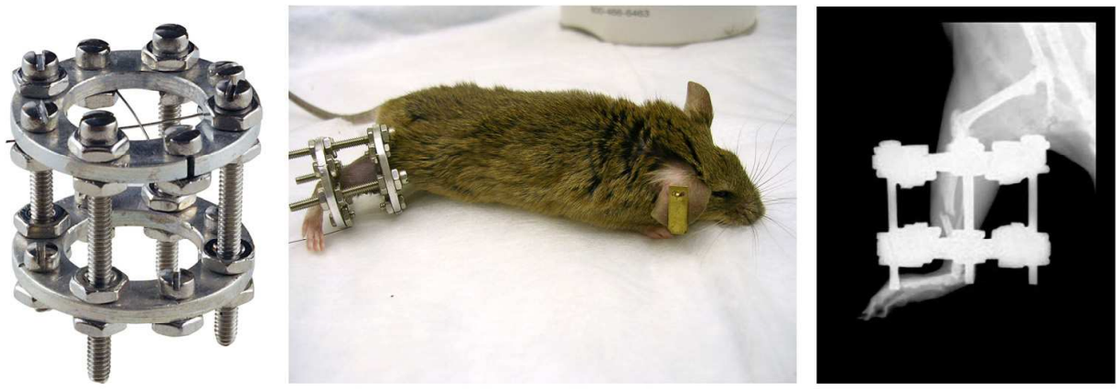


Fig. 10. Mini Ilizarov circular frame applied to a mouse model of DO in our laboratory , and developed by Tay et al. (Tay et al., 1998).

However, the main problem with the use of BMPs in humans remains the large doses that have to be used in order to obtain clinical significant results.

13.2 Issues associated with the use of large doses of BMPs in the clinic

The rapid clearance of BMPs from the site of application when locally applied in the absence of an adequate system for sustained delivery and the short half life of BMPs mandate the use of large, supraphysiological doses of BMPs in the milligram range to achieve satisfactory bone healing (Haidar et al., 2009b; Haidar et al., 2010b). Importantly, the minimal effective dose of recombinant BMPs (rhBMP2 or rhBMP7) currently used is equivalent to the sum of all BMPs present in 1000 human skeletons! These expensive large supraphysiological doses could have numerous side effects and unknown serious long-term effects as the expression of BMPs, including BMP2 and 7 is not exclusive to bones. BMPs have a neoplastic potential and although they have not been shown to directly cause malignancies, they are expressed in several tumors (Hsu et al., 2005). BMPs can cross the placenta, and, if given to patients in the child bearing age, may lead to serious teratologic sequelae. In addition, local and systemic immunological reactions could develop including local swelling and discharge (Dohin et al., 2009). Also, ectopic ossification could develop. In addition, the effects of BMPs on the growth plate remain unknown. Due to all these concerns, the use of BMPs is contraindicated in children and skeletally immature patients, thus precluding their use in a large population of patients that may benefit from BMPs.

Two compelling questions then arise: first, how can we decrease these large doses of exogenous BMPs without altering their efficiency? Second, as an alternative, would manipulating the endogenous BMP pathway in DO by decreasing BMP antagonist expression increase the bioavailability of endogenous BMPs and enhance osteogenesis (without having to apply exogenous BMPs). We believe that a local sustained and prolonged release of low doses of rhBMPs would address the first question. To provide a sustained and prolonged release of BMPs, several options are currently being investigated: gene therapy, cell therapy, multiple repeated injections of rhBMPs or cytokine therapy. Gene therapy is still in the experimental stages of research (Long et al., 2011). Multiple repeated injections of

rhBMPs are not an option from a practical point of view. Cell therapy is a viable option, as was previously mentioned. However, in order to be effective, bone marrow mesenchymal stem cells have to be cultured before local application. Cytokine therapy is feasible provided, there is a delivery system that would ensure a sustained and prolonged delivery of adequate protein concentrations to the desired site (Haidar et al., 2009b; Haidar et al., 2010b; Schmidmaier et al., 2008).

