Clinical and Experimental Studies of Osteogenesis in Dogs

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Voor Marsja, Thymen en Basse

Clinical and Experimental Studies of Osteogenesis in Dogs

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(met een samenvatting in het Nederlands)

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Chapter 1

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Bone has the unique property of complete regeneration after disruption of its architecture. The mechanisms behind this phenomenon are of great interest not only to understand the repair and maintenance processes of bone tissue, but also considering its implications for the regeneration of non-skeletal tissues. Growth factors are now generally accepted to play a crucial role in regulating bone formation and bone resorption. The maintenance of bone depends on the delicate equilibrium between formation and resorption. After disruption of the architecture of bone as occurs in skeletal fractures, new bone formation is critical to regain its supportive function. The general response of bone to fracture is formation of excessive fibrocartilage, which is mineralized and eventually forms a bony callus reuniting the fracture segments. Distraction osteogenesis, in which bone formation occurs under gradual distraction of two bone surfaces, relies on this ability of bone to repair itself. This technique allows for lengthening procedures of long bones and reconstruction of bone deficits by the induction of new bone. Distraction osteogenesis proved being an effective treatment option both in humans and in canine patients. In addition, distraction osteogenesis is a valuable asset in experimentally studying the intricate role of growth factors during bone formation.

The aim of this thesis was threefold. The first goal was to evaluate the clinical use of distraction osteogenesis in treating antebrachial growth deformities in the dog. The second aim was to investigate experimentally the role of bone growth factors during distraction-induced bone formation. The third goal was to study the effect of growth hormone treatment on bone regeneration in a critical sized bone defect model.

In the general introduction of this thesis (**Chapter 2**) an overview is given of bone histology and histiogenesis, growth plate injuries, healing of bone fractures, and distraction osteogenesis. Attention is then focused on circular external skeletal fixation systems, which are used in the clinical and experimental studies in this thesis. The hormonal regulation of bone is the next item in the general introduction. An extensive review is presented concerning the role of skeletal growth factors in bone formation and resorption. This overview is completed with a description of the most important markers of bone metabolism.

The following chapter of the thesis focuses on the clinical use of distraction osteogenesis in dogs. The outcome and prognostic factors are presented when treating antebrachial growth deformities with a lengthening procedure in canine patients. Emphasis is put on the role of incongruity of the elbow joint and antebrachiocarpal subluxation concomitant with antebrachial growth deformities (**Chapter 3**).

The second part of the thesis is concerned with growth factors which play a role during distraction osteogenesis. A cascade of growth factors is essential for bone formation both during distraction-induced bone regeneration and bone healing. These growth factors are expressed locally, but are also known to affect circulating levels of these factors. We hypothesized that the local expression of growth factors and systemic levels of factors associated with bone regeneration differ between distraction osteogenesis and osteotomy bone healing. A canine crural lengthening model was used to explore the local expression of insulin-like growth factor-I (IGF-I), insulin-like growth factor II (IGF-II), growth hormone (GH), growth hormone receptor (GHR), and bone morphogenetic protein-2 (BMP-2) in combination with the circulating levels of GH, IGF-I, IGF-II, insulin-like growth factor binding protein-4 (IGFBP-4), and insulin-like growth factor binding protein-6 (IGFBP-6) (**Chapter 4**).

In the past, monitoring of progression of osteogenesis during active distraction and subsequently in the maturation and consolidation phase of bone regenerate has depended mainly on repetitive radiographic examinations. Radiography evaluates the amount of mineralization in the newly formed callus. During active lengthening the bone regenerate mainly consists of fibrous tissue and blood vessels. Mineralization becomes evident in the consolidation phase of the bone regenerate. We hypothesized that markers of bone formation and bone resorption in plasma could effectively monitor osteogenesis comparing distraction osteogenesis with osteotomy bone healing. The bone markers under scrutiny were osteocalcin (OC) as a proposed marker of bone formation and carboxyterminal cross-linked telopeptide of type I collagen (ICTP) as a marker of bone resorption (**Chapter 5**).

By analogy with the use of bone markers, we evaluated delayed image bone scintigraphy to assess distraction-induced bone formation. Bone scintigraphy is a non-invasive, quantitative method to evaluate changes in the activity of bone metabolism. In contrast to radiography, which addresses the amount of mineralization, delayed image bone scintigraphy evaluates the uptake of Technetium-99m tracer by immature bone regenerate at places of increased bone

turnover and thus precedes the actual accretion of bone. We hypothesized that delayed image bone scintigraphy could effectively distinguish between the amounts of distraction-induced bone and bone formed during osteotomy healing (**Chapter 6**).

Although bone has the capability to completely regenerate under optimal circumstances, delayed or absent bone healing is a major problem in orthopedic patients. Growth hormone and growth factors, including IGF-I, BMP, and transforming growth factor-β (TGF-β) have been used with varying success to stimulate bone healing in fracture and osteotomy gap models. Very little information is available to date concerning their ability to stimulate bone regeneration in a critical-sized bone defect, i.e., a segmental defect, which will not heal spontaneously. We hypothesized that continuous infusion with GH could effectively induce bone formation and bone healing in a critical-sized bone defect model. In addition, we speculated that local GH application had the largest stimulatory effect on bone regeneration within the defect (**Chapter 7**).

The findings in this thesis are summarized and discussed in **Chapter 8**. The thesis is concluded with a summary in English (**Chapter 9**) and a summary in Dutch (**Chapter 10**).

Chapter 2

General Introduction

1. Introduction

Bone is a remarkable tissue with diverse functions. In close collaboration with ligaments, muscles, and tendons the skeleton literally forms the backbone of locomotion. In addition, the skeleton protects vital organs, including bone marrow, brain, spinal cord, heart, and lungs. And last but not least, bone has a metabolic function, acting as a reservoir of ions, especially calcium and phosphate, for the maintenance of serum homeostasis. Bone is a highly specialized connective tissue. The skeletal system consists of bone and cartilage. The rigidity of bone tissue relies on mineralization of the bone matrix with hydroxyapatite crystals, which are predominantly composed of $Ca_{10}(PO_4)_6(OH)_2$. There are two major families of bone cells, the osteoblast and the osteoclast lineages.[179,180] Osteoblasts and osteoclasts are responsible for the dynamic turnover of bone both during growth and in adult life. The osteoblast is the bone-lining cell responsible for the production of bone matrix, whereas the osteoclast is a multinucleated bone-lining cell responsible for bone resorption. During bone formation osteoblasts are embedded within the bone matrix in small lacunae to form osteocytes (Fig 1).
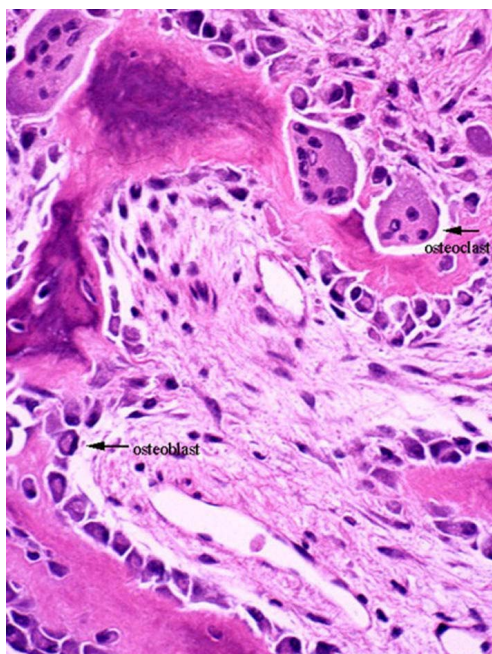

Fig 1. Osteoblasts are present on the lower left, lining a bone trabecula. Osteocytes can be seen embedded within the bone matrix. Osteoclasts are present at the right top corner.

Bone formation occurs through the coordinated production and mineralization of the osteoid matrix. Bone matrix components consist of three classes of macromolecules. The first group possesses repetitive structural motifs and includes collagen, hyaluronan, decorin and biglycan. The second group is characterized by a modular domain structure and includes versican, thrombospondin, fibronectin, osteonectin, and tenascin. The third group demonstrates no clear structural motifs and includes matrix γ-carboxylated protein, bone sialoprotein, osteopontin, and osteocalcin.[139] The complexity of the interactions between these various matrix components and bone cells conveys to bone its unique function of sustaining a stable yet dynamic structure. The major organic component of the extracellular bone matrix is type I collagen, accounting for up to 90% of the organic matter. Collagen I is composed of trimers of two α1 chains and one α2 chain, to form triple helical molecules. The collagen α chains are produced as procollagen which possesses amino- and carboxyl-terminal polypeptide extensions. Enzymatic removal of the noncollagenous N- and C-terminal extensions precedes collagen fibril formation. Collagen III is also found in bone matrix, but its role in bone metabolism is unclear.

2. Bone histology and histiogenesis

On gross observation of bone, cancellous and compact bone can be distinguished. Cancellous bone consists of many trabecular walls separating numerous interconnecting cavities filled with bone marrow or fat tissue. Compact bone does not show these cavities. Nevertheless, on microscopic examination both cancellous and compact bone have the same basic histologic structure. In long bones, compact bone is mainly found in the diaphysis whereas cancellous bone is located in the metaphyseal areas and the epiphysis, surrounded by a thin layer of compact bone.[192] Flat bones of the skull, scapula, and pelvis usually have a core of cancellous bone flanked by two plates of compact bone. On histology, there are two varieties of bone tissue: primary, immature or woven bone; and secondary, mature or lamellar bone. The difference between the two relies on the fact that collagen bundles are placed randomly in the first variety and organized into bone lamellae in the second.

Primary bone is the first bone tissue formed during bone formation. It is temporary and is readily replaced by secondary bone tissue in most places in the skeleton. In addition to the irregular deposition of collagen bundles, primary bone is characterized by a lower amount of mineralization and a larger amount of osteocytes compared to secondary bone.

Secondary bone is the variety mainly found in the mature skeleton. The bone lamellae, which characterize mature bone, are arranged parallel to each other or concentrically surrounding a central canal containing blood vessels and nerves. This complex of concentric lamellae is called the osteon or Haversian system and is the main building block of the skeleton (Fig 2). Between, and occasionally within the lamellae, lacunae containing osteocytes are encountered. These osteocytes have numerous long cell processes, which are in contact with other osteocytes and osteoblasts. Osteocytes are still capable of matrix production. The osteons communicate with each other, the endosteum, the marrow cavity, and the periosteum through transverse or oblique Volkmann's or perforating canals (Fig 2). These Volkmann's canals have no concentric lamellae and perforate the lamellae of the osteons. During growth and also in the adult skeleton continuous remodeling of osteons takes place. This explains the great variability in size and form of osteons.
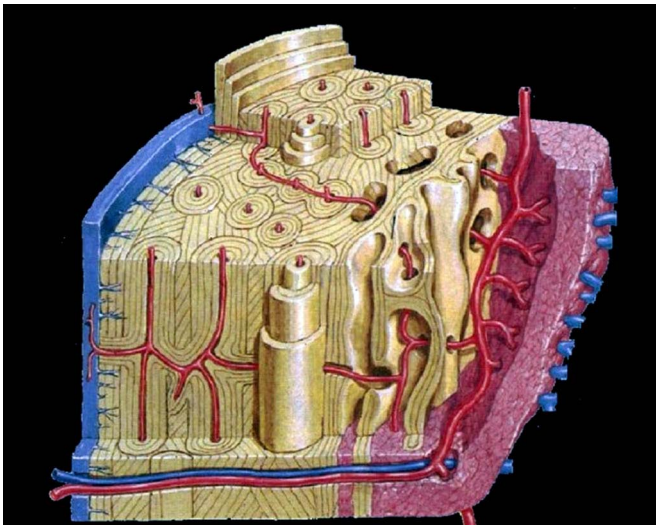
Fig 2. Representation of bone histology demonstrating the main building unit of bone i.e., the osteon.

Bone tissue is formed either by intramembranous ossification or by endochondral ossification.[87] Intramembranous ossification is characterized by direct bone formation within a layer or membrane of connective tissue i.e., periosteum. During endochondral ossification, a cartilaginous model precedes the actual accretion of bone tissue. In both intramembranous and endochondral ossification bone tissue is deposited first as primary or immature bone. Through

remodeling this primary bone is replaced by mature lamellar bone. Most of the bones of the skull, including parietal bones, frontal bones, mandible, and maxilla are formed by intramembranous ossification. Growth in width of short and long bones proceeds through intramembranous bone formation. Primary ossification starts within the connective tissue layers. Preosteoblasts differentiate into osteoblasts, which start producing osteoid.[179] Osteoid is mineralized in turn to form primary bone. The trabeculas of primary bone unite to create cancellous bone. Growing blood vessels and undifferentiated mesenchymal cells, which give rise to bone marrow cells, including preosteoclasts, penetrate the cancellous bone.

Endochondral ossification is responsible for the formation of most short and long bones. Endochondral ossification relies on the replacement of a hyaline cartilage and basically depends on two processes. The first process is hypertrophy and apoptosis of chondrocytes of the model of bone leaving expanded lacunas separated by septa of calcified cartilage matrix. In the second process, an osteogenic bud consisting of blood capillaries and osteogenic precursor cells penetrates into the lacunas left by the apoptotic chondrocytes. The undifferentiated cells give rise to osteoblasts, which lay down osteoid on the remnants of the calcified cartilage matrix. In this way, bone tissue appears at the site where there was cartilage.

Long bones are formed from cartilaginous models with a cylindrical shaft or diaphysis and enlarged extremities or epiphyses. The first bone to develop in the diaphysis is the bone collar, which surrounds the periphery of the cartilaginous matrix and thus forms the shaft of the bone. This bone collar is produced through intramembranous ossification. Within the forming bone collar, chondrocytes of the cartilage model start the process of hypertrophy, apoptosis, lacuna formation, and mineralization of the remaining cartilage matrix, also known as hypertrophication. Blood vessels of the osteogenic bud invade the lacuna and osteoblasts start to synthesize bone matrix. This ossification center, which appears in the diaphysis, is called the primary ossification center. At later stages of development a secondary ossification center arises at the end of the long bone to form an epiphysis or apophysis. Instead of the longitudinal growth of the primary ossification center, growth in the secondary center is radial. As bone formation in the primary and secondary ossification centers progresses, an epiphyseal plate or growth plate is formed between the diaphysis and epiphysis.[89] By analogy an apophyseal plate is formed between the diaphysis and apophysis. During adolescence, longitudinal bone growth continues in the growth plate through a highly coordinated type of endochondral ossification.[21]

In the epiphyseal plate five zones can be distinguished. Starting from the epiphyseal side these are: 1- the resting zone containing hyaline cartilage and small chondrocytes; 2- the proliferative zone with rapidly dividing chondrocytes, which

form columns of stacked cells parallel to the long axis on the bone; 3- the hyperthrophic cartilage zone with enlarged chondrocytes and interspersed thin septa of resorbed cartilaginous matrix; 4- the calcified cartilage zone with chondrocyte apoptosis. The cartilaginous matrix septa are mineralized with hydroxyapatite; 5- the ossification zone in which primary bone is formed. Blood vessels and osteoblasts invade the calcified cartilage matrix and deposit osteoid on the septa. The osteoid is mineralized, thus forming primary bone tissue. Longitudinal bone growth relies on the continuous cell division of chondrocytes in the proliferative zone and bone accretion at the metaphyseal side (Fig 3).
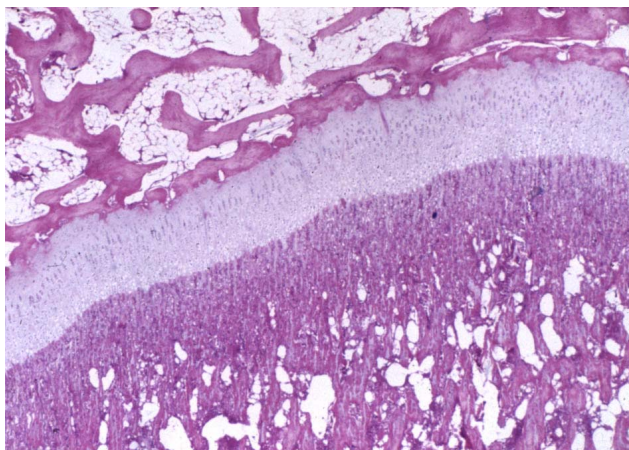
Fig 3. Microscopic section of the growth plate demonstrating endochondral bone formation. Courtesy of the Department of Pathobiology, Division Pathology, Faculty of Veterinary Medicine, Utrecht University.

3. Growth plate injuries.

Injuries of the growth plate can occur as long as the epiphyseal plate is not replaced by bone and usually occurs before he long bones have reached their full growth potential. Longitudinal bone growth in the dog ceases at approximately 10 months of age and is characterized by closure of the epiphyseal plates.[34] Growth plate fractures are classified according to the Salter-Harris system into 5 categories: type I, fracture through the growth plate; type II, fracture through the growth plate and metaphysis; type III, fracture through the growth plate and epiphysis; type IV, fracture through the growth plate, epiphysis and metaphysis, and type V, crush or compression injury of the growth plate.[21,145] The Salter-Harris system was designed to predict the prognosis of growth plate injuries. Based on clinical and experimental physeal fractures they postulated that most type I and type II fractures were restricted to the zone of hyperthrophic chondrocytes and thus should not

seriously affect longitudinal growth after careful reduction and stabilization. In dogs, the prognosis of these types of physeal fractures was proven to be less favorable. Disruption of the cells of the proliferative zone was found in the majority of traumatic growth plate injuries, which accounts for the high incidence of growth retardation in these patients.[59,89] In addition to decreased longitudinal growth, malformation of the limb is a common finding.

In dogs, growth deformity of the antebrachium is the most common limb malformation.[88] The antebrachium consists of the paired bones radius and ulna. Proximally these bones articulate with the humerus to form the elbow joint. The distal radius and ulna contribute to the antebrachiocarpal joint. During active growth the radius has a proximal and distal epiphyseal plate. The proximal growth plate contributes approximately 35%, whereas the distal plate contributes 75% to the total length of the radius.[34] The ulna has a proximal apophyseal growth plate, which accommodates for longitudinal growth of the olecranon. The ulna has only one distal epiphyseal growth plate, which is responsible for the entire longitudinal development of the ulnar diaphysis. This distal ulnar growth plate is shaped like an inverted cone to enlarge the proliferative zone area and thus the number of chondrocytes contributing to longitudinal growth. This adaptation is shape allows for synchronous longitudinal growth between radius and ulna. In short-legged dogs, this inverted cone shape is less obvious as growth rate in the radius and ulna is much slower.

By its high growth rate and configuration the distal ulnar growth plate is reported to be more vulnerable to trauma than the radial physes.[60,135] The typical presentation of growth retardation or premature closure of the distal ulnar growth plate is the radius curvus syndrome. An ulnar length deficit, cranial bowing of the radius, exorotation of the antebrachium, and valgus deviation of the distal limb in the carpus characterize this syndrome.[25,62,63] In the clinical situation, isolated disturbance of one physis is not common and usually both the distal radial and distal ulnar growth plates are involved.[128,140,183] The severity and localization of the growth disturbance within the radial and ulnar physes will vary, resulting in a heterogeneous presentation of the growth deformities. In addition to the growth deformity, asynchronous development of the radius and ulna can lead to incongruity of the elbow joint.[102,105,106,112] Asynchronous radial and ulnar growth can also result in carpal malalignment and subluxation.[61,117,122,134]

In summary, antebrachial growth deformities are characterized by a combination of length deficits, angular and rotational malalignment, elbow incongruity, and carpal subluxation. Osteoarthritis of the elbow and carpal joint is a common sequel of radial and ulnar growth deformities.

4. Healing of bone fractures

Bone fractures are characterized by discontinuity of bone architecture and loss of function. Bone healing is the reparative process by which bone regains its function. Two different mechanisms of bone repair can be distinguished, i.e., direct and indirect bone healing. Direct bone union is characterized by direct osteonal reconstruction, whereas indirect bone healing depends on the formation of an intermediate fibrous and cartilaginous callus.

Direct bone healing is achieved by internal remodeling of the Haversian systems without resorption of the fracture surfaces and intramembranous ossification. This type of healing is also known as contact healing and occurs with stable fixation and compression of the fracture surfaces. Under these conditions blood vessels can cross the fracture line followed by osteoclasts and osteoblasts, thus forming new Haversian systems. In small stable gaps of up to 0.2 mm in width direct union can also occur by direct deposition of lamellar bone. In larger stable gaps of up to 0.8 mm in width direct healing can proceed by the formation of cancellous bone.[41]

Indirect bone healing is characterized by the formation of an intermediate callus. This callus consists of fibrous and cartilaginous tissue. After bone fracture the sequence of events leading to bone healing can be described as hemorrhage in the fracture area, clot formation, inflammatory response, angiogenesis, proliferation of pluripotential mesenchymal cells, fibrous and cartilaginous callus formation, bone formation, and bone remodeling.[192] This sequence of events results in a gradual progressive stabilization of the fracture area with increasingly stronger and stiffer tissues. Callus formation can be subdivided on the basis of location into periosteal callus, intercortical callus, and medullary callus. The periphery of the callus consists of fibrous tissue, which encloses the more centrally located cartilaginous tissue of the callus. Bone formation in the callus proceeds from the periphery of the cartilaginous callus to the central area of the fracture zone. During this process of endochondral bone formation, cancellous bone is produced until the fracture gap is bridged. This cancellous bone is replaced by lamellar bone during remodeling, which can take up to several years. The extent of callus formation depends on several factors, including stability in the fracture area, age, and local blood supply. Increasing instability tends to result in a larger amount of callus formation. In contrast, excessive motion within the fracture zone will result in compromised angiogenesis and delayed union. Prolonged fracture union characterizes delayed union. Bone healing is progressive in delayed union and leads to full recovery of function. In nonunion, fracture healing stops altogether and results in either atrophic or hypertrophic nonunion. Atrophic or nonviable nonunion is characterized by resorption of the fracture ends without callus

formation. In hypertrophic or viable nonunion, callus formation is present at the ends of the fracture segments, but no bridging of the fracture gap occurs. Fractures can develop into nonunions by a variety of factors, including insufficient stability, inadequate reduction, interposition of soft tissues, compromised vascularity, and infection. In addition, systemic factors can contribute to the development of nonunion, including old age, hyperadrenocorticism, hypothyroidism, renal disease, osteoporosis, and GH deficiency.[75]

Fracture healing will proceed by a combination of direct and indirect bone union after osteosynthesis in the clinical situation. The cascade of events during fracture healing culminates in the recruitment of bone forming cells. Various angiogenic and osteogenic growth factors interact with pluripotential mesenchymal cells and their respective differentiated cell lineages during progression of fracture healing. These bone growth factors will be discussed in more detail in the next section of this introduction.

5. Distraction osteogenesis

Distraction osteogenesis is the formation of new bone under gradual mechanical distraction of two bone surfaces. Dr. Gavriil Abramovich Ilizarov was the first to develop distraction osteogenesis into a clinical treatment option, using a circular external skeletal fixation system.[84,85] The technique depends on a minimally invasive osteotomy of the bone while preserving soft tissues, periosteum, endosteum, bone marrow, and intramedullary blood vessels.[98] Stability of the external fixation is essential to allow new capillary blood vessels to bridge the osteotomy and allow for the lengthening procedure. After a latency period, the duration of which depends on the age of the patient and location of the osteotomy, gradual distraction is started with 1 mm per day, usually divided into 2 to 4 steps. New bone is formed in parallel columns extending from the osteotomy surfaces towards a central growth zone of the distraction gap. The growth zone that forms under the influence of tension-stress has features of both endochondral and intramembranous ossification.[5,8,9] After lengthening is ceased, the newly formed bone regenerate is allowed to mature and consolidate until the stage that the bone can support its physiologic load.

In humans, distraction osteogenesis has been used in treating a variety of skeletal conditions, including bone length deficits, growth deformities, bone loss after trauma or radical resection, and craniofacial surgery.[90,113,143,162] In dogs, distraction osteogenesis was introduced in the late 1980s mainly to treat growth deformities of the antebrachium and to a lesser extent of the crus.[105,106,116,117] In

addition to the clinical use of distraction osteogenesis, the dog has been used extensively as an experimental model.[4,8,56,57,95,100,118,129,155,186]

Distraction osteogenesis proved to be a successful model to study the role of growth factors during bone formation. Mechanical distraction stress induces the expression of transforming growth factor- β1 (TGF-β1), insulin-like growth factor-I (IGF-I), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) within the bone regenerate.[31,49,52,100,110,155,186,190] During distraction osteogenesis bone morphogenetic proteins-2 (BMP-2) and BMP-4 are produced by osteoblasts.[53,147] Recent interest has focused on angiogenesis of the distraction induced bone regenerate and the expression of vascular endothelial growth factor-A (VEGF-A), VEGF-D, angiopoietin-1 and angiopoietin-2.[28,44,51,77,82,121,130,142] These studies demonstrate the interdependence of the mechanical environment, angiogenesis, and bone formation during distraction osteogenesis. Expression of angiogenic genes and a proper mechanical environment are essential in supporting new vasculature for bone regeneration. Although our knowledge in this field is expanding, the complex cascade of growth factors regulating bone formation during distraction osteogenesis is still unclear.

6. Circular external skeletal fixation

External skeletal fixation systems have been used already for fracture stabilization from the mid-19th century onwards.[185] The concept of external fixation relies on fixation of bone by percutaneous pins linked with external connectors. External skeletal fixation systems can be used in various configurations. The three basic frame designs are the type I or unilateral configuration, the type II or bilateral fixation with transosseus pins, and the type III external fixation which combines types I and II to create a three dimensional frame.

Circular external skeletal fixation stands apart from the traditional external fixation systems as it is characterized by the use of metal rings surrounding the limb. Although circular external skeletal fixation systems were described in the early 20th century, Dr. Gavriil Abramovich Ilizarov was the first to develop this method of fixation into a clinical treatment modality.[84,85] Circular external skeletal fixation relies on the use of transosseus wires under tension rather than pins to connect the bone to the external fixation rings (Fig 4). The system is highly versatile and permits the use of partial rings and posts for transcutaneous pins thus encompassing the features of traditional external fixators. In addition, hybridization of the circular fixation with other external fixation configurations is possible. Initially, Dr. Ilizarov developed his circular fixation system to treat fractures and nonunion. In order to stabilize bone fractures under compression, threaded

connecting rods were supplemented to the system. Anecdotal, Dr. Ilizarov recognized new bone formation in a patient who accidentally distracted rather than compressed the fracture gap. This observation was the start of his pursuit culminating in the concept of distraction osteogenesis.
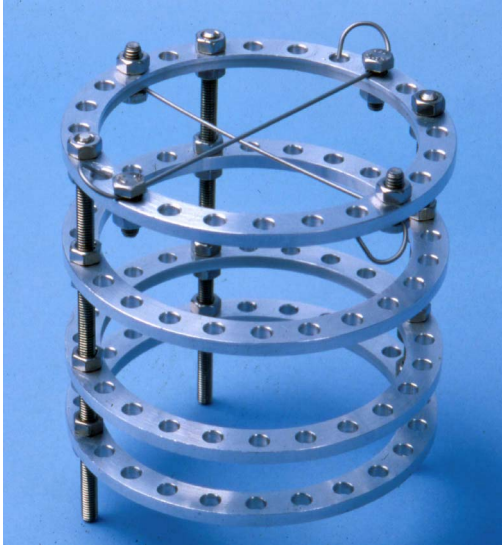
Fig 4. Circular external skeletal fixation system including rings, threaded connecting rods, and transosseus wires

Circular external skeletal fixation is used in veterinary medicine to treat a variety of fracture types.[105,106,108,115] Its use in companion animals is restricted mainly to fractures distal of the elbow and stifle joint. Although hybrid frame designs can be used on the humerus and femur, the use of transosseus wires proximal of the elbow and knee has high morbidity associated with the large muscle volume in these areas. In addition to fracture treatment, circular fixation has been used effectively to perform panarthrodesis of carpal and tarsal joints. The stability of the frame depends on several factors. The most important parameter is ring diameter.[107] Larger ring diameters require longer transosseus wires, which result in larger moments on the wire-bone and wire-bolt interface. Ring diameter is determined depending on the diameter of the limb adding approximately 2 cm on either side to allow for soft tissue swelling. Larger diameters will result in increased instability at the fracture site. The second most important parameter is wire tension.[7,107] Tensioning the transosseus wires will increase frame stability. Tension depends on the body weight of the patient and is applied by use of a dynamometric wire tensioner. An equivalent of 20 kg (±200 N) is used for dogs up

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to 10 kg in body weight, an equivalent of 40 kg (±400 N) for dogs between 10 and 20 kg, an equivalent of 60 kg (±600 N) for dogs between 20 and 30 kg, and an equivalent of 80 kg (±800 N) for dogs 30 and 40 kg in body weight. Equivalents of up to 120 kg (±1200 N) can be applied in giant breed dogs.[84,85,105,106] In the basic configuration of the circular frame two transosseus wires are used per ring. The divergence angle between the transosseus wires has little impact on axial stability and is usually maintained between 60-90 degrees. Angles of divergence smaller than 60 degrees will decrease rotational and bending stability. Slippage of wires at the wire-bolt interface is a common cause for loss of initial stability. It is essential to tighten the nuts correctly after tensioning and to retighten them after cutting excess wire length and bending of the protruding wire ends to prevent auto-mutilation.

The basic design of circular fixators includes two full rings proximal of the fracture area each with two tensioned transosseus wires and two full rings distally. The rings are connected with 3 or 4 threaded rods to create a very stable frame. Transosseus wires with a diameter ranging from 1.2 to 1.6 mm are typically used in companion animals. Circular external skeletal fixation is very resistant to strain in bending and torsion. In contrast, it allows for micro-motion in the axial direction. This dynamic feature of circular fixation is considered to stimulate bone formation and thus bone healing.[6,8]

By analogy with all other external skeletal fixation systems, infection at the transosseus wire-skin interface is the most common complication.[105,106,113] This complication usually occurs at sites where there are large soft tissue masses covering the bone. Movement of soft tissues especially muscles will result in sliding movement at the wire-skin interface thus creating an easy access for bacteria. Avoiding large muscle masses can reduce this complication to a minimum. Proper care of the circular external skeletal fixation system especially of the transosseus wires remains essential as wire-tract infection is painful and restricts function. Bandages are effective in stabilizing the soft tissues adjacent to the transosseus wires and in protecting the skin-wire interface. Wire-tract infection should be treated aggressively with antibiotics and local wound care to avoid progression of the infection to the transosseus wire-bone interface. Established infection will necessitate transosseus wire removal and revision of the frame design.

Circular external skeletal fixation is well tolerated with proper care. Dogs are able to bear weight shortly after surgery, which is beneficial for both bone healing and musculoskeletal function. Dogs can be managed on an outpatient basis with weekly checkups. Exercise should be limited to leash walks until bone has consolidated and the frame can be removed. Although circular external skeletal

fixation is a valuable asset in treating fractures, its strength lies in the management of growth deformities and bone deficits.[47,116,117]

7. Hormonal regulation of bone formation

The endocrine regulation of bone is mediated through several hormones, including growth hormone (GH), calcitonin, parathyroid hormone (PTH), calcitriol, androgen, and estrogen.[126,167,174] Growth hormone is essential for longitudinal growth occurring in the epiphyseal plate through the process of endochondral ossification.[12,33,54,125] Part of the actions of GH is mediated through the induction of IGF-I, but GH also directly promotes longitudinal bone growth.[158,188] The expression of growth hormone receptor (GHR) within the growth plate is consistent with this finding.[37,71,93] At least in humans, GH has a major role in the maintenance of bone mass in the adult skeleton by regulating bone remodeling through a complex interaction of circulating GH, insulin-like growth factors (IGFs), insulin-like growth factors binding proteins (IGFBPs), and locally produced IGFs and IGFBPs.[177] Relative or absolute GH deficiency thus results in osteoporosis.[18,146,159] In dogs, GH deficiency or estrogen depletion does not lead to osteoporosis.[176] Osteoporosis does occur in renal or alimentary induced hyperparathyroidism in dogs.[157]

Growth hormone has been used successfully in treating GH-deficiency related osteoporosis and postmenopausal osteoporosis in humans.[101,146] In view of its role in bone metabolism, GH has been used experimentally to stimulate osteogenesis. Systemic GH application effectively stimulated bone formation in fracture models in different species, including dogs.[10,149,191] In addition, GH was demonstrated to enhance consolidation of the bone regenerate after distraction osteogenesis.[11,136]

Calcitonin, PTH and calcitriol are crucial in maintaining calcium homeostasis.[123,166,174] Calcitonin is synthesized by the clear cells of the thyroid gland and stimulates calcium deposition while inhibiting bone resorption. Parathyroid hormone is synthesized by the parathyroid glands, which are located in or adjacent to the thyroid glands. The function of PTH is to mobilize calcium from bone and reabsorb calcium in the kidney. Continuously increased PTH levels increase osteoclast activity mediated through the shrinkage of osteoblasts thus exposing bone to osteoclasts, whereas intermittent treatment with PTH stimulates osteoblast activity and thus bone formation.[67]

Skeletal growth and puberty are connected since sex hormones estrogen and androgen play a role in cartilage growth and endochondral ossification. Early castration results in delayed closure of the growth plate with subsequent increased

final bone length.[144] In adult dogs, estrogen and androgen do not seem to be essential in maintaining bone mass. Ovariectomy or orchydectomy, even at a young age, does not result in clinical osteoporosis. Nevertheless, estrogen depletion after ovariectomy does negatively affect cancellous bone growth and increases cortical porosity.[156,193]

8. Skeletal growth factors

The family of insulin-like growth factors (IGF) consists of IGF-I, IGF-II and insulin. IGF-I and IGF-II are major constituents of both local and systemic growth factors.[125,196] There are three receptors each with the highest affinity for their specific IGF. These receptors, particularly for IGF-I, are present in almost all tissues. IGF-I is mainly produced in the liver, but also in peripheral tissues including bone.[46,158] Knowledge about the role of IGF-II in bone formation and bone healing remains limited.[13] IGF-I and IGF-II are important as local growth factors for osteoblast survival and apoptosis.[81] By modulating osteoblast-osteoclast interactions IGF-I and IGF-II are critical in bone remodeling and sustaining bone mass.[78,80] In vitro, IGF-I stimulates existing bone resorption by existing osteoclasts and the formation of new osteoclasts from precursor cells.[119] Upregulation of IGF-I and IGF-II expression occurs during bone healing.[164] IGF-I is also expressed in the bone regenerate during distraction osteogenesis.[170] Local treatment with IGF-I stimulates bone healing and bone consolidation after distraction osteogenesis.[97,165]

Six high-affinity IGF binding proteins (IGFBP) regulate the actions of IGF-I and IGF-II.[92,120] The IGFBPs are produced by osteoblast and IGFBP-4 and IGFBP-5 are the most abundant.[74,120] In general, IGFBP-1, -2, -4, and -6 inhibit and IGFBP-3 and -5 stimulate osteoblast function.[30,43,50] Overexpression of IGFBP-2 was demonstrated to impair long bone development in vivo by blocking the ability of IGF-I and IGF-II to promote cell proliferation and matrix synthesis.[58] Growth hormone treatment in postmenopausal women resulted in elevated serum levels of IGF-I, IGF-II, IGFBP-3, IGFBP-4, and decreased serum levels of IGFBP-1 and IGFBP-2. Serum levels of IGFBP-4 and IGFBP-5 correlated with bone mineral density in GH-deficient adults. Growth hormone replacement therapy increased both IGFBPs.[172]

Although IGFs and IGFBPs are important for bone maintenance and formation their precise role is still to be elucidated.

The transforming growth factor-β superfamily consists of the transforming growth factor-βs (TGF-βs) and the bone morphogenetic proteins (BMPs). The TGF-βs are multifunctional peptides expressed in mammals as highly homologous isoforms, TGF-β1, TGF-β2, and TGF-β3. The TGF-βs act via autocrine and

paracrine modes to control a variety of developmental processes.[14] TGF-βs stimulate osteoblast proliferation and matrix production. In this way they are important regulators of bone formation and fracture repair[17,19,163]An early decline in serum TGF-β1 levels was demonstrated in patients with delayed fracture healing.[197] TGF-βs have been used successfully to stimulate fracture healing. [16] In distraction osteogenesis, TGF-βs are typically expressed during the phase of active lengthening.[110,170] Experimental stimulation of bone consolidation with TGF-β1 during distraction osteogenesis was not successful indicating a role for TGF-βs especially in the early stages of osteogenesis.[138,151]

        BMPs were first described as factors capable of inducing new bone.[179]The group of BMPs comprises at least 15 growth factors, which are highly osteoinductive.[29] Besides their role in osteogenesis, BMPs exert essential functions during embryogenesis.[184] BMPs bind to two distinct types of transmembrane receptors with serine-threonine kinase activity. The activated receptors phosphorylate Smad proteins, which act as intracellular signal mediators.[24,104] These Smads regulate the expression of target genes. The TGF-βs signal through the same Smad pathways. The BMPs can induce endochondral bone formation through the proliferation and differentiation of chondrocytes and osteoblasts.[175,187] BMP-2, BMP-4, and BMP-7 expression was highest during the early phases of fracture healing.[161] The expression of these BMPs diminished after the initial phases of osteogenesis. By analogy, BMP-2, BMP-4, and BMP-7 expression was most prominent during the phase of active lengthening during distraction osteogenesis, to decrease during the phase of bone consolidation.[137] BMP-2 and BMP-7 are the most potent osteoinductive growth factors and have been used both experimentally and clinically to stimulate bone formation.[73,91,181] Part of the mitogenic action of BMP-7 is mediated through the modulation of IGF-II secretion and the balance between stimulatory and inhibitory IGFBPs.[96] BMP-2 was most effective in stimulating bone healing in an experimental fracture model when administered immediately postoperatively.[124]   The delivery systems by which BMPs are administered also play an important role in their effectiveness.[152] In dogs, BMPs have been used successfully to treat nonunions and segmental bone defects.[35,79]

        The group of fibroblast growth factors (FGFs) consists of 18 growth factors. Fibroblast growth factor-1 (FGF-1 or acidic FGF) and fibroblast growth factor-2 (FGF-2 or basic FGF) are the prototypic members of the FGF family. Fibroblast growth factors and their receptors have an important role in the control of endochondral and intramembranous bone formation.[14,72] The expression of FGFs is closely related to the expression of vascular endothelial growth factors (VEGFs). Both FGF-2 and VEGFs are expressed in the early stages of distraction osteogenesis and are considered essential for angiogenesis.[82,130,195] Expression of

VEGFs occurs at the osteogenic front during distraction osteogenesis and precedes the expression of BMPs by osteoblasts. This demonstrates that angiogenesis is induced before osteogenesis.[160] VEGF was capable of stimulating bone healing in an atrophic nonunion model.[45] In contrast, VEGF application was not effective in increasing bone consolidation in a distraction osteogenesis model.[44] Combined delivery of angiogenic (VEGF) and osteogenic (BMP) factors was more effective in enhancing new bone formation than application of the single factors.[83]

Platelet-derived growth factor (PDGF) was initially isolated from platelets, but was found to be expressed by skeletal and a variety of non-skeletal tissues.[19] Nevertheless, platelets are the major source of this growth factor, which is released following platelet aggregation. Aggregation of platelets typically occurs after fracture and soft tissue trauma. This emphasizes the role of PDGF in fracture repair. Platelet-derived growth factor increases the replication of cells of osteoblastic and osteoclastic lineages.[14]

Hepatocyte growth factor (HGF) was discovered as a potent growth-promoting agent in liver cells. HGF is expressed in most tissues and plays an important role in tissue repair. This growth factor stimulates both osteoclasts and osteoblasts, thus regulating bone remodeling. In addition, HGF is expressed at the fracture site and induces upregulation of BMP receptors.[86] In this way, HGF facilitates BMP signaling and stimulates bone healing.