14. Delivery systems for BMPs and other growth factors

There is no question that the efficacy of BMPs in enhancing bone formation is dependent on its mode of delivery (Boerckel et al., 2011; La et al., 2010). In the absence of a suitable delivery system, huge doses of BMPs have to be used in order to overcome the rapid clearance and the very short life of BMPs. In order to be able to use low doses of BMPs that would be equally effective as a single large dose, an adequate delivery system that would allow the slow and controlled release of adequate concentrations (in low doses) of BMPs over the desired period of time becomes necessary. Probably, there is no single delivery system that would be suitable for all conditions. Rather, different delivery systems will be required for different pathologies (for example an injectable system may be required for some cases, while in others a locally applied one at the time of surgery would be indicated).

Furthermore, as BMP2 and BMP7 have different temporal and spatial expression as well as different modes of action and different cellular targets, the use of a delivery system that would allow the sequential delivery of these two factors may present a huge step towards improving bone formation and a huge advantage over the use of a single factor.

The literature is abundant on studies describing various delivery systems for BMPs and other growth factors, including various biomaterials, scaffolds, gene delivery and numerous tissue engineering techniques. Recently, the use of nanoparticles as a delivery system has gained popularity (Facca et al., 2011; Yilgor et al., 2010).

14.1 Nanoparticles as delivery system

Besides the known advantages including the size, long shelf life and ability to entrap more drugs (Gref et al., 1994), nano-sized systems reside longer in circulation, therefore greatly extend the biological activity when compared to microparticles (Desai et al., 1996). The literature provides evidence that particles (<500 nm) cross membranes of epithelial cells through endocytosis while larger particles (>5 mm) are taken up via the lymphatic system (Dong and Feng, 2004; Zhang and Feng, 2006).

15. Ongoing research in our laboratory

Our current research is focused on the use of BMPs in DO, specifically in two areas: first, the development of a delivery system for BMPs that would allow sustained and prolonged delivery of low doses of exogenous BMPs, with the same osteogenic effect of a single large dose, and second, to investigate if suppression of BMP antagonists could up-regulate the biological activity of endogenous BMPs so as to decrease or avoid the use the use of exogenous BMPs.

15.1 Development of a delivery system for BMPs

Over the last 5 years, we have been working on the development of a unique hybrid core-shell nanoparticle layer-by-layer (chitosan-alginate) delivery system. The core is composed of charged large unilamellar liposomes (LUVs) and the shell is constructed through the layer-by-layer (L-b-L) self-assembly of alternating layers of sodium alginate and chitosan. Alginates are water-soluble linear un-branched anionic polymers (marine sources, algae). Chitosan is a linear cationic polysaccharide (derived by the N-deacetylation of chitin, a product found in the shells of crustaceans). It is a biocompatible, non-immunogenic, and biodegradable polymer with bioadhesive, wound healing, antimicrobial and even osteogenic properties; making it a favorable option for biomedical applications. Both, alginate and chitosan have been extensively studied for drug delivery in different forms, such as microcapsules, beads or even wound dressing membranes (Haidar et al., 2010b).

In our laboratory, we have successfully completed all the *in vitro* characterization, formulation (Figure 11) and release kinetics studies (Haidar et al., 2008; Haidar et al., 2010a) of our NP system. Then, we showed the capability of the novel core-shell NPs to efficiently encapsulate and release a range of concentrations of rhBMP7 up to 45 days (Haidar et al., 2009a) (Figure 12). In order to better understand the fate of the NPs and their targeted delivery, we have developed an imaging tool using quantum dots (QDs) and reported on the development of biocompatible chitosan-QD nanoparticles (Sandros et al., 2007). We then completed a toxicology study in rats, where we determined that our delivery system was safe and biocompatible (Haidar et al., 2010a). We then showed that a single injection of NPs loaded with low doses of rhBMP7 administered early in the distraction phase accelerated osteogenesis (Haidar et al., 2010b) and that some nanoparticles were detected in the distracted gap (Figure 13).

Ongoing research in our laboratory is focused now on the optimization of the timing and dosage of the nanoparticle–BMP injections.