The identification of the receptor activator of nuclear factor kappaB ligand (RANKL), its cognate receptor RANK, and its decoy receptor osteoprotegerin (OPG) resulted in a new perspective on osteoclast function and bone homeostasis.[169] Osteoclast precursors express RANK and differentiate into osteoclasts after recognizing RANKL through cell-to-cell interaction with osteoblasts and stromal cells.[166,171] Membrane bound colony-stimulating factor-1 (mCSF-1) plays an important role in osteoblast-mediated osteoclastogenesis.[189,194] Osteoprotegerin acts as a soluble receptor antagonist for RANKL that prevents it from binding to and activating RANK. Hepatitis C-associated osteosclerosis is characterized by diffuse osteosclerosis, decreased osteoclast numbers, and increased OPG serum levels in adult humans. An imbalance in the OPG/RANKL system is supposed to be responsible for these findings.[114] There is increasing evidence that the OPG/RANKL system links the skeletal with the vascular system.[141,148] These findings may lead to new therapeutic regimes in preventing bone resorption. For instance, GH replacement therapy increased OPG levels, which may lead to a positive bone balance by inhibiting osteoclastogenesis.[103,178]

Ghrelin, the endogenous ligand for the GH secretagogue receptor (GHS-R) is a recently discovered brain-gut peptide involved in GH secretion and energy homeostasis.[66] In addition, ghrelin is widely expressed in several tissues, where it might therefore act as a paracrine or autocrine factor. Ghrelin directly regulates

bone formation by stimulating osteoblast proliferation, differentiation, and function and by inhibiting apoptosis.[66,94,111] Ghrelin is also synthesized and secreted by chondrocytes.[23] In future, ghrelin and synthetic GHS-R ligands may be effective in modulating bone homeostasis.

9. Bone markers

Bone markers are used to monitor bone metabolism non-invasively. These markers can be divided into markers of bone formation and markers of bone resorption, respectively. Although the commercially available bone marker assays were designed initially for use in humans, several of these assays are also validated to monitor bone metabolism in laboratory animals, dogs and horses.[20,26,27,99]

The markers of bone metabolism can be subdivided into enzymatic markers and metabolic products of bone formation and resorption, respectively.[22,153,154] Enzymatic markers include bone-specific alkaline phosphatase (BAP) and tartrate-resistant acid phosphatase (TRAP)[90,127]. Bone-specific alkaline phosphatase is an osteoblast-related marker of bone formation.[38,69] Tartrate-resistant acid phosphatase-5b (TRAP-5b) is an osteoclast-related marker of bone resorption. [76,173] Serum or plasma markers of metabolic products of bone metabolism include osteocalcin (OC), carboxyterminal propeptide of type-I procollagen (PICP), aminoterminal propeptide of type-I procollagen (PINP), cross-linked carboxyterminal telopeptide of type-I collagen (ICTP), and C-terminal cross-linked telopeptide of type-I collagen (CTX). Osteocalcin is an osteoblast-related marker of bone formation, but its precise function is unknown.[168]. Increased bone formation was present in OC-deficient mice, indicating a suppressive role of OC on bone formation, possibly through inhibition of osteopontin.[42] Markers PICP and PINP are metabolic products of type-I collagen synthesis and hence bone formation.[36,133,167] Markers ICTP and CTX are products of type-I collagen breakdown and hence bone resorption.[2,20,132,150] Marker ICTP is released through the actions of matrix metalloproteinases (MMPs) and is therefore also known as CTX-MMP. Marker CTX is formed from collagen I by cysteine proteinases, including cathepsin K.[70] The collagen type I C-telopeptide is susceptible to molecular rearrangement. In newly synthesized collagen this site is in the native form, but during aging a spontaneous reaction occurs, resulting in three age-modified isomerized and racemized fragments. These modified forms of CTX may provide new diagnostic and monitoring tools in evaluating bone disease.[32]

Bone markers have been used successfully in humans to monitor metabolic bone disease and the effect of treatment. In rheumatoid arthritis, characterized by increased bone resorption, ICTP levels were elevated while OC levels were

decreased.[1] Growth hormone treatment in GH-deficient patients resulted in transient changes in BAP, OC, and ICTP levels.[18,146,159] Serum BAP and PINP were the most sensitive markers for monitoring treatment efficacy in Paget's disease.[3] In Cushing's syndrome, the markers OC and CTX decreased consistent with increased bone resorption.[36] The markers BAP, OC, PINP, and CTX were most effective in monitoring osteoporosis.[39,40] Although bone mass measurements at the present time still supply most information about fracture risk, markers of bone resorption may be useful in predicting fracture risk in osteoporosis.[68] In chronic renal failure, PICP and ICTP have been used as markers of bone formation and resorption, respectively.[133] Bone markers have also been used to monitor bone growth. In pubertal boys and girls, markers of bone metabolism related positively to growth velocity.[182] This finding could be of great interest in monitoring the rapid growth phase in dogs.

In dogs, normal values for BAP, OC and ICTP have been established and are not affected by breed size.[20,99] By analogy with humans, bone markers demonstrate a circadian rhythm in the dog.[99,109,131] Therefore, sampling should be standardized both in an experimental and clinical setting. Bone markers have not been used extensively to monitor bone pathology in dogs. Experimentally induced osteomyelitis increased ICTP serum levels.[132] Orchydectomy in male beagle dogs resulted in decreased BAP and OC levels indicating an imbalance in bone metabolism with diminished bone formation.[65] In canine appendicular osteosarcoma, BAP proved to be a prognostic factor in predicting survival time.[48] Experimentally, OC was used effectively to monitor new bone formation following distraction osteogenesis.[15,55,100,155] Although bone markers can be an adjunct in monitoring bone metabolism, they cannot replace bone histomorphometry in orthopaedic research at the present time.

References

1.   Al Awadhi A, Olusi S, Al Zaid N, Prabha K. Serum concentrations of interleukin 6, osteocalcin, intact parathyroid hormone, and markers of bone resorption in patients with rheumatoid arthritis. *J Rheumatol* 1999;26:1250-1256.
2.   Allen MJ, Hoffmann WE, Richardson DC, Breur GJ. Serum markers of bone metabolism in dogs. *Am J Vet Res* 1998;59:250-254.
3.   Alvarez L, Guanabens N, Peris P, Vidal S, Ros I, Monegal A, Bedini JL, Deulofeu R, Pons F, Munoz-Gomez J, Ballesta AM. Usefulness of biochemical markers of bone turnover in assessing response to the treatment of Paget's disease. *Bone* 2001;29:447-452.
4.   Aronson J. Experimental and clinical experience with distraction osteogenesis. *Cleft Palate Craniofac J* 1994;31:473-481.
5.   Aronson J, Good B, Stewart C, Harrison B, Harp J. Preliminary studies of mineralization during distraction osteogenesis. *Clin Orthop* 1990;250:43-49.
6.   Aronson J, Harp JH. Mechanical forces as predictors of healing during tibial lengthening by distraction osteogenesis. *Clin Orthop* 1994;310:73-79.
7.   Aronson J, Harrison B, Boyd CM, Cannon DJ, Lubansky HJ. Mechanical induction of osteogenesis: the importance of pin rigidity. *J Pediatr Orthop* 1988;8:396-401.
8.   Aronson J, Harrison B, Boyd CM, Cannon DJ, Lubansky HJ, Stewart C. Mechanical induction of Osteogenesis. Preliminary studies. *Ann Clin Lab Sci* 1988;18:195-203.
9.   Aronson J, Harrison BH, Stewart CL, Harp JH, Jr. The histology of distraction osteogenesis using different external fixators. *Clin Orthop* 1989;241:106-116.
10.   Bail HJ, Kolbeck S, Krummrey G, Schmidmaier G, Haas NP, Raschke MJ. Systemic application of growth hormone for enhancement of secondary and intramembranous fracture healing. *Horm Res* 2002;58 Suppl 3:39-42.
11.   Bail HJ, Kolbeck S, Lindner T, Dahne M, Weiler A, Windhagen HJ, Raun K, Skjaerbaek C, Flyvbjerg A, Orskov H, Haas NP, Raschke MJ. The effect of growth hormone on insulin-like growth factor I and bone metabolism in distraction osteogenesis. *Growth Horm IGF Res* 2001;11:314-323.
12.   Baroncelli GI, Bertelloni S, Ceccarelli C, Cupelli D, Saggese G. Dynamics of bone turnover in children with GH deficiency treated with GH until final height. *Eur J Endocrinol* 2000;142:549-556.
13.   Bautista CM, Mohan S, Baylink DJ. Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. *Metabolism* 1990;39:96-100.
14.   Baylink DJ, Finkelman RD, Mohan S. Growth factors to stimulate bone formation. *J Bone Miner Res* 1993;8 Suppl 2:565-572.
15.   Blair HC, Robinson LJ, Zaidi M. Osteoclast signalling pathways. *Biochem Biophys Res Commun* 2005;328:728-738.
16.   Blumenfeld I, Srouji S, Lanir Y, Laufer D, Livne E. Enhancement of bone defect healing in old rats by TGF-beta and IGF-1. *Exp Gerontol* 2002;37:553-565.
17.   Bolander ME. Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med* 1992;200:165-170.
18.   Bollerslev J, Moller J, Thomas S, Djoseland O, Christiansen JS. Dose-dependent effects of recombinant human growth hormone on biochemical markers of bone and collagen metabolism in adult growth hormone deficiency. *Eur J Endocrinol* 1996;135:666-671.
19.   Bourque WT, Gross M, Hall BK. Expression of four growth factors during fracture repair. *Int J Dev Biol* 1993;37:573-579.

20. Breur GJ, Allen MJ, Carlson SJ, Richardson DC. Markers of bone metabolism in dog breeds of different size. *Res Vet Sci* 2004;76:53-55.
21. Brown JH, DeLuca SA. Growth plate injuries: Salter-Harris classification. *Am Fam Physician* 1992;46:1180-1184.
22. Calvo MS, Eyre DR, Gundberg CM. Molecular basis and clinical application of biological markers of bone turnover. *Endocr Rev* 1996;17:333-368.
23. Caminos JE, Gualillo O, Lago F, Otero M, Blanco M, Gallego R, Garcia-Caballero T, Goldring MB, Casanueva FF, Gomez-Reino JJ, Dieguez C. The endogenous growth hormone secretagogue (ghrelin) is synthesized and secreted by chondrocytes. *Endocrinology* 2005;146:1285-1292.
24. Cao X, Chen D. The BMP signaling and in vivo bone formation. *Gene* 2005;357:1-8.
25. Carmichael S. Current concepts in the management of growth plate abnormalities. *Vet Ann* 1992;32:47-60.
26. Carstanjen B, Hoyle NR, Gabriel A, Hars O, Sandersen C, Amory H, Remy B. Evaluation of plasma carboxy-terminal cross-linking telopeptide of type I collagen concentration in horses. *Am J Vet Res* 2004;65:104-109.
27. Carstanjen B, Sulon J, Banga-Mboko H, Beckers JF, Remy B. Development and validation of a specific radioimmunoassay for equine osteocalcin. *Domest Anim Endocrinol* 2003;24:31-41.
28. Carvalho RS, Einhorn TA, Lehmann W, Edgar C, Al Yamani A, Apazidis A, Pacicca D, Clemens TL, Gerstenfeld LC. The role of angiogenesis in a murine tibial model of distraction osteogenesis. *Bone* 2004;34:849-861.
29. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. *Growth Factors* 2004;22:233-241.
30. Chevalley T, Strong DD, Mohan S, Baylink D, Linkhart TA. Evidence for a role for insulin-like growth factor binding proteins in glucocorticoid inhibition of normal human osteoblast-like cell proliferation. *Eur J Endocrinol* 1996;134:591-601.
31. Cillo JE, Jr., Gassner R, Koepsel RR, Buckley MJ. Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: implications for distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000;90:147-154.
32. Cloos PA, Fledelius C, Christgau S, Christiansen C, Engsig M, Delmas P, Body JJ, Garnero P. Investigation of bone disease using isomerized and racemized fragments of type I collagen. *Calcif Tissue Int* 2003;72:8-17.
33. Conzemius MG, Brown DC, Brabec M, Smith GK, Washabau R, LaFond E, Chakraborty PK. Correlation between longitudinal bone growth, growth hormone, and insulin-like growth factor-I in prepubertal dogs. *Am J Vet Res* 1998;59:1608-1612.
34. Conzemius MG, Smith GK, Brighton CT, Marion MJ, Gregor TP. Analysis of physeal growths in dogs, using biplanar radiography. *Am J Vet Res* 1994;55:22-27.
35. Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC. Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. *Clin Orthop* 1994;301:302-312.
36. Cortet B, Cortet C, Blanckaert F, d'Herbomez M, Marchandise X, Wemeau JL, Decoulx M, Dewailly D. Quantitative ultrasound of bone and markers of bone turnover in Cushing's syndrome. *Osteoporos Int* 2001;12:117-123.
37. Cruickshank J, Grossman DI, Peng RK, Famula TR, Oberbauer AM. Spatial distribution of growth hormone receptor, insulin-like growth factor-I receptor and apoptotic chondrocytes during growth plate development. *J Endocrinol* 2005;184:543-553.
38. de Vernejoul MC. Markers of bone remodelling in metabolic bone disease. *Drugs Aging* 1998;12 Suppl 1:9-14.

39.   Delmas PD. Markers of bone turnover for monitoring treatment of osteoporosis with antiresorptive drugs. *Osteoporos Int* 2000;11 Suppl 6:66-76.
40.   Delmas PD, Eastell R, Garnero P, Seibel MJ, Stepan J. The use of biochemical markers of bone turnover in osteoporosis. *Osteoporos In* 2000;11 Suppl 6:2-17.
41.   Draenert Y, Draenert K. Gap healing of compact bone. *Scan Electron Microsc* 1980;103-111.
42.   Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996;382:448-452.
43.   Durham SK, Riggs BL, Conover CA. The insulin-like growth factor-binding protein-4 (IGFBP-4)-IGFBP-4 protease system in normal human osteoblast-like cells: regulation by transforming growth factor-beta. *J Clin Endocrinol Metab* 1994;79:1752-1758.
44.   Eckardt H, Bundgaard KG, Christensen KS, Lind M, Hansen ES, Hvid I. Effects of locally applied vascular endothelial growth factor (VEGF) and VEGF-inhibitor to the rabbit tibia during distraction osteogenesis. *J Orthop Res* 2003;21:335-340.
45.   Eckardt H, Ding M, Lind M, Hansen ES, Christensen KS, Hvid I. Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model. *J Bone Joint Surg Br* 2005;87:1434-1438.
46.   Edwall D, Schalling M, Jennische E, Norstedt G. Induction of insulin-like growth factor I messenger ribonucleic acid during regeneration of rat skeletal muscle. *Endocrinology* 1989;124:820-825.
47.   Ehrhart N. Longitudinal bone transport for treatment of primary bone tumors in dogs: technique description and outcome in 9 dogs. *Vet Surg* 2005;34:24-34.
48.   Ehrhart N, Dernell WS, Hoffmann WE, Weigel RM, Powers BE, Withrow SJ. Prognostic importance of alkaline phosphatase activity in serum from dogs with appendicular osteosarcoma: 75 cases (1990-1996). *J Am Vet Med Assoc* 1998;213:1002-1006.
49.   Eingartner C, Coerper S, Fritz J, Gaissmaier C, Koveker G, Weise K. Growth factors in distraction osteogenesis. Immuno-histological pattern of TGF-beta1 and IGF-I in human callus induced by distraction osteogenesis. *Int Orthop* 1999;23:253-259.
50.   Ernst M, Rodan GA. Increased activity of insulin-like growth factor (IGF) in osteoblastic cells in the presence of growth hormone (GH): positive correlation with the presence of the GH-induced IGF-binding protein BP-3. *Endocrinology* 1990;127:807-814.
51.   Fang TD, Salim A, Xia W, Nacamuli RP, Guccione S, Song HM, Carano RA, Filvaroff EH, Bednarski MD, Giaccia AJ, Longaker MT. Angiogenesis is required for successful bone induction during distraction osteogenesis. *J Bone Miner Res* 2005;20:1114-1124.
52.   Farhadieh RD, Dickinson R, Yu Y, Gianoutsos MP, Walsh WR. The role of transforming growth factor-beta, insulin-like growth factor I, and basic fibroblast growth factor in distraction osteogenesis of the mandible. *J Craniofac Surg* 1999;10:80-86.
53.   noutsos MP, Yu Y, Walsh WR. The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. *J Craniofac Surg* 2004;15:714-718.
54.   Favier RP, Mol JA, Kooistra HS, Rijnberk A. Large body size in the dog is associated with transient GH excess at a young age. *J Endocrinol* 2001;170:479-484.
55.   Fink B, Fox F, Singer J, Skripitz R, Feldkamp J. Monitoring of bone formation during distraction osteogenesis via osteocalcin: a time sequence study in dogs. *J Orthop Sci* 2002;7:557-561.
56.   Fink B, Schwinger G, Singer J, Sager M, Wilke C, Braunstein S. The effect of tibial lengthening using the Ilizarov method on the cartilage and the menisci of the knee joint. *J Orthop Res* 2001;19:665-670.