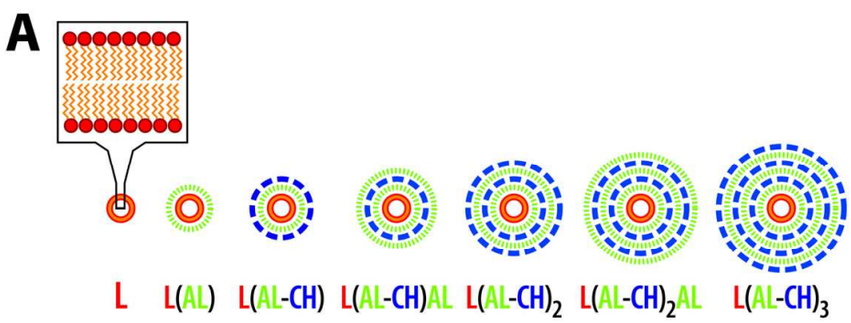


Fig. 11. Layer-by-Layer self-assembly of Alginate (AL) and Chitosan (CH) on Liposomes (L) up to six layers are shown. Reprint with kind permission from Springer Science+Business Media B.V; *Biotechnol Lett.* 2009 Dec;31(12):1817-24. *Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part A: Current challenges in BMP delivery. Haidar ZS, Hamdy RC, Tabrizian M.*

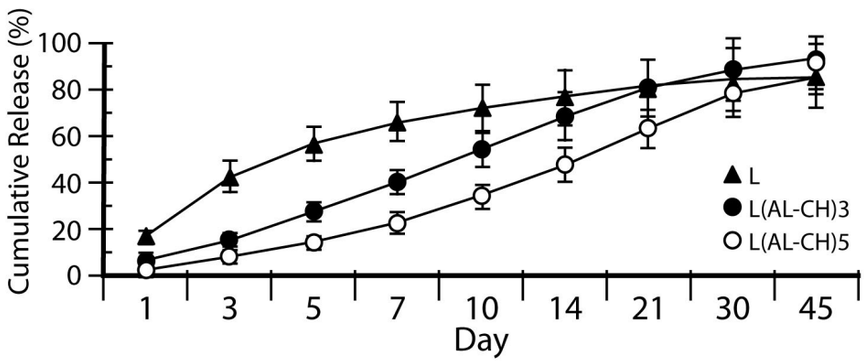


Fig. 12. Cumulative rhBMP7 release kinetic profile for uncoated liposomes (L), 3 bi-layered [L(AL-CH)3 or NPs3] and 5 bi-layered [L9AL-CH)5 or NPs5] core-shell NPs over an extended period of 45 days, *in vitro*. Controlled initial burst and rhBMP7 release from NPs is evident. *Reprint with permission from J Biomed Mater Res A. 2009 Dec;91(3):919-28.*

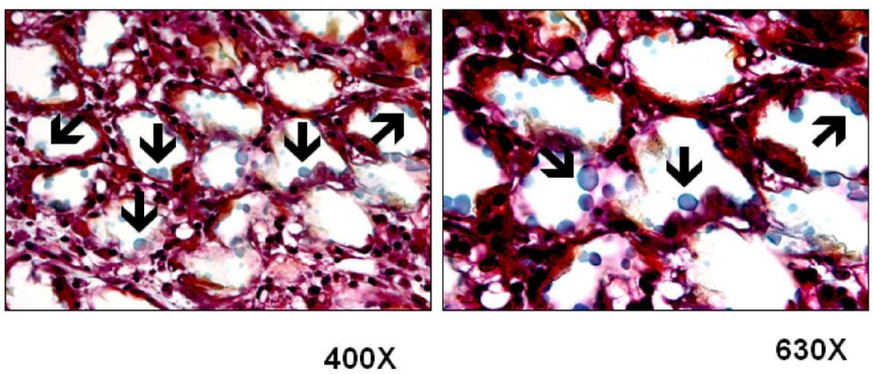


Fig. 13. Histological images (Goldner-Trichrome stain) of the distracted gap of a rabbit model of DO that received a single injection of NPs with 0.5 μg of rhBMP7 two weeks post-surgery, showing the persistence of NPs as indicated by the arrows. Animals were sacrificed five weeks after surgery (Data not published).