57.   Fink B, Schwinger G, Singer J, Schmielau G, Ruther W. Biomechanical properties of tendons during lower-leg lengthening in dogs using the Ilizarov method. *J Biomech* 1999;32:763-768.
58.   Fisher MC, Meyer C, Garber G, Dealy CN. Role of IGFBP2, IGF-I and IGF-II in regulating long bone growth. *Bone* 2005;37:741-750.
59.   Fjeld TO. Growth plate injury and retardation of growth in the canine radius and ulna Traumatiske epifyseskiveskader og vekstretardasjon i radius og ulna hos hund. *Norsk Vet* 1986;98:513-519.
60.   Fjeld TO. Patterns of traumatic premature closure of growth plates in the canine radius and ulna
De ulike typer av traumatisk betinget prematur epifyseskivelukning i radius og ulna hos hund. *Norsk Vet* 1986;98:633-641.
61.   Forell EB, Schwarz PD. Use of external skeletal fixation for treatment of angular deformity secondary to premature distal ulnar physeal closure. *J Am Anim Hosp Assoc* 1993;29:460-476.
62.   Fox SM. Premature closure of distal radial and ulnar physes in the dog. I. Pathogenesis and diagnosis. *Compend Contin Educ Pract Vet* 1984;6:128-138.
63.   Fox SM. Premature closure of distal radial and ulnar physes in the dog. II. Treatment. *Comp Cont Educ Pract Vet* 1984;6:212-221.
64.   Frierson M, Ibrahim K, Boles M, Bote H, Ganey T. Distraction osteogenesis. A comparison of corticotomy techniques. *Clin Orthop* 1994;301:19-24.
65.   Fukuda S, Iida H. Effects of orchidectomy on bone metabolism in beagle dogs. *J Vet Med Sci* 2000;62:69-73.
66.   Fukushima N, Hanada R, Teranishi H, Fukue Y, Tachibana T, Ishikawa H, Takeda S, Takeuchi Y, Fukumoto S, Kangawa K, Nagata K, Kojima M. Ghrelin directly regulates bone formation. *J Bone Miner Res* 2005;20:790-798.
67.   Gabet Y, Kohavi D, Muller R, Chorev M, Bab I. Intermittently administered parathyroid hormone 1-34 reverses bone loss and structural impairment in orchiectomized adult rats. *Osteoporos Int* 2005;16:1436-1443.
68.   Garnero P. Markers of bone turnover for the prediction of fracture risk. *Osteoporos Int* 2000;11 Suppl 6:55-65.
69.   Garnero P, Delmas PD. Bone markers. *Baillieres Clin Rheumatol* 1997;11:517-537.
70.   Garnero P, Ferreras M, Karsdal MA, Nicamhlaoibh R, Risteli J, Borel O, Qvist P, Delmas PD, Foged NT, Delaisse JM. The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. *J Bone Miner Res* 2003;18:859-867.
71.   Gevers EF, van der Eerden BC, Karperien M, Raap AK, Robinson IC, Wit JM. Localization and regulation of the growth hormone receptor and growth hormone-binding protein in the rat growth plate. *J Bone Miner Res* 2002;17:1408-1419.
72.   Goodman SB, Song Y, Yoo JY, Fox N, Trindade MC, Kajiyama G, Ma T, Regula D, Brown J, Smith RL. Local infusion of FGF-2 enhances bone ingrowth in rabbit chambers in the presence of polyethylene particles. *J Biomed Mater Res A* 2003;65:454-461.
73.   Govender PV, Rampersaud YR, Rickards L, Fehlings MG. Use of osteogenic protein-1 in spinal fusion: literature review and preliminary results in a prospective series of high-risk cases. *Neurosurg Focus* 2002;13 e4:1-6.
74.   Govoni KE, Baylink DJ, Mohan S. The multi-functional role of insulin-like growth factor binding proteins in bone. *Pediatr Nephrol* 2005;20:261-268.
75.   Green E, Lubahn JD, Evans J. Risk factors, treatment, and outcomes associated with nonunion of the midshaft humerus fracture. *J Surg Orthop Adv* 2005;14:64-72.
76.   Halleen JM, Alatalo SL, Suominen H, Cheng S, Janckila AJ, Vaananen HK. Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption. *J Bone Miner Res* 2000;15:1337-1345.

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77.   Hansen-Algenstaedt N, Algenstaedt P, Bottcher A, Joscheck C, Schwarzloh B, Schaefer C, Muller I, Koike C, Ruther W, Fink B. Bilaterally increased VEGF-levels in muscles during experimental unilateral callus distraction. *J Orthop Res* 2003;21:805-812.

78.   Hayden JM, Mohan S, Baylink DJ. The insulin-like growth factor system and the coupling of formation to resorption. *Bone* 1995;17:93-98.

79.   Heckman JD, Boyan BD, Aufdemorte TB, Abbott JT. The use of bone morphogenetic protein in the treatment of non-union in a canine model. *J Bone Joint Surg Am* 1991;73:750-764.

80.   Hill PA, Reynolds JJ, Meikle MC. Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. *Endocrinology* 1995;136:124-131.

81.   Hill PA, Tumber A, Meikle MC. Multiple extracellular signals promote osteoblast survival and apoptosis. *Endocrinology* 1997;138:3849-3858.

82.   Hu J, Zou S, Li J, Chen Y, Wang D, Gao Z. Temporospatial expression of vascular endothelial growth factor and basic fibroblast growth factor during mandibular distraction osteogenesis. *J Craniomaxillofac Surg* 2003;31:238-243.

83.   Huang YC, Kaigler D, Rice KG, Krebsbach PH, Mooney DJ. Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration. *J Bone Miner Res* 2005;20:848-857.

84.   Ilizarov GA. The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation. *Clin Orthop* 1989;238:249-281.

85   Ilizarov GA. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. *Clin Orthop* 1989;239:263-285.

86.   Imai Y, Terai H, Nomura-Furuwatari C, Mizuno S, Matsumoto K, Nakamura T, Takaoka K. Hepatocyte Growth Factor Contributes to Fracture Repair by Upregulating the Expression of BMP Receptors. *J Bone Miner Res* 2005;20:1723-1730.

87.   Jazrawi LM, Majeska RJ, Klein ML, Kagel E, Stromberg L, Einhorn TA. Bone and cartilage formation in an experimental model of distraction osteogenesis. *J Orthop Trauma* 1998;12:111-116.

88.   Johnson JA, Austin C, Breur GJ. Incidence of canine appendicular musculoskeletal disorders in 16 veterinary teaching hospitals from 1980 through 1989. *Vet Comp Orthop Traumatol* 1994;7:56-69.

89.   Johnson JM, Johnson AL, Eurell JA. Histological appearance of naturally occurring canine physeal fractures. *Vet Surg* 1994;23:81-86.

90.   Kabata T, Tsuchiya H, Sakurakichi K, Yamashiro T, Watanabe K, Tomita K. Reconstruction with distraction osteogenesis for juxta-articular nonunions with bone loss. *J Trauma* 2005;58:1213-1222.

91.   Kain MS, Einhorn TA. Recombinant human bone morphogenetic proteins in the treatment of fractures. *Foot Ankle Clin* 2005;10:639-650.

92.   Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM, Rosenfeld RG. Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. *Int J Biochem Cell Biol* 1996;28:619-637.

93.   Kelly PA, Finidori J, Moulin S, Kedzia C, Binart N. Growth hormone receptor signalling and actions in bone growth. *Horm Res* 2001;55 Suppl 2:14-17.

94.   Kim SW, Her SJ, Park SJ, Kim D, Park KS, Lee HK, Han BH, Kim MS, Shin CS, Kim SY. Ghrelin stimulates proliferation and differentiation and inhibits apoptosis in osteoblastic MC3T3-E1 cells. *Bone* 2005;37:359-369.

95.   Klotch DW, Ganey TM, Slater-Haase A, Sasse J. Assessment of bone formation during osteoneogenesis: a canine model. *Otolaryngol Head Neck Surg* 1995;112:291-302.

96.   Knutsen R, Honda Y, Strong DD, Sampath TK, Baylink DJ, Mohan S. Regulation of insulin-like growth factor system components by osteogenic protein-1 in human bone cells. *Endocrinology* 1995;136:857-865.

97.   Kobayashi K, Agrawal K, Jackson IT, Vega JB. The effect of insulin-like growth factor 1 on craniofacial bone healing. *Plast Reconstr Surg* 1996;97:1129-1135.

98.   Kojimoto H, Yasui N, Goto T, Matsuda S, Shimomura Y. Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. *J Bone Joint Surg Br* 1988;70:543-549.

99.   Ladlow JF, Hoffmann WE, Breur GJ, Richardson DC, Allen MJ. Biological variability in serum and urinary indices of bone formation and resorption in dogs. *Calcif Tissue Int* 2002;70:186-193.

100. L ammens J, Liu Z, Aerssens J, Dequeker J, Fabry G. Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. *J Bone Miner Res* 1998;13:279-286.

101. Landin-Wilhelmsen K, Nilsson A, Bosaeus I, Bengtsson BA. Growth hormone increases bone mineral content in postmenopausal osteoporosis: a randomized placebo-controlled trial. *J Bone Miner Res* 2003;18:393-405.

102. Langley-Hobbs SJ, Carmichael S, Pead MJ, Torrington AM. Management of antebrachial deformity and shortening secondary to a synostosis in a dog. *J Small Anim Pract* 1996;37:359-363.

103. Lanzi R, Losa M, Villa I, Gatti E, Sirtori M, Dal Fiume C, Rubinacci A. GH replacement therapy increases plasma osteoprotegerin levels in GH-deficient adults. *Eur J Endocrinol* 2003;148:185-191.

104. Larsson J, Karlsson S. The role of Smad signaling in hematopoiesis. *Oncogene* 2005;24:5676-5692.

105. Latte Y. Application of the Ilizarov method in veterinary orthopaedic surgery (part 1). *Eur J Comp Anim Pract* 1997;7:26-50.

106. Latte Y. 75 applications of the Ilizarov method (part 2). *Eur J Comp Anim Pract* 1998;8:64-81.

107. Lewis DD, Bronson DG, Cross AR, Welch RD, Kubilis PS. Axial characteristics of circular external skeletal fixator single ring constructs. *Vet Surg* 2001;30:386-394.

108. Lewis DD, Cross AR, Carmichael S, Anderson MA. Recent advances in external skeletal fixation. *J Small Anim Pract* 2001;42:103-112.

109. Liesegang A, Reutter R, Sassi ML, Risteli J, Kraenzlin M, Riond JL, Wanner M. Diurnal variation in concentrations of various markers of bone metabolism in dogs. *Am J Vet Res* 1999;60:949-953.

110. Liu Z, Luyten FP, Lammens J, Dequeker J. Molecular signaling in bone fracture healing and distraction osteogenesis. *Histol Histopathol* 1999;14:587-595.

111. Maccarinelli G, Sibilia V, Torsello A, Raimondo F, Pitto M, Giustina A, Netti C, Cocchi D. Ghrelin regulates proliferation and differentiation of osteoblastic cells. *J Endocrinol* 2005;184:249-256.

112. MacPherson GC, Lewis DD, Johnson KA, Allen GS, Yovich JC. Fragmented coronoid process associated with premature distal radial physeal closure in four dogs. *Vet Comp Orthop Traumatol* 1992;5:93-99.

113. Maffulli N, Lombari C, Matarazzo L, Nele U, Pagnotta G, Fixsen JA. A review of 240 patients undergoing distraction osteogenesis for congenital post-traumatic or postinfective lower limb length discrepancy. *J Am Coll Surg* 1996;182:394-402.

114. Manganelli P, Giuliani N, Fietta P, Mancini C, Lazzaretti M, Pollini A, Quaini F, Pedrazzoni M. OPG/RANKL system imbalance in a case of hepatitis C-associated osteosclerosis: the pathogenetic key? *Clin Rheumatol* 2005;24:296-300.

115. Marcellin-Little DJ. Fracture treatment with circular external fixation. *Vet Clin North Am Small Anim Pract* 1999;29:1153-70.
116. Marcellin-Little DJ. Treating bone deformities with circular external skeletal fixation. *Compend Contin Educ Pract Vet* 1999;21:481-491.
117. Marcellin-Little DJ, Ferretti A, Roe SC, DeYoung DJ. Hinged Ilizarov external fixation for correction of antebrachial deformities. *Vet Surg* 1998;27:231-245.
118. Matsuyama J, Ohnishi I, Kageyama T, Oshida H, Suwabe T, Nakamura K. Osteogenesis and angiogenesis in regenerating bone during transverse distraction: quantitative evaluation using a canine model. *Clin Orthop Relat Res* 2005;433:243-250.
119. Mochizuki H, Hakeda Y, Wakatsuki N, Usui N, Akashi S, Sato T, Tanaka K, Kumegawa M. Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology* 1992;131:1075-1080.
120. Mohan S. Insulin-like growth factor binding proteins in bone cell regulation. *Growth Regul* 1993;3:67-70.
121. Moore DC, Leblanc CW, Muller R, Crisco JJ, III, Ehrlich MG. Physiologic weight-bearing increases new vessel formation during distraction osteogenesis: a micro-tomographic imaging study. *J Orthop Res* 2003;21:489-496.
122. Morgan PW, Miller CW. Osteotomy for correction of premature growth plate closure in 24 dogs. *Vet Comp Orthop Traumatol* 1994;7:129-135.
123. Muhlbauer RC, Fleisch H. The diurnal rhythm of bone resorption in the rat. Effect of feeding habits and pharmacological inhibitors. *J Clin Invest* 1995;95:1933-1940.
124. Murnaghan M, McIlmurray L, Mushipe MT, Li G. Time for treating bone fracture using rhBMP-2: a randomised placebo controlled mouse fracture trial. *J Orthop Res* 2005;23:625-631.
125. Nap RC, Mol JA, Hazewinkel HA. Age-related plasma concentrations of growth hormone (GH) and insulin- like growth factor I(IGF-I) in Great Dane pups fed different dietary levels of protein. *Domest Anim Endocrinol* 1993;10:237-247.
126.   Niu T, Rosen CJ. The insulin-like growth factor-I gene and osteoporosis: A critical appraisal. *Gene* 2005;361:38-56.
127. Ohashi S, Ohnishi I, Kageyama T, Fukuda S, Tsuchiya A, Imai K, Matsuyama J, Nakamura K. Effect of Vascularity on Canine Distracted Tibial Callus Consolidation. *Clin Orthop Relat Res* 2005;438:253-259.
128. Olson NC, Brinker WO, Carrig CB. Premature closure of the distal radial physis in two dogs. *J Am Vet Med Assoc* 1980;176:906-910.
129. Orbay JL, Frankel VH, Finkle JE, Kummer FJ. Canine leg lengthening by the Ilizarov technique. A biomechanical, radiologic, and morphologic study. *Clin Orthop* 1992;278:265-273.
130. Pacicca DM, Patel N, Lee C, Salisbury K, Lehmann W, Carvalho R, Gerstenfeld LC, Einhorn TA. Expression of angiogenic factors during distraction osteogenesis. *Bone* 2003;33:889-898.
131. Panteghini M, Pagani F. Biological variation in bone-derived biochemical markers in serum. *Scand J Clin Lab Invest* 1995;55:609-616.
132. Philipov JP, Pascalev MD, Aminkov BY, Grosev CD. Changes in serum carboxyterminal telopeptide of type I collagen in an experimental model of canine osteomyelitis. *Calcif Tissue Int* 1995;57:152-154.
133. Polak-Jonkisz D, Zwolinska D, Bednorz R, Owczarek H, Szymanska A, Nahaczewska W. Procollagen I carboxyterminal propeptide (PICP) as a bone formation marker and carboxyterminal telopeptide of type I collagen (ICTP) as a bone degradation marker in children with chronic renal failure under conservative therapy. *Med Sci Monit* 2003;9:19-23.

134. Quinn MK, Ehrhart N, Johnson AL, Schaeffer DJ. Realignment of the radius in canine antebrachial growth deformities treated with corrective osteotomy and bilateral (type II) external fixation. *Vet Surg* 2000;29:558-563.
135. Ramadan RO, Vaughan LC. Premature closure of the distal ulnar growth plate in dogs - a review of 58 cases. *J Small Anim Pract* 1978;19:647-667.
136. Raschke MJ, Bail H, Windhagen HJ, Kolbeck SF, Weiler A, Raun K, Kappelgard A, Skiaerbaek C, Haas NP. Recombinant growth hormone accelerates bone regenerate consolidation in distraction osteogenesis. *Bone* 1999;24:81-88.
137. Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R. Temporal and spatial expression of bone morphogenetic protein-2, -4, and -7 during distraction osteogenesis in rabbits. *Bone* 2000;27:453-459.
138. Rauch F, Lauzier D, Travers R, Glorieux F, Hamdy R. Effects of locally applied transforming growth factor-beta1 on distraction osteogenesis in a rabbit limb-lengthening model. *Bone* 2000;26:619-624.
139. Robey PG. Vertebrate mineralized matrix proteins: structure and function. *Connect Tissue Res* 1996;35:131-136.
140. Robins G. The management of distal radial growth plate closure. *Austr Vet Pract* 1987;17:143-144.
141. Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFkappaB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. *Bone* 2002;30:340-346.
142. Rowe NM, Mehrara BJ, Luchs JS, Dudziak ME, Steinbrech DS, Illei PB, Fernandez GJ, Gittes GK, Longaker MT. Angiogenesis during mandibular distraction osteogenesis. *Ann Plast Surg* 1999;42:470-475.
143. Sabharwal S, Paley D, Bhave A, Herzenberg JE. Growth patterns after lengthening of congenitally short lower limbs in young children. *J Pediatr Orthop* 2000;20:137-145.
144. Salmeri KR, Bloomberg MS, Scruggs SL, Shille V. Gonadectomy in immature dogs: effects on skeletal, physical, and behavioral development. *J Am Vet Med Assoc* 1991;198:1193-1203.
145. Salter RB, Harris WR. Injuries involving the epiphyseal plate. *J Bone Joint Surg Am* 1963;45:587-622.
146. Sartorio A, Ortolani S, Galbiati E, Conte G, Vangeli V, Arosio M, Porretti S, Faglia G. Effects of 12-month GH treatment on bone metabolism and bone mineral density in adults with adult-onset GH deficiency. *J Endocrinol Invest* 2001;24:224-230.
147. Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. *J Bone Miner Res* 1999;14:1084-1095.
148. Sattler AM, Schoppet M, Schaefer JR, Hofbauer LC. Novel aspects on RANK ligand and osteoprotegerin in osteoporosis and vascular disease. *Calcif Tissue Int* 2004;74:103-106.
149. Schmidmaier G, Wildemann B, Heeger J, Gabelein T, Flyvbjerg A, Bail HJ, Raschke M. Improvement of fracture healing by systemic administration of growth hormone and local application of insulin-like growth factor-1 and transforming growth factor-beta1. *Bone* 2002;31:165-172.
150. Schoenmakers I, Hazewinkel HAW, Voorhout G, Carlson CS, Richardson D. Effect of diets with different calcium and phosphorus contents on the skeletal development and blood chemistry of growing Great Danes. *Vet Rec* 2000;147:652-660.
151. Sciadini MF, Dawson JM, Banit D, Juliao SF, Johnson KD, Lennington WJ, Schwartz HS. Growth factor modulation of distraction osteogenesis in a segmental defect model. *Clin Orthop* 2000;381:266-277.

152. Seeherman H, Wozney JM. Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. *Cytokine Growth Factor Rev* 2005;16:329-345.
153. Seibel MJ. Molecular markers of bone turnover: biochemical, technical and analytical aspects. *Osteoporos Int* 2000;11 Suppl 6:18-29.
154. Seibel MJ, Woitge HW. Basic principles and clinical applications of biochemical markers of bone metabolism: biochemical and technical aspects. *J Clin Densitom* 1999;2:299-321.
155. Shevtsov VI, Asonova SN. Ultrastructural changes of articular cartilage following joint immobilization with the Ilizarov apparatus. *Bull Hosp Jt Dis* 1995;54:69-75.
156. Shih LY, Shih HN, Chen TH. The effects of sex and estrogen therapy on bone ingrowth into porous coated implant. *J Orthop Res* 2003;21:1033-1040.
157. Sietsema WK. Animal models of cortical porosity. *Bone* 1995;17:297-305.
158. Sjogren K, Jansson JO, Isaksson OG, Ohlsson C. A model for tissue-specific inducible insulin-like growth factor-I (IGF-I) inactivation to determine the physiological role of liver-derived IGF-I. *Endocrine* 2002;19:249-256.
159. Sneppen SB, Hoeck HC, Kollerup G, Sorensen OH, Laurberg P, Feldt-Rasmussen U. Bone mineral content and bone metabolism during physiological GH treatment in GH-deficient adults--an 18-month randomised, placebo-controlled, double blinded trial. *Eur J Endocrinol* 2002;146:187-195.
160. Sojo K, Sawaki Y, Hattori H, Mizutani H, Ueda M. Immunohistochemical study of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2, -4 (BMP-2, -4) on lengthened rat femurs. *J Craniomaxillofac Surg* 2005;33:238-245.
161. Spector JA, Luchs JS, Mehrara BJ, Greenwald JA, Smith LP, Longaker MT. Expression of bone morphogenetic proteins during membranous bone healing. *Plast Reconstr Surg* 2001;107:124-134.
162. Stanitski DF, Shahcheraghi H, Nicker DA, Armstrong PF. Results of tibial lengthening with the Ilizarov technique. *J Pediatr Orthop* 1996;16:168-172.
163. Steinbrech DS, Mehrara BJ, Rowe NM, Dudziak ME, Luchs JS, Saadeh PB, Gittes GK, Longaker MT. Gene expression of TGF-beta, TGF-beta receptor, and extracellular matrix proteins during membranous bone healing in rats. *Plast Reconstr Surg* 2000;105:2028-2038.
164. Steinbrech DS, Mehrara BJ, Rowe NM, Dudziak ME, Saadeh PB, Gittes GK, Longaker MT. Gene expression of insulin-like growth factors I and II in rat membranous osteotomy healing. *Ann Plast Surg* 1999;42:481-487.
165. Stewart KJ, Weyand B, van't Hof RJ, White SA, Lvoff GO, Maffulli N, Poole MD. A quantitative analysis of the effect of insulin-like growth factor-1 infusion during mandibular distraction osteogenesis in rabbits. *Br J Plast Surg* 1999;52:343-350.
166. Suda T, Ueno Y, Fujii K, Shinki T. Vitamin D and bone. *J Cell Biochem* 2003;88:259-266.
167. Syed F, Khosla S. Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun* 2005;328:688-696.
168. Szulc P, Seeman E, Delmas PD. Biochemical measurements of bone turnover in children and adolescents. *Osteoporos Int* 2000;11:281-294.
169. Takahashi N, Udagawa N, Suda T. A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. *Biochem Biophys Res Commun* 1999;256:449-455.
170. Tavakoli K, Yu Y, Shahidi S, Bonar F, Walsh WR, Poole MD. Expression of growth factors in the mandibular distraction zone: a sheep study. *Br J Plast Surg* 1999;52:434-439.
171. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine Growth Factor Rev* 2004;15:457-475.

172. Thoren M, Hilding A, Brismar T, Magnusson P, Degerblad M, Larsson L, Saaf M, Baylink DJ, Mohan S. Serum levels of insulin-like growth factor binding proteins (IGFBP)-4 and -5 correlate with bone mineral density in growth hormone (GH)- deficient adults and increase with GH replacement therapy. *J Bone Miner Res* 1998;13:891-899.

173. Torres R, de la PC, Rapado A. Clinical usefulness of serum tartrate-resistant acid phosphatase in Paget's disease of bone: correlation with other biochemical markers of bone remodelling. *Calcif Tissue Int* 1991;49:14-16.

174. Tryfonidou MA, Holl MS, Vastenburg M, Oosterlaken-Dijksterhuis MA, Birkenhager-Frenkel DH, van den Brom WE, Hazewinkel HA. Hormonal regulation of calcium homeostasis in two breeds of dogs during growth at different rates. *J Anim Sci* 2003;81:1568-1580.

175. Tsumaki N, Yoshikawa H. The role of bone morphogenetic proteins in endochondral bone formation. *Cytokine Growth Factor Rev* 2005;16:279-285.

176. Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD. Animal models for osteoporosis. *Rev Endocr Metab Disord* 2001;2:117-127.

177. Ueland T. Bone metabolism in relation to alterations in systemic growth hormone. *Growth Horm IGF Res* 2004;14:404-417.

178. Ueland T. GH/IGF-I and bone resorption in vivo and in vitro. *Eur J Endocrinol* 2005;152:327-332.

179. Urist MR, DeLange RJ, Finerman GA. Bone cell differentiation and growth factors. *Science* 1983;220:680-686.

180. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. *J Cell Sci* 2000;113:377-381.

181. Valentin-Opran A, Wozney J, Csimma C, Lilly L, Riedel GE. Clinical evaluation of recombinant human bone morphogenetic protein-2. *Clin Orthop* 2002;395:110-120.

182. van Coeverden SC, Netelenbos JC, de Ridder CM, Roos JC, Popp-Snijders C, Delemarre-van-de-Waal HA. Bone metabolism markers and bone mass in healthy pubertal boys and girls. *Clin Endocrinol (Oxf)* 2002;57:107-116.

183. Vandewater A, Olmstead ML. Premature closure of the distal radial physis in the dog. A review of eleven cases. *Vet Surg* 1983;12:7-12.

184. Varga AC, Wrana JL. The disparate role of BMP in stem cell biology. *Oncogene* 2005;24:5713-5721.

185. Vidal J. External fixation. Yesterday, today, and tomorrow. *Clin Orthop Relat Res* 1983;7-14.

186. Walsh WR, Hamdy RC, Ehrlich MG. Biomechanical and physical properties of lengthened bone in a canine model. *Clin Orthop* 1994;306:230-238.

187. Wan M, Cao X. BMP signaling in skeletal development. *Biochem Biophys Res Commun* 2005;328:651-657.

188. Wang J, Zhou J, Cheng CM, Kopchick JJ, Bondy CA. Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. *J Endocrinol* 2004;180:247-255.

189. Wei S, Lightwood D, Ladyman H, Cross S, Neale H, Griffiths M, Adams R, Marshall D, Lawson A, McKnight AJ, Stanley ER. Modulation of CSF-1-regulated post-natal development with anti-CSF-1 antibody. *Immunobiology* 2005;210:109-119.

190. Weiss S, Baumgart R, Jochum M, Strasburger CJ, Bidlingmaier M. Systemic regulation of distraction osteogenesis: a cascade of biochemical factors. *J Bone Miner Res* 2002;17:1280-1289.

191. Wilkens BE, Millis DL, Daniel GB, Munson L, Patel KR, Buonomo FC. Metabolic and histologic effects of recombinant canine somatotropin on bone healing in dogs, using an unstable ostectomy gap model. *Am J Vet Res* 1996;57:1395-1401.

192.   Willenegger H, Perren SM, Schenk R. [Primary and secondary healing of bone fractures]. *Chirurg* 1971;42:241-252.
193.   Wilson AK, Bhattacharyya MH, Miller S, Mani A, Sacco-Gibson N. Ovariectomy-induced changes in aged beagles: histomorphometry of rib cortical bone. *Calcif Tissue Int* 1998;62:237-243.
194.   Yao GQ, Sun BH, Weir EC, Insogna KL. A role for cell-surface CSF-1 in osteoblast-mediated osteoclastogenesis. *Calcif Tissue Int* 2002;70:339-346.
195.   Yeung HY, Lee SK, Fung KP, Leung KS. Expression of basic fibroblast growth factor during distraction osteogenesis. *Clin Orthop Relat Res* 2001;385:219-229.
196.   Zangger I, Zapf J, Froesch ER. Insulin-like growth factor I and II in 14 animal species and man as determined by three radioligand assays and two bioassays. *Acta Endocrinol (Copenh)* 1987;114:107-112.
197.   Zimmermann G, Henle P, Kusswetter M, Moghaddam A, Wentzensen A, Richter W, Weiss S. TGF-beta1 as a marker of delayed fracture healing. *Bone* 2005;36:779-785.

Chapter 3

Prognostic Factors in Treating Antebrachial Growth Deformities with a Lengthening Procedure Using a Circular External Skeletal Fixation System in Dogs

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Abstract

The aim of this prospective clinical study was to evaluate the treatment of antebrachial growth deformities (AGD) with a lengthening procedure using a circular external skeletal fixation (CESF) system and to determine prognostic factors. The study included thirty-four dogs with unilateral AGD. Length deficits, angular and rotational deformities, elbow incongruity (EI), osteoarthritis (OA) of the elbow and carpal joint, function and cosmesis were determined before and after a CESF lengthening procedure. At presentation, EI (21/34 dogs; 62%), OA of the elbow joint (17/34 dogs; 50%), carpal OA (12/34 dogs; 35%) and concomitant elbow and carpal OA (5/34 dogs; 7%) were common findings. Treatment significantly improved function (normal in 20/34 dogs; 60%) and cosmesis (normal in 22/34 dogs; 65%). Angular and rotational deformities were almost completely corrected with small remaining length deficits. Elbow and carpal OA increased significantly during the follow-up period. Significant correlations were demonstrated between initial elbow OA and final function (R = 0.42, P = .02), initial function and final function (R = 0.41, P = 0.02), and initial ulnar and radial deficit and final cosmesis (R = 0.58, P = .0001 and R = 0.45, P = .008). Treatment of AGD with a CESF lengthening procedure was successful despite small remaining length deficits. Initial elbow OA, initial function, and ulnar and radial length deficits are prognostic factors in predicting the functional outcome of treatment of AGD with a CESF lengthening procedure in dogs.

Introduction

Antebrachial growth deformities (AGD) are the most common limb malformation in dogs.[23] Various causes for AGD have been described, including trauma of the antebrachial physes, chondrodystrophia, genetically induced deformities, metabolic disease and unbalanced nutrition.[11,16,28,38,46] Growth deformities are characterized by a combination of antebrachial length deficit, angular and rotational malalignment, elbow incongruity (EI), and carpal subluxation. The secondary effects may include osteoarthritis (OA) of the elbow and carpal joints. Further, EI has been associated with the occurrence of fragmented medial coronoid process (FCP) and ununited anconeal process (UAP).[31,49,50,51] Clinically, AGD will compromise limb function and cosmesis. The reduced function is characterized by lameness because of a combination of joint pain, decreased range of motion and antebrachial length deficit. The treatment of AGD is directed at correcting the complexity of malalignment, length deficit, and joint function and at preventing secondary degenerative changes.

The introduction of the concept of distraction osteogenesis, using circular external skeletal fixation (CESF), has revolutionized the treatment of AGD as lengthening procedures have now enabled the dynamic correction of length deficits.[1,20,21,36] In veterinary orthopedics, CESF has proven to be a highly dynamic system in treating length deficits, angular and rotational deformities, and EI.[26,32,41,48] Although several previous reports have described the use of CESF in dogs, these studies usually included a limited number of cases with AGD.[7,27,30,33] Our purpose was to evaluate the correction of AGD with CESF in a large group of dogs and to determine preoperative factors with a prognostic significance.

Material and Methods

Dogs

Thirty-four canine patients with unilateral AGD were evaluated prospectively during the period 1994-2002. The patient data included breed, age at treatment, gender, and body weight (BW). Functional and cosmetic grading of the affected limb was performed.[10,12] Limb function was graded by the degree of lameness: 0 = clinically normal, 1 = slight lameness, 2 = moderate lameness, 3 = marked lameness, intermittently non-weight-bearing, and 4 = continuous non-weight-bearing lameness. Limb cosmesis was graded by the severity of the deformity in comparison with the non-affected limb: 0 = no detectable deformity, 1 = minor alteration but difficult to detect, 2 = noticeable deviation from normal limb

appearance, and 3 = severe changes from normal appearance. The minimum follow-up period after frame removal was 10 weeks.

Assessment of the antebrachial growth deformity

The rotational deformity of the antebrachium was assessed clinically by determining the planes of flexion and extension of the elbow and carpal joint and by measuring the angle between these planes with increments of 5°.[30] Radiographic examination of both antebrachia was performed preoperatively, immediately after surgery, immediately before and after removal of the CESF, and after a minimum follow-up period of 10 weeks. Final evaluation was always performed after closure of the proximal and distal physes of the radius and ulna in the contralateral antebrachium and termination of longitudinal growth.

Radiographs of the right and left antebrachium were made, including craniocaudal (CrCd) and mediolateral (ML) views centered on the elbow and carpal joints. The appearance of the antebrachial physes was assessed to determine the primary location of compromised longitudinal growth (Fig 1). Radiographs were also evaluated for the presence of a synostosis between the radius and ulna. Functional radial length was determined on the ML radiograph by measuring the distance from the center of the proximal radial joint surface (fovea capitis) to the center of the distal radial joint surface. Ulnar length was measured, using the same radiograph, from the proximal aspect of the olecranon to the end of the ulnar styloid process. Radial and ulnar length deficits were determined and expressed as a percentage of the longitudinal measurements of the non-affected side. The final extent of radial distraction was determined by measuring the width of the distraction gap and expressed as a percentage of the initial radial length. The overall increase in radial length after correction was determined and expressed as a percentage of the initial radial length.

Angular deformity was assessed in the ML and CrCd planes. The ML antebrachiocarpal joint angle was determined on the CrCd radiograph. The angle between a line drawn through the long-axis of the radius proximal to the deformity and a line parallel to the antebrachiocarpal joint was measured in increments of 5°. Carpal valgus was indicated with a positive grading and carpal varus with a negative grading. The CrCd antebrachiocarpal joint angle was determined on the ML radiograph. The angle between a line drawn through the center of the proximal and distal joint surfaces of the radius and a line parallel to the distal radial joint surface was measured in increments of 5°. Caudal subluxation of the carpus was indicated with positive grading and cranial carpal subluxation with a negative grading.

Fig 1. Antebrachial growth deformity with premature closure of the distal radial physis, resulting in a length deficit, incongruity of the elbow joint, and fragmentation of the medial coronoid process.

The median CrCd antebrachiocarpal joint angle in the non-affected limb was 15° (range 10 – 20°). Angular deformities were determined by comparing the measurements of the affected side with the normal contralateral limb angles.

ML and CrCd radiographs of the elbow joint were taken and evaluated for the presence of EI, FCP, UAP, and OA. Incongruity of the elbow was determined on the ML radiograph, using a template with circles of increasing diameter to match the trochlear notch of the ulna and radial head, respectively. The position of the circles was delineated on the radiograph and the difference in overlap between both circles was measured (mm) and corrected for the magnification factor to establish the size of the step-defect (Fig 2).

Fig 2   Mediolateral radiograph of the elbow joint with superposed templates to determine the amount of elbow incongruity (same dog as in Fig 1).

Incongruity because of a radial length deficit was given a negative mark and EI due to an ulnar length deficit a positive mark. Elbow OA was graded from 0 to 3 according to the guidelines of the International Elbow Working Group (IEWG). In this system grade 0 typifies no OA, grade 1 osteophytes < 2mm, grade 2 osteophytes between 2-5mm, and grade 3 osteophytes > 5mm at well-defined locations.[15] Carpal OA was graded in a similar way by measuring the size of the osteophytes on the cranial, caudal, lateral and medial aspect of the antebrachiocarpal joint.

Frame design

The CESF was assembled using the Polyfix® system (Polyfix, Grenoble, France) supplemented with IMEX[TM] parts (IMEX Veterinary Inc., Longview, TX, USA). The system included aluminum full, three-quarter, and half rings with diameters ranging from 60 to 110 mm, stainless steel connecting bars with a 6 mm diameter and a 1 mm pitch, stainless steel connecting bolts and nuts, hinges and angular motors. Depending on the size of the dog transosseus wires ranged from 1.2 to 1.6 mm in diameter. No stopper or olive wires were used in these dogs. The basic CESF frame design included a proximal and distal full ring adjacent to the radial osteotomy and secured to the radius with 2 tensioned transosseus wires per

ring and a proximal three-quarter ring secured to the radius with 1 tensioned transosseus wire. Tension was applied with a dynamometric wire tensioner (Hofmann SaS, Monza, Italy) with an equivalent of 20 kg in dogs with a BW up to 10 kg, an equivalent of 40 kg in dogs with a BW between 10 and 20 kg, an equivalent of 60 kg in dogs with a BW between 20 and 30 kg, and an equivalent of 80 kg in dogs with a BW between 30 and 40 kg. To reduce EI dynamically an extension or flag was incorporated into the frame design. This flag consisted of two treaded rods with one or two partial rings, which were attached to the proximal ulna with one or two tensioned transosseus wires. For additional stability, a supplemental proximal ring on the radius was used in selected cases (Fig 3). Care was taken to allow free motion in flexion of the elbow joint, which was accomplished by applying a partial radial ring when necessary. In two large and heavy dogs (BW > 38 kg), a 2nd distal radial ring was placed to enhance frame stability.

Fig 3. Circular external fixation system during the lengthening procedure with flag on the ulna to correct the incongruity of the elbow joint. The frame includes a proximal and distal full ring on the radius adjacent to the distraction zone, a three-quarter ring on the proximal radius for additional stability, and a flag with a half ring mounted on the proximal ulna (same dog as in Fig 1).

Surgical treatment

Correction of the deformity included rotational correction, angular correction, dynamic reduction of the elbow joint incongruity, and antebrachial lengthening, based on the preoperative data. The procedure was started with the partial ulnectomy in the distal third of the ulna without the use of a fat graft. In dogs diagnosed with a FCP, a medial arthrotomy of the elbow joint was performed, the medial and lateral coronoid process was examined, and fragments were removed. After placing the proximal ring without attaching is to the bone, the distal ring was secured, using two transosseus wires, perpendicular to the longitudinal axis of the distal radius. The position of the distal radial ring was determined based on the radiographic angular deformities in the ML and CrCd planes. When applicable a second distal ring was placed parallel to and at a distance of 4 cm of the first ring.

Correction of the rotational deformity was planned and performed as a 1-stage procedure in all cases. Correction of the angular deformity was performed as a 1-stage procedure, using a closing wedge osteotomy, in the first 24 cases. Dynamic correction with a hinge and angular motor configuration was available from case 25 onward. The hinge and angular motor configuration was used depending on the size of the patient and severity of the angular deformity. The location of the hinges and angular motor were determined as described.[33] Osteotomy of the radius was performed as close as possible to the center of the deformity through a medial approach using an oscillating saw and ample lavage. The periosteum was preserved, while minimizing trauma to the soft tissues. The frame was secured to the proximal radius with 2 transosseus wires on the full ring and 1 transosseus wire on the three-quarter ring. If EI had to be addressed, a flag on the ulna was incorporated into the frame design.

Postoperative care consisted of analgesics (buprenorphine, 10μg/kg 4 times daily subcutaneously) for 3 days, a full-leg bandage for the first 3 days, and a protective bandage incorporating the CESF only, thereafter. Exercise was restricted to leash walks for the duration of the treatment.

Correction of the EI was performed by either raising or lowering the ulnar flag at a rate of 0.5 mm twice daily, and was started the day after surgery. The measured amount of EI, corrected for the radiographic magnification factor, was restored and the result was evaluated on the ML radiograph of the elbow joint using the template, immediately after the expected reduction of the step-defect (Fig 4 and 5). After a latency period of 3 days, the lengthening procedure was executed by distracting the radius at a rate of 0.5 mm twice daily. The distraction rate of the angular motor was adjusted to match an elongation of 1 mm a day at the opening side of the osteotomy. Distraction was verified radiographically on a weekly basis

until the determined amount of lengthening was accomplished. Dogs were discharged after correction of EI and the remainder of the radial distraction was performed by the owners. The distraction of the antebrachium was stopped when the length deficit was corrected or when the carpal joint showed early signs of carpal flexor contracture with the inability to extend the joint.

After lengthening was stopped, consolidation of the distraction zone was evaluated every 2 weeks until the bone was judged strong enough to allow for frame removal. The duration of treatment from CESF placement to frame removal was determined. The occurrence of complications concerning the CESF, transosseus bone wires, or soft tissues were recorded. After consolidation of the distraction zone, the frame and transosseus wires were removed under sedation and a protective bandage was applied for 2 days. Exercise was limited to leash walks for at least another 2 weeks. The result of the lengthening procedure was evaluated by measuring the length of the distraction zone, using the radiographs taken immediately after frame removal. The final results of the correction of the AGD and the lengthening procedure were evaluated when longitudinal growth in the non-affected leg had ceased by measuring radial and ulnar length, joint angles, EI and elbow and carpal OA as described earlier. At final follow-up, function and cosmesis were graded.

Fig 4. Mediolateral elbow radiograph centered on the joint after correction of the elbow incongruity (same dog as in Fig 1).