16. Suppression of BMP antagonists

As stated previously, the use of exogenous BMPs to enhance bone repair suffers from several drawbacks. Instead of administering exogenous BMPs to speed to up the regeneration process, an alternative strategy envisioned would be to inhibit BMP antagonists. This would allow increased levels of biologically active endogenous BMPs, which would in turn speed up the osteogenesis repair process. Attenuating BMP antagonist expression might also help to reduce the effective dose of exogenously applied BMP. This raises the question: do BMP antagonists play a role in fracture healing and new bone formation in DO?

BMP antagonists have been identified as a broad class of molecules, (Figure 14), which control BMP activity through a negative feedback mechanism. Some BMP antagonists, such as Noggin and Chordin, interact directly with BMP ligands (mostly BMP2, 4, 6 and 7) to restrict their

biological activities extracellularly. Once bound to BMP antagonists, BMPs are prevented from interacting with their cognate membrane receptors and inducing intracellular signaling. BMP antagonists may also act at the membrane level (such as Bambi) or intracellularly (Smad 6 and 7) (Cao and Chen, 2005; Gazzerro and Canalis, 2006; Rosen, 2006).

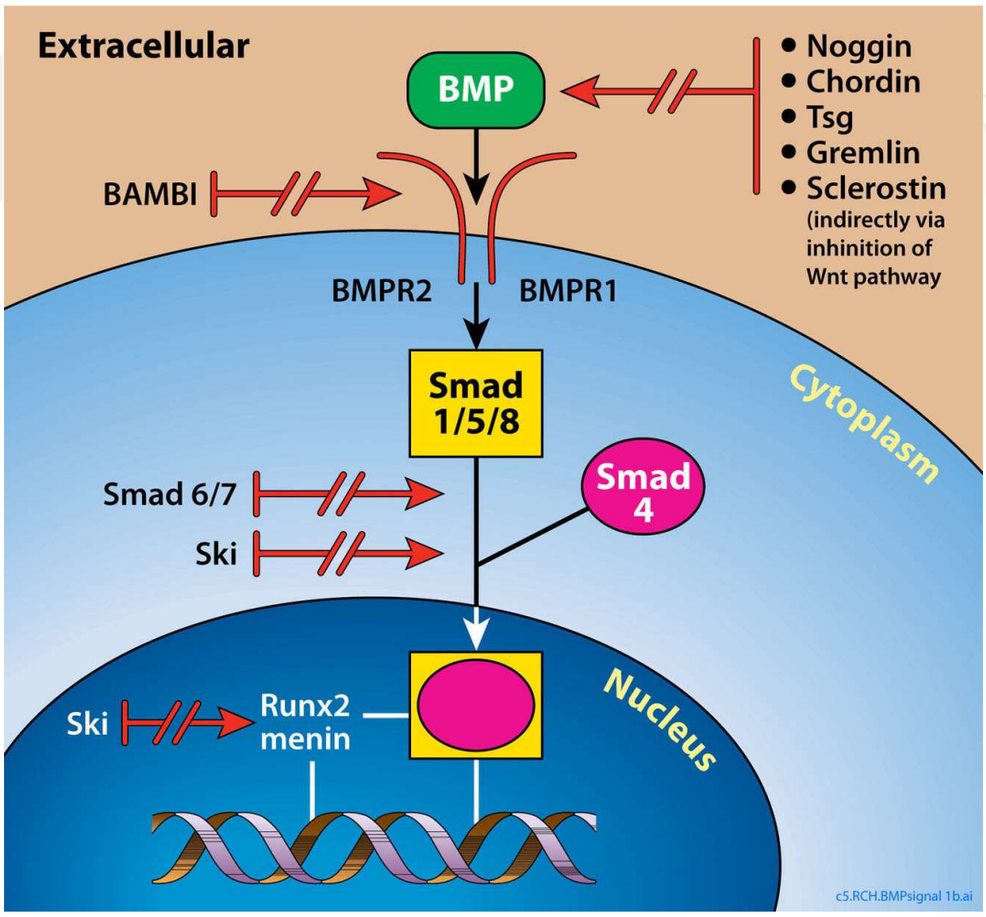


Fig. 14. BMP Signaling and BMP Antagonists.