Fig 5. Result of lengthening procedure and correction of elbow incongruity at 4 months postoperative (same dog as in Fig 1). The distraction zone is still distinguishable despite advanced remodeling. There is minimal osteoarthritis (OA) in the elbow joint and no OA in the carpal joint.

Evaluation of prognostic factors

To identify factors, which could predict the outcome of treatment of AGD with a CESF, correlations between initial function and cosmesis, age, rotational, CrCd, and ML angular deformities, radial and ulnar length deficits, EI, FCP, initial OA of the elbow and carpal joints, and function, cosmesis, and OA at the end of the follow-up period were assessed. Correlations between final function, final elbow and carpal OA, final radial and ulnar deficits, remaining angular deformities

and EI, radial distraction, overall lengthening, duration of treatment and follow-up were also determined.

Statistical analysis

Preoperative radial and ulnar deficits, ML, CrCd, and rotational angles, EI, OA grade of the elbow and carpal joints, cosmesis and function were compared with the results of these parameters after treatment, using a Wilcoxon sign rank test. Correlations between the non-parametric data were determined using a Spearman test. The effect of elbow OA on function was corrected for the influence of carpal OA and vice versa. All statistical analyses were performed, using computer software (SPSS 10.1, SPSS Inc., Chicago, IL, USA). A P-value < .05 was considered significant. Results were reported as mean ± SD.

Results

The study included 32 dogs with normal skeletal proportions except for the AGD and 2 chondrodystrophic dogs, both Basset Hounds. Labrador (n = 6, 18%) and Golden retriever (n = 4, 12%) dogs were the most common breeds. On admission, the mean age of the dogs was 7 months (range, 3 - 19 months) with 24 dogs (70%) < 7 months of age and 10 dogs (30%) > 8 months. The mean weight was 21 kg (range, 6 - 40 kg). Gender distribution was 15 females (44%) and 19 males (56%). Function was impaired in all dogs and cosmesis was graded abnormal in all but one. Initial function was scored as grade 1 lameness in one dog, grade 2 lameness in 27 dogs (79%), and grade 3 lameness in 6 dogs (18%). Initial cosmesis was scored as no deformity in one dog, grade 1 deformity in 6 dogs (18%), grade 2 deformity in 15 dogs (44%), and grade 3 deformity in 12 dogs (35%). Rotational deformity was present in 15 dogs (44%).

All dogs demonstrated a combined growth disturbance with involvement of both the distal radial and ulnar growth plates, resulting in radial and ulnar length deficits. In 20 dogs (59%), the distal radial physis was the most affected growth plate, while the distal ulnar physis was affected primarily in 13 dogs (38%). In one dog, the proximal radial physis was the most affected growth plate. In all cases, the onset of the AGD could be contributed to a traumatic event. Synostosis was present in 5 dogs (15%). Angular deformity in the ML and CrCd plane was present in 33 dogs (97%). ML angular deformity was found in 27 dogs (79%) with carpal valgus in 23 and carpal varus in 4 of these dogs (85% and 15%, respectively). Valgus

deformity could be attributed primarily to the distal ulnar physis in 12 dogs (52%) and to the distal radial physis in 11 dogs (48%).

CrCd angular deformity was present in 30 dogs (88%) and caudal carpal subluxation in 23 dogs and with cranial carpal subluxation in 7 dogs (77% and 23%, respectively). Caudal subluxation of the carpus could be attributed to the distal ulnar physes in 11 of 13 dogs (85%) and to the radial physes in 12 of 21 dogs (57%). Cranial subluxation of the carpus was demonstrated in 6 of 21 dogs (29%) with radial length deficits. External rotation of the radius was found in 11 of 13 dogs (85%) primarily with growth arrest of the distal ulnar physis, whereas only 4 of 21 dogs (20%) had external rotation from a primarily radial length deficit.

EI was demonstrated in 21 dogs (62%) with AGD and was caused by radial length deficits in 17 (81%) and by ulnar length deficits in 4 dogs (19%). A combination of EI and synostosis was present in 2 dogs (6%). The measurements of radial and ulnar deficits, angular and rotational deformities, and EI are presented in Table 1.

Table 1. Radial and ulnar length deficits, angular and rotational deformities, and elbow incongruity (EI) in 34 dogs with unilateral antebrachial growth deformities (AGD) before surgical correction.

AGD	Range	Median	Mean ± SD
Radial deficit (%)	2 - 25	13.4	12.8 ± 5.5
Ulnar deficit (%)	1 - 19	7.9	8.5 ± 4.5
ML angle (^{o})	-40 - 50	20	15.4 ± 21.8
CrCd angle (^{o})	-30 - 50	10	12.2 ± 17.9
Rotational angle (^{o})	0 - 80	0	17.2 ± 23.3
EI (mm)	- 10 - +7	-1	- 0.4 ± 4.5

ML angle is the angulation of the antebrachiocarpal joint in the mediolateral (ML) plane with negative values indicating carpal varus and positive values indicating carpal valgus.
CrCd angle is the angulation in the antebrachiocarpal joint in the craniocaudal (CrCd) plane with negative values indicating cranial carpal subluxation and positive values indicating caudal carpal subluxation.
EI is presented with negative values to indicate a radial length deficit and with positive values to indicate an ulnar length deficit.

Fragmentation of the coronoid process was diagnosed radiographically in 9 dogs (27%), and was always found in combination with EI caused by a radial length deficit. The breed distribution of FCP was 3 Bernese Mountain dogs, 2 Labrador Retrievers, a miniature Schnauzer, a Beagle and 2 small mongrel dogs.

UAP was not found in this patient group. OA of the elbow joint occurred in 17 dogs (50%) and was found concurrently with EI in 16 of 21 dogs (76%). Initial elbow OA was scored as grade 1 osteophyte formation in 15 dogs (44%), and grade 2 osteophyte formation in 2 dogs (6%). Carpal OA occurred in 12 dogs (35%), and all of these dogs had grade 1 osteophyte formation. In 5 dogs, concurrent OA of the elbow and carpal joint was present (5/34; 14%).

Treatment of AGD, using the basic CESF frame design with a closing wedge osteotomy in 30 dogs (88%) and a hinge-and-motor CESF with dynamic correction of the angular deformity in the remaining 4 dogs (12%), was uneventful. A lengthening procedure was performed in all dogs. After completing dynamic distraction, the consolidation of the bone regenerate progressed without serious complications. No differences were found between dogs treated with the closing wedge technique or the hinge-and-motor CESF for any of the variables investigated. Treatment duration was 6.3 ± 1.5 weeks, with a follow-up period of 17 ± 12 weeks. The most common complication was wire tract infection, which occurred in 20 dogs (59%). Wire tract infections were almost completely limited to the bone wires of the flag on the proximal ulna and usually started in the 3rd week after surgery. Breakage of transosseus wires occurred in 2 dogs (6%), and included the wires mounted on the distal radial ring. In both dogs, the CESF was caught on an object and when the dog struggled to break free, the transosseus wires were damaged. Immediate replacement of the transosseus wires was sufficient to re-establish CESF stability.

After treatment, function and cosmesis had improved significantly. Function was determined to be normal in 20 dogs (60%), while 14 dogs (40%) had grade 1 lameness. Lameness could be attributed to the elbow joint in 5 dogs (35%), to the carpal joint in 2 dogs (15%) and to a combination of the elbow and carpal joints in 7 dogs (50%). An FCP had been removed in 5 dogs with elbow joint lameness (60%). Cosmesis was restored in 22 dogs (65%), while 12 dogs (35%) had grade 1 deformity and 7 of these dogs (58%) had carpal valgus despite correction of the angles of ML deformity, indicative of malformation within the carpus.

Radial and ulnar deficits, angular and rotational deformities, and EI improved significantly after treatment (Table 2). Radial distraction was 12 ± 7 mm (range, 2 - 26 mm) corresponding to $11 \pm 7\%$ (range, 1 - 27%) of the initial radial length. The result of the lengthening procedure was 15 ± 9 mm (range, 2 - 39 mm), corresponding to $13 \pm 10\%$ (range, 1 - 40%) of initial radial length. Radial and ulnar length deficits were corrected completely in 4 dogs (12%) and 5 dogs (15%), respectively, whereas minor deficits were present in the remaining dogs (88% and 85%, respectively). ML and CrCd angular and rotational deformities were restored in 27 dogs (79%), 22 dogs (65%) and 32 dogs (94%), respectively. EI was

corrected in 20 of 21 dogs (95%). Final function was graded as clinically normal in 11 of these dogs (52%).

Radiographic diagnosis of FCP was confirmed during arthrotomy in 9 dogs, and the FCP was removed. In 7 of these dogs, fragmentation involved both medial and lateral coronoid processes. At final follow-up, OA of the elbow and carpal joint was found in 20 dogs (58%) and 19 dogs (56%), respectively, with a significant increase in comparison with preoperative OA scores. Concurrent OA of the elbow and carpal joint was present in 11 dogs (32%). Final elbow OA was scored as grade 1 osteophyte formation in 14 dogs (41%) and grade 2 osteophyte formation in 6 dogs (18%). In the dogs with EI, only 5 of 21 (24%) were considered free of OA at follow-up. None of these dogs was diagnosed with FCP and the median age of these dogs was 6 months (range, 4-6 months). Final carpal OA was scored as grade 1 osteophyte formation in all 19 dogs.

Table 2. Radial and ulnar length deficits, angular and rotational deformities, and elbow incongruity (EI) in 34 dogs with unilateral antebrachial growth deformities (AGD) after surgical correction, using a lengthening procedure with a circular external skeletal fixation (CESF) system.

AGD	Range	Median	Mean ± SD
Radial deficit (%)	-1 - 16	6.7	7.1 ± 5.1[*]
Ulnar deficit (%)	-9 - 14	4.6	4.8 ± 4.6[*]
ML angle (^{o})	0 - 10	0	2.1 ± 3.9[*]
CrCd angle (^{o})	-10 - 15	0	-0.1 ± 5.6[*]
Rotational angle (^{o})	0 - 20	0	1.2 ± 4.8[*]
EI (mm)	-1 - 0	0	-0.1 ± 0.2[*]

ML angle is the angulation of the antebrachiocarpal joint in the mediolateral (ML) plane with negative values indicating carpal varus and positive values indicating carpal valgus.

CrCd angle is the angulation in the antebrachiocarpal joint in the craniocaudal (CrCd) plane with negative values indicating cranial carpal subluxation and positive values indicating caudal carpal subluxation.

EI is presented with negative values indicating a radial length deficit and positive values indicating an ulnar length deficit.

[*] Significant improvement in comparison with these values before correction of the AGD (Table 1) ($P < .001$).

Significant positive correlations were demonstrated between initial elbow OA and final function (R = 0.42, P = .02) and between initial function and final function (R = 0.41, P = .02). In other words, a higher initial OA grade resulted in a higher final function grade and thus more severe lameness. By analogy, a higher

initial function grade resulted in more severe lameness at final follow-up. Initial ulnar and radial deficits (R = 0.58, P = .0001 and R = 0.45, P = .008, respectively) and final ulnar and radial deficits (R = 0.51, P = .002 and R = 0.35, P = .04, respectively) were positively correlated with final cosmesis. In addition, initial EI (R = 0.49, P = .003) and FCP (R = 0.48, P = .004) showed a positive correlation with final elbow OA. Initial EI was positively correlated with the presence of an FCP (R = 0.57, P = .0001). Final elbow OA was more severe with increasing age on admission (R = 0.37, P = .03) In other words, treatment at a later age resulted in more severe elbow OA at follow-up. Increasing degrees of ML angular deformity resulted in more severe final carpal OA (R = 0.34, P = .04). A negative correlation was found between the ML and CrCd angular deformity and final elbow OA (R = -0.42, P = .01 and R = -0.37, P = .03, respectively), indicating that more severe initial carpal angular deformity resulted in less OA in the elbow joint. No significant correlation was found between initial EI and final function. Final function correlated positively with final elbow OA (R = 0.51, P = .002) and final cosmesis (R = 0.38, P = .02), but not final carpal OA. Final carpal OA was positively correlated with both the amount of radial distraction and overall lengthening (R = 0.45, P = .008 and R = 0.38, P = .02, respectively).

Discussion

AGDs vary considerably in the extent of malformation, which contributes to a heterogeneous patient group. The common factor in our study was that all dogs had unilateral AGD caused by a traumatic event. On admission, 70% of the dogs were < 7 months of age. Cessation of radial growth usually occurs between 8 and 9 months of age, which implies functional growth plates and thus growth potential in the contralateral antebrachium of these dogs with AGD.[5] Assessment of growth potential in the affected limb is essential during the planning of correction of length deficits. Unfortunately, there is no accurate way to predict the amount of remaining growth of the antebrachium during and after treatment, and close radiographic monitoring of longitudinal growth is the best option.[40] Another problem is unequal growth within the physis, which can result in relapse deformity of the limb after correction. These general considerations would favor treatment of the patients at an age when length growth has ceased, but this would discount the deleterious effects of growth deformities on joint function and the development of OA. Normal joint function can only be achieved by realignment of the mechanical axis of joint movement and reducing EI and carpal subluxation as soon as possible. In our study, OA was already present in these dogs on admission and proved to be a major factor in outcome.

Gender distribution slightly favored male dogs, which, in combination with behavioral aspects, may indicate that higher growth rates render the physes more susceptible to traumatic disturbance. As function and cosmesis were clearly comprised and most dogs still had significant growth potential, correction of the AGD with a CESF lengthening procedure was indicated.

Synostosis of the radius and ulna usually is a consequence of high-impact antebrachial fractures and has been described in young dogs as a contributing factor in the development of AGD.[25] The incidence of synostosis of 15% in our study was considerable and is of importance as removal of the synostosis is critical when restoring antebrachial alignment and in correcting EI and carpal subluxation. Furthermore, synostosis will restrict pronation and supination of the antebrachium and thus compromise function.

In the antebrachium, the distal growth plate of the ulna by its configuration and subsequent high growth rate is reported to be more vulnerable to trauma than the radial physes.[38] This will typically result in radius curvus syndrome with cranial bowing of the radius, exorotation of the antebrachium, and valgus of the carpus. In the clinical situation, isolated disturbance of just 1 physis is not a common finding and usually both the distal radial and distal ulnar growth plates will be affected. The degree of growth disturbance within the radial and ulnar physes will vary from patient to patient, resulting in a heterogeneous presentation of AGD.

We demonstrated a higher prevalence radial physeal growth disturbance, which may be attributed to the fact that we not only looked at the radiographic appearance of the physes but also measured the effect of growth disturbance on radial and ulnar length. Although the radial longitudinal growth deficit was more substantial than the ulnar growth deficit, the overall result was valgus deformity in most dogs, which is consistent with other reports.[10,38] This observation suggests that trauma to the distal radial physis in most cases elicits an asymmetrical growth arrest, which is more severe on the lateral side of the physis.[17,39,46] Carpal varus was not a common finding Compromised longitudinal growth of the distal radius may results in cranial subluxation of the carpus as we found. In contrast, compromised growth in the distal ulnar physis can cause caudal subluxation of the carpus. Internal rotation of the antebrachium was not encountered, and external rotation was typically found in conjunction with primarily ulnar length deficits.

EI in conjunction with AGDs has been reported in several studies.[25,26,27,31] Radiographic evaluation of in vitro-created EI was associated with relatively poor sensitivity and specificity.[34] However, EI in pathological cases is characterized by malformation of the humeroulnar, humeroradial, and radioulnar joint surfaces with subsequent OA. In our study, the high prevalence of EI was critical for both initial lameness and elbow OA after treatment. A major problem in evaluating EI is the lack of a uniform grading system. The grading system that we used was effective in

expressing the severity of EI. It is clear that this method is an oversimplification of EI and only focuses on the step defect between radius and ulna in the elbow joint. Malformation of the elbow joint cannot be assessed with this radiographic technique. In our opinion, the AP radiograph of the elbow joint was not useful for evaluation of EI as the step defect is obscured in this projection.[34] Nevertheless, our method proved to be helpful in determining the amount of correction required to restore congruity and to assess the result of treatment.

The correlation between EI and FCP seems to support the hypothesis that an abnormal weight distribution on the ulna is a pathogenic factor for FCP.[22,49] In small breed dogs, the occurrence of a FCP is a rare finding, but FCP coincided with EI in 4 small dogs in our study. The fact that fragmentation of the lateral coronoid process was also found frequently is consistent with this concept of overloading of the ulnar joint surface. As FCP, especially in young dogs, can be difficult to diagnose radiographically, an underestimation of the concurrence of FCP with EI in dogs with AGD is to be expected.[15] In contrast, FCP with or without EI is a regular finding in Bernese Mountain dogs and Labrador Retrievers as part of elbow dysplasia. Ubbink showed that FCP and EI have a genetic basis in Bernese Mountain dogs, but are distinct entities, which nuances the former hypothesis of overloading.[45] Critical evaluation of both EI and FCP is essential, as both were shown to cause progressive elbow OA. Three-dimensional imaging techniques are invaluable in assessing EI, but need further study.

Angular deformities of the antebrachium will lead to abnormal loading of the carpal joint and subsequent OA. Although most dogs presented with a combination of valgus or varus with cranial or caudal carpal subluxation, OA in the carpal joint was not as common as in the elbow. A possible explanation for this finding might be that the dog compensates for the angular deformity by altering foot placement and thus to some extend normalizes joint loading. Compensation for the EI cannot be achieved in this respect. Although the importance of deviation of the carpal joint axis is recognized in literature, very little is reported on the impact of this problem on carpal development, function, and secondary OA.[4,10,35] Literature concerning the treatment of AGD, focuses on the correction of the ML and CrCd carpal joint angles, but does not address the occurrence of carpal deformity.[26,27,32,33,37] Malformation of the carpal bones during growth can lead to angular deformity within the joint, which is not susceptible to correction (Fig 6). In our study, carpal malformation was judged responsible for carpal valgus, which occurred despite proper realignment of the distal antebrachial joint surface.

By analogy with EI, there is no grading system to classify carpal malformation. We have focused on the secondary carpal OA resulting from abnormal joint loading and carpal malformation. The high prevalence of carpal OA that we found has not

been reported previously, but is in accordance with loss of carpal range of motion reported previously.[27,33]

Restoration of the functional alignment of the elbow and carpal joints is critical in treating AGD. Although dynamic correction of rotational deformities is possible, this requires an elaborate frame design and the character, and the character and nature of dogs do not make this a feasible option.[19,47] In view of this, we performed correction of rotational deformity acutely during surgery. Correction of angular deformities and limb lengthening were executed successfully, using a closing wedge technique or dynamic correction with the hinge and motor configuration. The closing wedge correction of the angular and rotational deformities had the advantage of direct visualization of joint alignment. Further, the closing wedge osteotomy allowed for a large area of contact between the proximal and distal parts of the radius. This is advantageous for revascularization of the osteotomy zone before distraction. In an open wedge osteotomy with limited bone-to-bone contact, revascularization of the distraction zone will take longer requiring an extended latency period. A disadvantage of the closing wedge technique was the initial bone loss, which had to be compensated for during distraction.

Fig 6.   Malformation of the carpal bones, resulting in valgus deformity within the carpus. The mediolateral angle of the antebrachiocarpal joint is near normal. This type of malformation is not amenable to angular correction of the antebrachium.

Although the hinge and motor configuration had the advantage of dynamic correction of the angular deformity, its size restricted its use in smaller dogs. Another problem, especially in caudal subluxation of the carpus, was assessing the ability of the antebrachial flexor tendons and muscles to adapt to the strain put on these structures during dynamic correction of the deformity. The major concern was relative flexor tendon contracture, occurring before realignment of the antebrachiocarpal joint was accomplished, leading to carpal flexion, abnormal weight bearing, and possibly joint damage. In an experimental study in dogs involving femoral lengthening, cartilage fibrillation and even necrosis in the stifle joint was reported.[42] Compression of the joint because of the distraction was held responsible for this finding as the combination of simultaneous lengthening and joint distraction was able to prevent this consequence.[9,43] Managing established carpal flexor contracture can be very frustrating and prevention is critical.[18] Acute correction of the antebrachiocarpal joint angles should be considered in treating severe caudal carpal subluxation with a restricted ability to extent the carpus.

Although Frierson reported an adverse effect of using an oscillating saw on bone regeneration in a canine tibial lengthening model, this observation is contrary to other experimental studies.[6,13,24] We used an oscillating saw in combination with adequate cooling by lavage, and it seemingly did not have an adverse effect on osteogenesis as the duration of treatment and the frequency percentage of complications were similar to other reports.[7,26,27,32,33] Osteogenesis and consolidation progressed rapidly in these dogs, which is assumed to be related to their young age, metaphyseal osteotomies, and increased blood flow during distraction.[2,3]

Most wire tract infections were encountered at the site of the ulnar flag. The ulnar flag restricts normal supination and pronation of the antebrachium, which may cause irritation and infection of soft tissues and subsequent loosening of the ulnar transosseus wires. A flag design, allowing free pronation and supination, might overcome this problem.[39] Breakage of transosseus wires was always associated with external trauma to the CESF, but proper care should prevent this complication. In larger and heavier dogs, the frame design should preferably incorporate a 4-ring construct with 2 tensioned bone wires/ring. In view of the distal location of most AGDs and the small size of distal radial segment, we typically used a frame with a single distal radial ring. This frame design had no distinguishable adverse effect on the incidence of complications.[29]

The major disadvantage of traditional external fixators and bone plates in comparison with CESF is the inability of these methods to correct antebrachial length deficits and EI dynamically.[4,10,12,35,37] Nevertheless, angular deformities have been corrected successfully using these methods with comparable results of angular realignment as we found.[4] The results of the lengthening procedure and the

correction of the angular and rotational deformities were in accordance with previous reports on the treatment of AGD with CESF.[7,25,26,27,32,33] The limiting factor of the lengthening procedure was the inability of the flexor tendons and muscles to keep up with the distraction rate. Although physiotherapy and supporting bandages may promote extension of the carpal joint, imminent flexor contracture necessitated the end of distraction before complete correction of the length deficit was accomplished.[8]

The distinction between the amount of active radial distraction and the result of the lengthening procedure was made for the following reasons. Firstly, correction of angular and rotational deformities in itself can result in an increase of the functional length measured. Secondly, the growth potential of still active radial physes can contribute to the overall result of lengthening. The result of the correction of AGD will depend on the combination of realignment, active distraction, and growth potential of the antebrachium. The remaining length deficits had a predictable negative effect on the cosmetic appearance, but not on final function. This may be explained by the ability of the animal to compensate for length deficits of up to 15% by extending the shoulder and elbow joint.[26,27] Although small length deficits do not seem to affect functional outcome, the goal should be to restore antebrachial length completely.

Treatment of EI was successful, and no correlation was found between the initial amount of EI and final function. Nevertheless, EI was associated with progressive elbow OA, which may affect function on long-term evaluation. Although a dynamic proximal ulnar osteotomy was reported to be effective in treating EI because of ulnar length deficits, this method is not applicable for dynamical correction of EI in radial length deficits.[14] Again, it has to be emphasized that only the step defect between radius and ulna was treated and evaluated.

Malformation of the elbow joint was a common finding even after correction of EI. In view of this, the positive correlations between initial EI, FCP, and age versus final OA and the progression of pre-existing OA were to be expected. Furthermore, successful surgical removal of FCP and restoration of function will coincide with progression of elbow OA as we demonstrated before.[44] The present study suggests that correction of EI was most favorable in dogs at a young age, with remaining growth potential, without pre-existing OA, and without FCP. The growth potential of the adjacent epiphyses is proposed to be essential as an adaptive mechanism during correction of EI. In mature dogs, established malformation of the joint will lead to disappointing results. The negative correlation between the initial angular deformities and final elbow OA shows that dogs with more severe angular deformity in the antebrachium are less likely to develop elbow OA because of EI.

Carpal OA progressed despite correction of angular deformities. Carpal malformation could play an important role in this process. The initial ML angular deformity was demonstrated to have a larger impact on OA and presumably carpal malformation than CrCd carpal subluxation. The correlation between the amount of distraction and final carpal OA suggests that the tension on the flexor tendons and muscles may aggravate the progression of OA.[43] The progression of elbow and carpal OA is expected to have a negative effect on long-term function, but this observation was outside the scope of our study.

Initial elbow OA and initial function proved to be effective predictors of functional outcome, which is consistent with earlier reports on AGD and EI.[10,14] The final function was mainly dependent on elbow OA. Preventing elbow OA is therefore critical in treating dogs with developing AGD. The final cosmetic appearance was predicted by the amount of initial radial and ulnar length deficits and not by the angular and rotational deformities. Final cosmesis was mainly influenced by length deficits remaining after the lengthening procedure and carpal malformation.

Summarily, AGDs can be treated successfully with a CESF lengthening procedure despite small remaining length deficits. Treatment limitations are mainly determined by the pre-existing OA and malformation in the elbow and carpal joints. Initial elbow OA and initial function are prognostic factors in predicting functional outcome. The cosmetic appearance after treatment is determined by the magnitude of the initial radial and ulnar length deficits. Progression of elbow and carpal OA may have a negative effect on the long-term outcome of treatment of AGD.

References

1. Aronson J. Experimental and clinical experience with distraction osteogenesis. *Cleft Palate Craniofac J* 1994;31:473-481.
2. Aronson J. Temporal and spatial increases in blood flow during distraction osteogenesis. *Clin Orthop* 1994;301:124-131.
3. Aronson J, Shen X. Experimental healing of distraction osteogenesis comparing metaphyseal with diaphyseal sites. *Clin Orthop* 1994;301:25-30.
4. Balfour RJ, Boudrieau RJ, Gores BR. T-plate fixation of distal radial closing wedge osteotomies for treatment of angular limb deformities in 18 dogs. *Vet Surg* 2000;29:207-217.
5. Conzemius MG, Smith GK, Brighton CT, Marion MJ, Gregor TP. Analysis of physeal growths in dogs, using biplanar radiography. *Am J Vet Res* 1994;55:22-27.
6. Delloye C, Delefortrie G, Coutelier L, Vincent A. Bone regenerate formation in cortical bone during distraction lengthening. An experimental study. *Clin Orthop* 1990;250:34-42.
7. Elkins AD, Morandi M, Zembo M. Distraction osteogenesis in the dog using the Ilizarov external ring fixator. *J Am Anim Hosp Assoc* 1993;29:419-426.
8. Fink B, Braunstein S, Singer J, Schmielau G, Ruther W. Behavior of tendons during lower-leg lengthening in dogs using the Ilizarov method. *J Pediatr Orthop* 1999;19:380-385.
9. Fink B, Schwinger G, Singer J, Sager M, Wilke C, Braunstein S. The effect of tibial lengthening using the Ilizarov method on the cartilage and the menisci of the knee joint. *J Orthop Res* 2001;19:665-670.
10. Forell EB, Schwarz PD. Use of external skeletal fixation for treatment of angular deformity secondary to premature distal ulnar physeal closure. *J Am Anim Hosp Assoc* 1993;29:460-476.
11. Fox SM. Premature closure of distal radial and ulnar physes in the dog. I. Pathogenesis and diagnosis. *Compend Contin Educ Pract Vet* 1984;6:128-138.
12. Fox SM, Bray JC, Guerin SR, Burbridge HM. Antebrachial deformities in the dog: treatment with external fixation. *J Small Anim Pract* 1995;36:315-320.
13. Frierson M, Ibrahim K, Boles M, Bote H, Ganey T. Distraction osteogenesis. A comparison of corticotomy techniques. *Clin Orthop* 1994;301:19-24.
14. Gilson SD, Piermattei DL, Schwarz PD. Treatment of humeroulnar subluxation with a dynamic proximal ulnar osteotomy. A review of 13 cases. *Vet Surg* 1989;18:114-122.
15. Hazewinkel HAW, Meij BP, Nap RC, Dijkshoorn NE, Ubbink G, Wolvekamp WTC. Radiographic views for elbow dysplasia screening in Bernese Mountain dogs. *Vet Comp Orthop Traumatol* 1996;9:66-68.
16. Hazewinkel HAW, Schoenmakers I. Influence of protein, minerals and vitamin D on skeletal development of dogs. *Vet Clin Nutr* 1995;2:93-99.
17. Henney LHS, Gambardella PC. Partial ulnar ostectomy for treatment of premature closure of the proximal and distal radial physes in the dog. *J Am Anim Hosp Assoc* 1990;26:183-188.
18. Herzenberg JE, Davis JR, Paley D, Bhave A. Mechanical distraction for treatment of severe knee flexion contractures. *Clin Orthop* 1994;301:80-88.
19. Herzenberg JE, Smith JD, Paley D. Correcting torsional deformities with Ilizarov's apparatus. *Clin Orthop* 1994;302:36-41.
20. Ilizarov GA. The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation. *Clin Orthop* 1989;238:249-281.
21. Ilizarov GA. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. *Clin Orthop* 1989;239:263-285.
22. Johnson AL. Treatment of growth deformities with external skeletal fixation. *Vet Clin North A, Small Anim Pract* 1992;22:209-223.

23.   Johnson JA, Austin C, Breur GJ. Incidence of canine appendicular musculoskeletal disorders in 16 veterinary teaching hospitals from 1980 through 1989. *Vet Comp Orthop Traumatol* 1994;7:56-69.

24.   Kojimoto H, Yasui N, Goto T, Matsuda S, Shimomura Y. Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. *J Bone Joint Surg Br* 1988;70:543-549.

25.   Langley-Hobbs SJ, Carmichael S, Pead MJ, Torrington AM. Management of antebrachial deformity and shortening secondary to a synostosis in a dog. *J Small Anim Pract* 1996;37:359-363.

26.   Latte Y. Application of the Ilizarov method in veterinary orthopaedic surgery (part 1). *Eur J Comp Anim Pract* 1997;7:26-50.

27.   Latte Y. 75 applications of the Ilizarov method (part 2). *Eur J Comp Anim Pract* 1998;8:64-81.

28.   Lau RE. Inherited premature closure of the distal ulnar physis. *J Am Anim Hosp Assoc* 1977;13:609-612.

29.   Lewis DD, Bronson DG, Cross AR, Welch RD, Kubilis PS. Axial characteristics of circular external skeletal fixator single ring constructs. *Vet Surg* 2001;30:386-394.

30.   Lewis DD, Radasch RM, Beale BS, Stallings JT, Welch RD, Samchukov ML, Lanz OI. Initial clinical experience with the IMEXTM Circular External Skeletal Fixation System. Part II: use in bone lengthening and correction of angular and rotational deformities. *Vet Comp Orthop Traumatol* 1999;12:118-127.

31.   MacPherson GC, Lewis DD, Johnson KA, Allen GS, Yovich JC. Fragmented coronoid process associated with premature distal radial physeal closure in four dogs. *Vet Comp Orthop Traumatol* 1992;5:93-99.

32.   Marcellin-Little DJ. Treating bone deformities with circular external skeletal fixation. *Compend Contin Educ Pract Vet* 1999;21:481-491.

33.   Marcellin-Little DJ, Ferretti A, Roe SC, DeYoung DJ. Hinged Ilizarov external fixation for correction of antebrachial deformities. *Vet Surg* 1998;27:231-245.

34.   Mason DR, Schulz KS, Samii VF, Fujita Y, Hornof WJ, Herrgesell EJ, Long CD, Morgan JP, Kass PH. Sensitivity of radiographic evaluation of radio-ulnar incongruence in the dog in vitro. *Vet Surg* 2002;31:125-132.

35.   Morgan PW, Miller CW. Osteotomy for correction of premature growth plate closure in 24 dogs. *Vet Comp Orthop Traumatol* 1994;7:129-135.

36.   Orbay JL, Frankel VH, Finkle JE, Kummer FJ. Canine leg lengthening by the Ilizarov technique. A biomechanical, radiologic, and morphologic study. *Clin Orthop* 1992;265-273.

37.   Quinn MK, Ehrhart N, Johnson AL, Schaeffer DJ. Realignment of the radius in canine antebrachial growth deformities treated with corrective osteotomy and bilateral (type II) external fixation. *Vet Surg* 2000;29:558-563.

38.   Ramadan RO, Vaughan LC. Premature closure of the distal ulnar growth plate in dogs - a review of 58 cases. *J Small Anim Pract* 1978;19:647-667.

39.   Robins G. The management of distal radial growth plate closure. *Aust Vet Pract* 1987;17:143-144.

40.   Sabharwal S, Paley D, Bhave A, Herzenberg JE. Growth patterns after lengthening of congenitally short lower limbs in young children. *J Pediatr Orthop* 2000;20:137-145.

41.   Stallings JT, Lewis DD, Welch RD, Samchukov M, Marcellin-Little DJ. An introduction to distraction osteogenesis and the principles of the Ilizarov method. *Vet Comp Orthop Traumatol* 1998;11:59-67.

42.   Stanitski DF. The effect of limb lengthening on articular cartilage. An experimental study. *Clin Orthop* 1994;301:68-72.

43.   Stanitski DF, Rossman K, Torosian M. The effect of femoral lengthening on knee articular cartilage: the role of apparatus extension across the joint. *J Pediatr Orthop* 1996;16:151-154.

44.   Theyse LFH, Hazewinkel HAW, Brom-WE vd, van den Brom WE. Force plate analyses before and after surgical treatment of unilateral fragmented coronoid process. *Vet Comp Orthop Traumatol* 2000;13:135-140.

45.   Ubbink GJ, Hazewinkel HAW, Broek JJ, Rothuizen J, van de BJ. Familial clustering and risk analysis for fragmented coronoid process and elbow joint incongruity in Bernese Mountain Dogs in The Netherlands. *Am J Vet Res* 1999;60:1082-1087.

46.   Vandewater A, Olmstead ML. Premature closure of the distal radial physis in the dog. A review of eleven cases. *Vet Surg* 1983;12:7-12.

47.   Waanders NA, Herzenberg JE. The theoretical application of inclined hinges with the Ilizarov external fixator for simultaneous angulation and rotation correction. *Bull Hosp Jt Dis* 1992;52:27-35.

48.   Welch RD, Lewis DD. Distraction osteogenesis. *Vet Clin North Am Small Anim Pract* 1999;29:1187-1205.

49.   Wind AP. Incidence and radiographic appearance of fragmented coronoid process. *Calif Vet* 1982;36:19-25.

50.   Wind AP. Elbow incongruity and developmental elbow diseases in the dog: Part I. *J Am Anim Hosp Assoc* 1986;22:711-724.

51.   Wind AP, Packard ME. Elbow incongruity and developmental elbow diseases in the dog: Part II. *J Am Anim Hosp Assoc* 1986;22:725-730.

Chapter 4

Expression of Osteotropic Growth Factors and Growth Hormone Receptor in a Canine Distraction Osteogenesis Model

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Abstract

Osteotropic growth factors play an important role in bone metabolism. Nevertheless, knowledge about their expression in relation to distraction osteogenesis remains limited. The aim of the present study was to determine the expression of growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), and bone morphogenetic protein-2 (BMP-2) in distraction-induced bone regenerate. Expression of these factors was assessed during the consolidation phase, comparing distraction osteogenesis with osteotomy-induced bone formation. Real time PCR was performed as a semi-quantitative measurement of mRNA and the relative expression levels of these factors were determined. In addition, plasma GH profiles and plasma concentrations of IGF-I, IGF-II, and insulin-like growth factor binding protein- 4 and -6 (IGFBP-4 and -6) were measured to assess their potential systemic role during bone formation. Expression of GHR, IGF-I, and BMP-2 had significantly increased in comparison with the expression of these factors in mature bone. Expression of GHR was significantly higher in distraction-induced bone regenerate than in osteotomy-induced bone. No significant differences were found for the expression of IGF-I and BMP-2 between distraction and osteotomy. Plasma concentrations of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 did not demonstrate any significant differences between treatment groups and controls. Up-regulation of GHR expression in distraction osteogenesis may enhance sensitivity to endogenous systemic GH and thus promote consolidation of the bone regenerate. Changes in the systemic osteotropic growth factors GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 do not seem to be of importance during distraction osteogenesis.

Introduction

Distraction osteogenesis is a widely used method in limb lengthening, deformity correction, bone segment transport, and cosmetic craniofacial surgery. The principle allows for the formation of new bone following slow distraction of an osteotomy.[23,24] Although the radiographic, histological and ultra-structural changes associated with this process have been delineated, knowledge about the interactions of the growth factors governing this process is still insufficient.[1,38,42] In accordance with bone fracture models, the expression of several osteotropic growth factors, including insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and bone morphogenetic proteins (BMP) has been demonstrated in distraction osteogenesis.[12,14,31,40,52] Growth hormone (GH), IGF-I and insulin-like growth factors-II (IGF-II) play a critical role during bone growth and bone accretion.[35] The actions of IGF-I and -II are modulated through their six high-affinity binding proteins (IGFBPs).[34] In general, IGFBP-1, -2, -4, and -6 inhibit and IGFBP-3 and -5 stimulate osteoblast function.[26,34] More recently, focus has been on angiogenesis and the role of vascular endothelial growth factor (VEGF) and VEGF receptor-1 and -2 (VEGFR-1 and -2).[5] Osteotropic and angiogenic factors, including GH, IGF-I, TGF-β, BMP-4, FGF-2, and VEGF have been used experimentally to stimulate bone formation during distraction osteogenesis.[10,13,28,37,39,41,47] Aim of these studies was to shorten the protracted treatment period, required for lengthening and subsequent consolidation of the bone regenerate in the clinical situation.

During distraction osteogenesis osteotropic and angiogenic factors are produced which exert their effect both locally and systemically.[18,22,25,30,51] In view of the complex interactions of the osteotropic factors and close relations between GH, GHR, IGFs, and IGF binding proteins (IGFBP) we decided to focus on the GH-IGF axis and include BMP-2 as a potent stimulator of osteogenesis.[43] Aim of the present study was to assess the local and systemic role of GH, GHR, IGF-I, IGF-II, IGFBP-4 and IGFBP-6 in distraction osteogenesis. Densitometric image analysis was used to quantify the amount of newly formed bone.

Materials and Methods

Animals

The Utrecht University Ethics Committee for Animal Care and Use approved all procedures in this study. Eighteen mature Labrador retriever dogs

were used, including 14 females and 4 males, with a mean age of 19 months (range; 12 - 31 months), and a mean body weight of 26 kg (range; 21 - 32 kg). The dogs were allocated to three groups of 6 animals each. A standard commercial dog food was fed twice a day and water was available ad libitum. This feeding regime was continued throughout the entire study and food was not withheld prior to or during blood sample collection. The body weight of the dogs was determined once a week to assess potential weight loss.

Collection of blood plasma samples

Jugular vein blood samples were collected into ice-chilled EDTA tubes and kept on ice prior to centrifugation for 15 min at 3.9 g and 4°C, within 60 min after collection. Plasma samples were stored at -20°C until analysis. GH profiles for each individual animal were determined by collecting plasma samples every 15 minutes over an 8-hour period in the week prior to surgery and during the first, second, fourth, and sixth week after surgery. Plasma samples of IGF-I, IGF-II, IGFBP-4, and IGFBP-6 were obtained twice per week, starting in the week prior to surgery for the duration of the study. To rule out diurnal variations all blood samples were taken at the same time of the day (i.e. 0800 hours).