Many *in vitro* studies have been conducted to investigate the role of BMP antagonists on osteoblast function and various aspects of osteogenesis and chondrogenesis. Noggin and Chordin have been particularly well studied in this context. Exogenous addition of Noggin or Chordin to osteoblast cell culture models results in the inhibition of expression for bone-specific genes, alkaline phosphatase, osteocalcin and bone sialoprotein, with a concomitant decrease in mineralization potential (Tsialogiannis et al., 2009).

Using transgenic and knockout animal models, many studies have revealed the crucial roles played by BMP antagonists in bone formation and repair. Over-expression of BMP antagonists has been shown to have detrimental effects on various bone parameters (Wu et al., 2003). In contrast, suppression of BMP antagonists using RNA interference caused increased osteogenic differentiation of cultured pre-osteoblastic cells, stromal cells and myoblastic cells (Kwong et al., 2008; Takayama et al., 2009). These findings strongly suggest that Noggin suppression via RNA interference stimulates bone formation in a mouse model

and may potentiate the effects of endogenous BMPs. Furthermore, as Noggin binds to several BMPs (BMP2, 4, 6 and 7), reducing Noggin expression may lead to increased availability of several, and not one BMP, thus reconstituting the normal biological environment, as compared to the exogenous application of BMPs, where only a single BMP, - either BMP2 or BMP7 can be locally applied.

Numerous reports have emphasized the role BMP antagonists may play in fracture healing and the potential acceleration of new bone formation by inhibiting the inhibitors (Tsialogiannis et al., 2009). The expression of various BMP antagonists has been reported in human specimen of fracture healing and cases of non-unions (Kloen et al., 2002). However, there have been very few studies in humans, analysing the effects of blocking BMP antagonists on fracture healing (Lissenberg-Thunnissen et al., 2011).

In the context of DO, we have previously shown in a mouse model of DO, that BMP antagonists, including BMP3, Noggin, Chordin and Inhibin showed significant increases in expression during the distraction process (Haque et al., 2008).

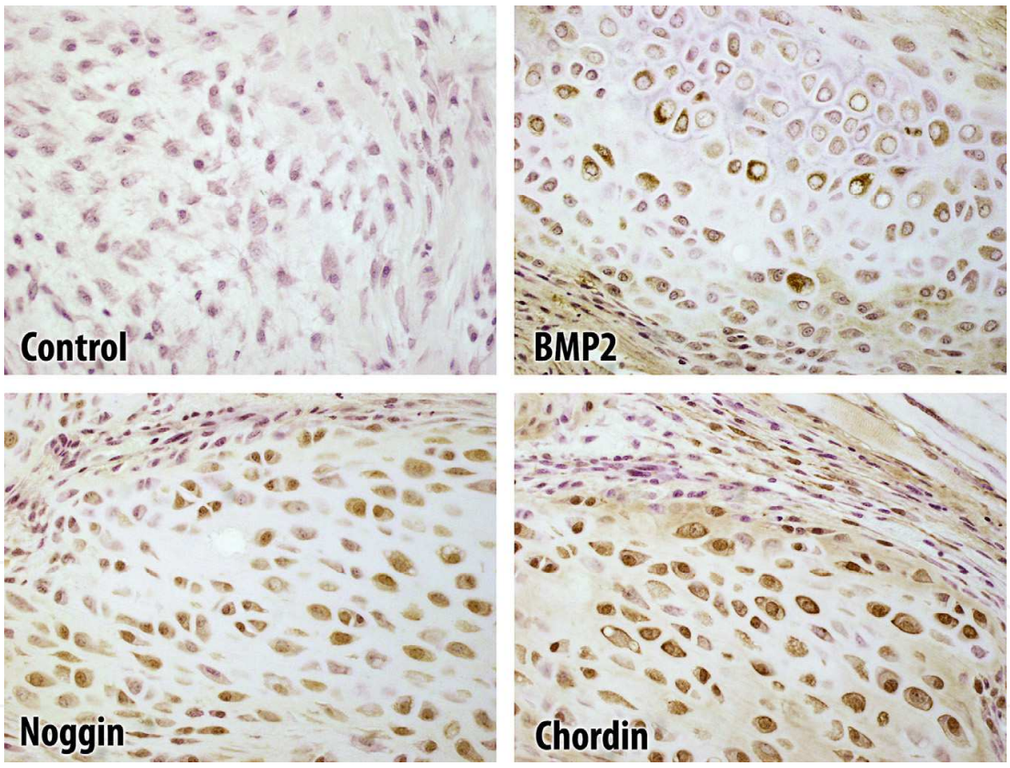


Fig. 15. Immunohistochemical staining of regenerate bone in the distracted gap in a rabbit model of DO, five weeks after osteotomy of the tibia and distraction of 2.0 cms, showing intense staining for BMP antagonists Noggin and Chordin in cartilage-like cells.

Based on these results, and a review of other reports in the literature, it would seem logical to hypothesize that these antagonists would be ideal candidates for therapeutic manipulation; blocking or inhibiting these antagonists, would in turn lead to up-regulation of BMPs and hence increased osteogenesis. The question then arises: which BMP antagonists to block and how to block them?

16.1 Methods to block BMP antagonists

These include naturally occurring substances, such as Heparan sulphate, monoclonal antibodies, and small interfering RNAs. In our laboratory, we first investigated if locally applied heparin sulphate in a mouse model of DO would enhance bone formation. Our results did not show any benefit with the use of heparin sulphate (results submitted for publication). We then decided to attempt inhibit BMP antagonists with the use of RNA technology.

16.1.1 RNA interference

As a method to block BMP antagonists, the use of RNA interference techniques is of particular interest. RNA interference is a powerful new strategy that has been broadly utilized in recent years to elucidate gene function (Pushparaj et al., 2008). It consists of delivering gene-specific double stranded RNA into cells that eventually causes silencing (knockdown) of the expression of a target gene. The mechanism leading to mRNA degradation involves a complex series of steps mediated by the host cell post-transcriptional machinery (Rana, 2007). Although this technology is easily applicable *in vitro*, directly by transfecting cells with synthetic double stranded small interfering RNA (siRNA), it requires a different delivery system for it to be used *in vivo*. Viral-mediated gene delivery of small hairpin RNA (shRNA) is one of the methods of choice to achieve gene silencing *in vivo*.

Lentiviruses represent a very flexible tool to modulate sustained gene expression both *in vitro* and *in vivo*. Lentiviral vectors are a method of choice because they: 1- infect both dividing and nondividing cells, 2- are less immunogenic because they are not encapsulated, 3- have a wide tropism, 4- can be concentrated to high titers, and 5-incorporate into the host genome (Kootstra and Verma, 2003). Lentiviruses have been used and showed that they can infect osteoblasts with high efficiency *in vitro*. When directly applied *in vivo* into surgically created lesions of the mandible and tibia, lentiviruses were found to infect most cell types present in bone, especially in the regenerating chondrocytic callus of the tibia (Wazen et al., 2006). A recent study showed success with lentiviral vectors as delivery system for shRNA-mediated RNA interference in MC3T3 cells (Moffatt et al., 2008).

Ongoing research in our laboratory aims at investigating the effects of blocking the BMP antagonists Noggin and Chordin using siRNA (small interfering RNA). We have chosen to focus our attention on Noggin and Chordin mainly because shRNA sequences against Noggin and Chordin have been identified previously to repress their expression with efficiencies greater than 80%. Furthermore, previous studies using knockout and transgenic mice revealed that Noggin suppression via RNA interference stimulates bone formation in a mouse model and may potentiate the effects of endogenous BMPs.As a first step and as proof of concept, we are using lentivirus to deliver siRNA to our mouse model of DO, as this delivery vehicle has been shown to have high transfection rate. Future research will aim at using our nanoparticle system for the delivery of siRNA in the context of DO.

17. Conclusion and future perspectives in distraction osteogenesis research

DO is a fascinating technique, that has become a standard method for bone regeneration used worldwide for the treatment of numerous orthopaedic conditions associated with bone loss, deficiencies and poor bone formation. The clinical importance of DO also extends to

the fields of craniofacial surgery and dentistry, where it has revolutionized the treatment of numerous previously untreatable pathologies.

DO is not only of interest to clinicians, but is equally attractive to many scientists and researchers in various specialties including developmental and molecular biologists, chemists, protein scientists as well biomedical and tissue engineers.

Opportunities for future research in DO are immense and include not only efforts aiming at enhancing bone formation but also the development of new devices for distracting bones, both internal and external, and novel methods to assess the quality and quantity of newly formed bone.

In this chapter, various methods used to accelerate bone formation in DO were reviewed and the use of BMPs was specifically addressed. BMPs are probably the most potent osteogenic factors known to date, however, the huge doses that need to be used in humans may pose serious problems and adverse effects that limit their widespread use. How to improve the efficacy of BMPs in order to optimize their clinical use is the challenge that we, scientists and clinicians, are facing. Can we meet this challenge? Much more work needs to be done in that respect. Only two avenues, as pertained to our research program were discussed here: the development of an adequate delivery system and methods to inhibit the BMP antagonists. However, there are many more methods to increase the efficacy of BMPs that have been recently reported. BMP2 and BMP7 are the two most extensively studied BMPs, however, less known BMPs such as BMP6 and BMP9 may possess similar or even greater osteogenic properties than BMP2 and 7 and more experiments are needed in that area. Wnt signaling is emerging as a new and powerful osteogenic pathway that needs to be investigated, specifically in the context of DO. More experiments need to be performed in order to assess if the combined local application of growth factors BMP2 and BMP7 or BMP and TGFB may be more effective than the use of a single growth factor. The sequential application of BMP2 and BMP7 via a suitable delivery system is a very attractive concept that needs to be further analyzed in order to determine its clinical efficacy.

There is no doubt that improving the technique of DO will have a huge impact medically, on the quality of life of patients and financially, on patients, their families and the health care system. Not only patients undergoing DO will benefit from advances in that field, but also patients with delayed fracture healing, non-unions and possibly also in patients with poor bony conditions, such as osteoporosis.

More than half a century after the Magician of Kurgan, Ilizarov, unraveled the biological principles of DO, many questions still remain to be answered.

18. Acknowledgment

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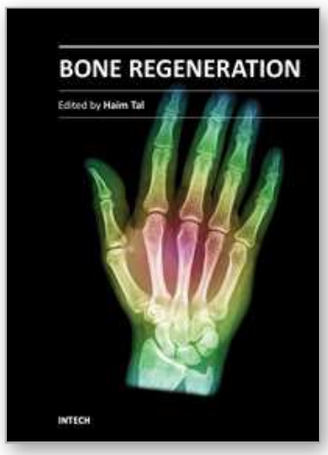

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Bone is a specialized connective tissue, most prominently characterized by its mineralized organic matrix that imparts the physical properties that allow bone tissue to resist load, to support functional organs, and to protect highly sensitive body parts. Bone loss and bone damage may occur as a result of genetic conditions, infectious diseases, tumours, and trauma. Bone healing and repair, involves integrative activity of native tissues and living cells, and lends itself to the incorporation of naturally derived or biocompatible synthetic scaffolds, aimed at replacing missing or damaged osseous tissues. There are several modalities of bone regeneration including tissue engineering, guided bone regeneration, distraction ontogenesis, and bone grafting. This book concentrates on such procedures that may well be counted among the recent outstanding breakthroughs in bone regenerative therapy.

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