Surgery and distraction

In all dogs, a circular external skeletal fixation system (CESF) was applied to the right tibia (Imex Veterinary Inc., Longview, TX, USA) in all dogs. All frames were identical and consisted of two proximal and two distal full rings with a 100 mm diameter, connected by three treaded rods with a one mm pitch. The CESF was assembled prior to surgery and steam sterilized.

The dogs received medetomidine (Domitor®, Pfizer Animal Health B.V., Capelle a/d IJssel, The Netherlands) as a pre-anesthetic sedative and anesthesia was induced intravenously with propofol (Rapinovet®, Schering-Plough Animal Health N.V., Bruxelles, Belgium). General inhalation anesthesia was accomplished with nitrous oxide, oxygen, and isoflurane. Amoxicilline with clavulanic acid (Augmentin®, SmithKline Beecham Farma B.V., Rijswijk, The Netherlands) was administered intravenously (20 mg/kg bw) prior to surgery. The right hind limb was prepared in a standard sterile fashion. The CESF was placed on the right crus and the proximal and distal ring were attached to the tibia, using two 1.5 mm diameter transosseus wires, whereas a single 1.5 mm diameter transosseus wire was used for the central rings. All wires were tensioned with a dynamometric tensioner (Hofmann SaS, Monza, Italy), to an equivalent of 60 kg. After elevating

the periosteum, the tibia and fibula in the distraction (n=6) and osteotomy (n=6) group were osteotomized with an oscillating saw and ample lavage for cooling. In the control dogs (n=6) the periosteum was also elevated after placement of the CESF. The periosteum was closed with an absorbable suture material and closure of the subcutis and skin was in a routine fashion. A protective bandage was applied for 3 days. The dogs received buprenorphine (Temgesic®, Schering-Plough B.V., Utrecht, The Netherlands) as a postoperative analgesic (4dd 10 μg/kg bw) for three days. Full weight bearing of the legs was permitted immediately after surgery. In the distraction group, lengthening was started after a 4-day latency at a rate of 0.5 mm twice a day for a period of 10 consecutive days.

Biopsies of the distraction, osteotomy, or control zone were taken at the right side under general anesthesia in one dog per group at 2 and 4 weeks after surgery, respectively. These biopsies were immediately frozen in liquid nitrogen, stored at −70° C and used for mRNA isolation to test the primers for the PCR amplification (data not shown). All dogs were euthanatized with an overdose of barbiturates at 6 weeks after surgery. Segmental biopsies of the right and left tibia-fibula with a length of 3 cm, including the distraction, osteotomy, or control zone were obtained and spit longitudinally. One halve was immediately frozen in liquid nitrogen and stored at −70° C until required for RNA isolation, while the other halve was processed for histological examination (data not shown).

Biochemical analysis

Plasma GH concentrations were measured in a homologous RIA as previously described.[11] The Pulsar program developed by Merriam and Wachter was used to analyze the GH profiles.[32] The values extracted from this program included the mean of the smoothed baseline (GH-B), the area under the curve (AUC) for GH above the zero-level (GH-AUC-0), and the AUC for GH above the baseline (GH-AUC-B).

Total plasma IGF-I and IGF-II concentrations were measured after acid-ethanol extraction to remove interfering IGFBPs.[15] The absence of interfering IGFBPs was confirmed according to the protocol of Sota *et al.*[45] IGF-I concentrations were measured in a heterologous RIA validated for the dog.[36] IGF-II concentrations were determined with a heterologous RIA, using monoclonal antibodies against rat IGF-II (Amano Enzyme U.S.A. Co., Lombard, IL, USA) as described previously.[15]

Plasma IGFBP-4 and IGFBP-6 concentrations were determined with a specific heterologous RIA, using polyclonal antiserum WKZ8209 and WKZ6278,

respectively.[48,49] The mean concentration of the in duplicate plasma samples was determined for each week.

Gene expression

Frozen bone regenerate was ground in liquid nitrogen pre-frozen cups of a micro-dismembrator (Micro-Dismembrator U, B. Braun Biotech International GmbH, Melsungen, Germany). Five hundred milligram of milled bone tissue was resuspended in Qiagen lysis buffer (Qiagen GmbH, Hilden, Germany) and centrifuged for 10 min at 5 *g*. The supernatant was applied to a Qiagen midi-column (Qiagen GmbH, Hilden, Germany) and total RNA was isolated according to the manufacturer's protocol. After DNase I treatment (DNA*free*[TM] kit, Ambion, Austin, TX, USA) the isolated total RNA (130 ± 58 ng; mean ± SEM) was amplified, using the SMART[TM] PCR cDNA synthesis protocol (Clontech Laboratories, Inc., Palo Alto, CA, USA). The yield of SMART[TM] cDNA was 2.2 ± 0.2 µg out of 25 ng total RNA. SMART[TM] cDNA was purified by use of silica-gel membrane spin-columns (QIAquick PCR Purification kit, Qiagen GmbH, Hilden, Germany) and brought to a concentration of 1 ng/µl in water.

Real time PCR based on the high affinity double-stranded DNA-binding dye SYBR green I was performed in triplicate in a spectrofluorimetric thermal cycler (iCycler, BioRad, Hercules, CA, USA). Data were collected and analyzed with the provided application software. In addition, except for BMP-2, intron-spanning primer pairs were designed. β-Actin was used as endogenous reference. In order to confirm that β-actin levels were not influenced by the experimental manipulation and to further rule out interference of β-actin pseudogenes, GAPDH and β-actin, using a primer pair that distinguishes cDNA from pseudogene DNA, were also tested as internal standards.

For each real time PCR reaction, 10 ng of SMART[TM] cDNA was used in a reaction volume of 50 µl, containing 1x PCR buffer, 3 mM MgCl$_2$, 1:100,000 dilution of SYBR[®] green I (BMA, Rockland, ME, USA), 10 nM fluorescein calibration dye (BioRad, Hercules, CA, USA), 200 µM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, Branchburg, NJ, USA). Cycling conditions were 5 min at 95° C followed by 45 cycles of 15 sec at 95° C, 30 sec at 60° C, and 40 sec at 72° C. Primer pairs were designed, using PrimerSelect software (DNASTAR Inc., Madison, WI, USA) (Table 1).

Melt curves (iCycler) and agarose gel electrophoresis were used to examine each sample for purity and standard sequencing procedures (ABI PRISM[TM] 310 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) were

used to verify the analytical specificity of the PCR products. The individual melting curves proved that a single, unique product was amplified. Standard curves constructed by plotting the log of starting amount versus the threshold cycle were generated using serial 10-fold dilutions of known amounts of PCR products (from a conventional PCR). The amplification efficiency, $E (\%) = (10^{(1/-s)} - 1) \times 100$ (s = slope), of each standard curve was determined and appeared to be >90% over a large dynamic range (6-8 orders of magnitude). Serial dilutions of SMART cDNA were also tested resulting in similar E values over 2-4 orders of magnitude and optimal input values of 10 ng of cDNA per reaction.

Table 1. Primer pairs used in the PCR amplification of cDNA generated from total mRNA in the various bone biopsies.

Gene	Primer (5' – 3')		Exon	Amplicon length (bp)
β-actin	F:	TGGCACCACACCTTCTACAACGAG	3	180
	R:	AGAGGCATACAGGGACAGGACAGC	4	
BMP-2	F:	CAGAAATGAGTGGGAAAACAAC	3	207
	R:	GTCTGGTCACGGGGAACTT	3	
IGF-I	F:	ATGTCCTCCTCGCATCTCTT	3	355
	R:	TCCCTCTACTTGCGTTCTTC	5	
IGF-II	F:	CGCAGCCGTGGCATCGTTGAGGAG	5	200
	R:	CTGCGCAGGCGCTGGGTGGACT	6	
GH	F:	CCTGATGCGGGAGCTGGAAGATG	4,5	130
	R:	GAAGCAGGAGAGCAGCCCGTAGTT	5	
GH-R	F:	GATCCACCCATTGGCCTC	6	471
	R:	AATCTTTGGAACTGGAACTGGG	9	

For each experimental sample the amount of target (GH, GHR, IGF-I, IGF-II, and BMP-2), with β-actin and GAPDH as endogenous references, was determined from the appropriate standard curve. The amount of target was divided by the amount of endogenous reference to obtain a normalized target value. Each of the normalized target values was divided by the normalized target value of the calibrator (osteotomy group) to generate *n*-fold relative expression levels.

Densitometric image analysis

Densitometric image analysis was used in order to quantify the amount of bone regenerate and to evaluate the distraction procedure. Immediately after surgery standardized radiographs of the right crus were obtained in a caudocranial (CdCr) and lateromedial (LM) direction and on a weekly basis thereafter. The radiographs included two rulers and an aluminum step-wedge, consisting of a total of ten 2 mm thick aluminum slabs mounted in an overlapping manner. Radiographs were recorded with a Sony b/w CCD camera type XC-77CE and digitized for image analysis (frame size 752 x 574 pixels; 256 gray levels) with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the amount of regenerated bone. Geometric and densitometric calibration of each radiograph was performed, using the ruler and aluminum step-wedge images. Densitometric calibration was executed by measuring the mean optical density of a square area of 50 x 50 pixels in 6 steps of the aluminum model (0,2,4,6,8, and 10 mm). The optical density values supplied a polygonal fit with the aluminum values to produce a transformation table, which enabled to express the amount of newly formed bone in equivalents of cubic mm of aluminum. The bone regenerate was delineated on the digitalized CdCr and LM images and densitometric analyses for mean bone area and mean bone amount were performed.

Statistical analysis

Real time PCR data were evaluated, using the Student's *t*-test for analysis of the log-transformed normalized target values. Growth factor data were evaluated with a general linear model for repeated measures and a paired sample T-test. Bone area and bone amount were evaluated using a Student's *t*-test and a paired samples T-test. *P* values < .05 were considered significant. All data analyses were performed, using the SPSS 10.1 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Biochemical analysis

The GH profiles of the dogs were very similar and demonstrated no significant differences between the groups at any time. Prior to surgery, GH-B, GH-AUC-0, and GH-AUC-B levels were 1.4 ± 0.3, 23.8 ± 3.6, and 11.5 ± 2.0,

respectively (mean ± SEM). The cumulative means of GH-B, GH-AUC-0, and GH-AUC-B from week 1 to 6 in the distraction group were 1.5 ± 0.2, 25.2 ± 2.7, and 13.2 ± 1.8; in the osteotomy group 1.9 ± 0.3, 29.1 ± 3.2, and 14.0 ± 1.9; and in the control group 1.5 ± 0.2, 28.6 ± 2.4, and 16.4 ± 2.2, respectively.

The IGF-I plasma concentrations revealed a significant decrease in the first weeks following surgery, which was similar in magnitude in all groups (Fig. 1A). IGF-II and IGFBP-4 plasma concentrations remained relatively constant during the experimental period and did not differ between groups (Fig. 1B and 1C). IGFBP-6 plasma concentrations demonstrated a decline in comparison with pre-operative levels, which was most pronounced in the distraction group (Fig. 1D).
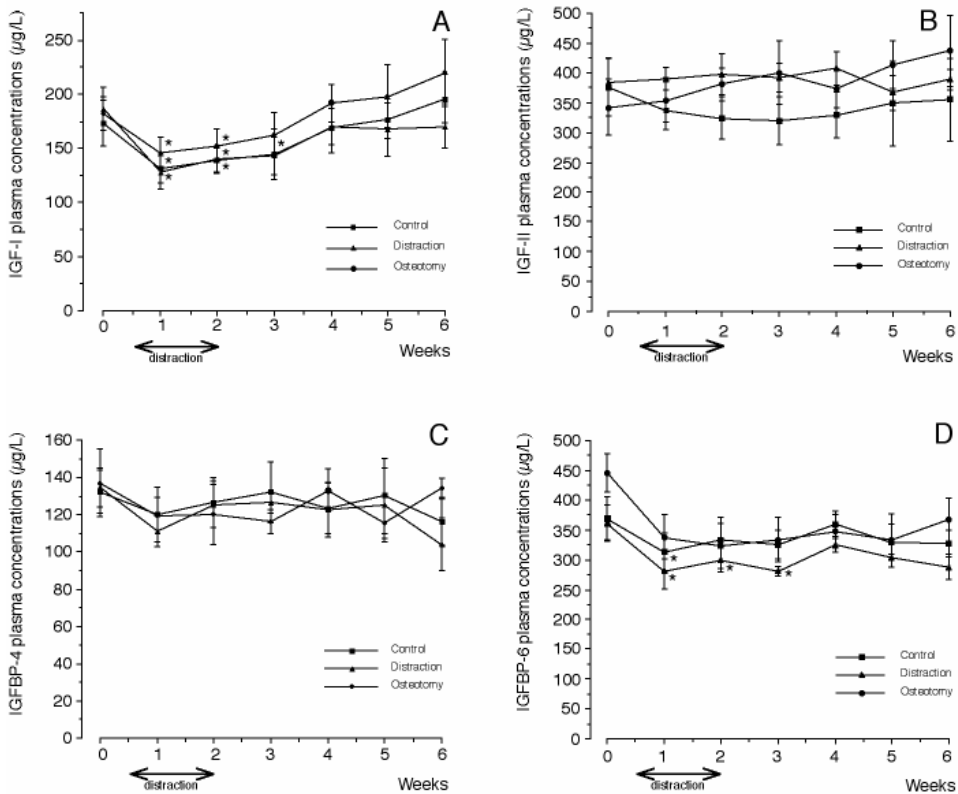

Figure 1. IGF-I, IGF-II, IGFBP-4, and IGFBP-6 plasma concentrations in the control, distraction, and osteotomy group during the 6-week study period.
Data are presented as the mean ± SEM. Week 0 shows the pre-operative plasma concentrations.
* indicates significant difference in comparison with preoperative levels of week 0 ($P < .05$).

Table 2. Normalized target values of BMP-2, IGF-I, and GHR gene expression determined by real time PCR in biopsies of osteotomy- and distraction-induced bone regenerate.

Bone biopsy	BMP-2	IGF-I	GHR
Osteotomy			
Median	0.54	0.97	0.03
Range	0.11 - 0.71	0.44 - 2.36	0.01 - 2.90
n-Fold (Calibrator)	1	1	1
Distraction			
Median	0.18	1.53	2.34
Range	0.08 - 0.43	0.80 - 2.32	0.10 - 5.44
n-Fold	0.3	1.6	78[*]

n-Fold is the relative expression level of bone morphogenetic protein-2 (BMP-2), insulin-like growth factor-I (IGF-I), and growth hormone receptor (GHR) in the distraction-induced bone regenerate with the osteotomy bone regenerate serving as the calibrator. [*] indicates a significant difference ($P < .05$).

Gene expression

In the right and left bone biopsies of the control as well as in the left specimens of the distraction and osteotomy group, the expression levels of GH, GHR, IGF-I, IGF-II, and BMP-2 were hard to determine, due to very low amounts of total RNA. The mRNA levels of GH and IGF-II were insufficient for reliable measurements, in spite of the fact that both GH and IGF-II mRNA could be clearly detected, using cDNA prepared from a similar amount of total mRNA originating from juvenile bone, still containing the epiphyseal growth plate (data not shown). Expression of GHR, IGF-I, and BMP-2 had significantly increased in comparison with the expression of these factors in the control bone segments. Compared to the control values (data not shown), BMP-2 expression in the biopsies of the osteotomy group at week 6 was significantly increased (10-fold), whereas the rise in BMP-2 expression in the distraction group was less pronounced (5-fold).

Comparing the expression levels of BMP-2, IGF-I, and GHR in the distraction group with the osteotomy group (calibrator) demonstrated a significant

78-fold up-regulation of the relative expression level of GHR (Table 2). The differences in BMP-2 and IGF-I relative expression levels were not significant. Similar results were obtained when GAPDH and β-actin were used as endogenous references (results not shown).

Distraction and densitometric image analysis

The distraction procedure was uneventful and no serious complications were encountered. Weight loss was not observed in the post-surgical period. Periosteal new bone formation adjacent to osteotomy site was already present radiographically as early as one week postoperatively in both the distraction and osteotomy group. The amount and density of the periosteal bone increased over time. In the distraction zone, new bone formation was visible at 3 weeks postoperative and the periosteal bone had merged with the bone in the distraction zone at 4 weeks. Bridging of the osteotomy was not seen before 6 weeks after surgery in the osteotomy group. Periosteal elevation in the sham-operated control group did not lead to periosteal new bone formation.

Densitometry revealed a significantly larger bone area and bone amount in the distraction group in comparison with the osteotomy group both at 5 and 6 weeks after surgery. Within the distraction group both bone area and bone amount had increased significantly during this period (Table 3).

Table 3. Densitometric image analysis of the bone formation in the distraction and osteotomy group.

	Bone area (mm^2)		Bone amount (mm^3 Al x 10^3)	
	Week 5	Week 6	Week 5	Week 6
Distraction	$277.6 \pm 39.7^*$	$366.6 \pm 52.8^{*,\#}$	$278.0 \pm 50.7^*$	$365.6 \pm 67.0^{*,\#}$
Osteotomy	120.6 ± 18.8	149.0 ± 26.7	132.9 ± 24.5	$166.5 \pm 32.7^{\#}$

Data are presented as the mean ± SEM. Bone amount is given in equivalents of Aluminum (Al) in mm^3 x 10^3. * Significant differences between groups at the corresponding time ($P < .05$). # Significant increase in time within the group ($P < .05$).

Discussion

In the present study, radiographic data and densitometric image analysis were consistent with a successful lengthening procedure in analogy with previous models of distraction osteogenesis in dogs.[9,23,24,30,38] Distraction osteogenesis is characterized by intramembranous ossification, whereas endochondral bone formation is encountered during healing of an osteotomy. This could lead to differences in the expression levels of GH, GHR, IGF-I, IGF-II, and BMP-2 in the bone regenerate. In addition, alterations in the local production of osteotropic growth factors could result in a shift of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 plasma concentrations.[30]

Expression of GH mRNA has been demonstrated in the metaphyseal areas adjacent to growth plate in dogs.[29] In the present study, GH expression in the bone regenerate was very low and did not seem to play an important role at this stage of bone formation. Although the role of GHR in the growth plate is slowly elucidated, very little is known about its role in distraction osteogenesis and bone healing.[8,17,27]. In the present study, GHR expression was also demonstrated in the osteotomy-induced callus, but there was a clear up-regulation in the distraction-induced bone regenerate. Mechanical tension-stress could be responsible for the increased GHR expression levels in the distraction-induced bone regenerate.[6] Raising the local sensitivity to GH may contribute in enhancing bone consolidation after distraction osteogenesis.[2,39] In the present study, up-regulation of GHR without alterations in IGF-I expression and IGF-I plasma concentrations could suggest a direct effect of endogenous GH on consolidation of the bone regenerate.[19,50]

Hypophyseal GH is presumed to represent the major constituent of GH in the circulation and GH release and IGF-I production are closely interconnected.[7,44] In the present study, changes in the GH 8-hour secretory profiles, including GH-B, GH-AUC-0, and GH-AUC-B, could not account for the initially decreased IGF-I plasma concentrations. Reports on IGF-I plasma levels during distraction osteogenesis have been contradictory and seem to be related to the duration and amount of lengthening.[4,30,33,51] In the present study, the initial decline in IGF-I plasma levels was similar in all groups. Although post-operative weight loss was excluded as a cause of decreased IGF-I production, a temporary relative insensitivity to GH due to stress could play a role in this finding. In accordance with earlier reports, IGF-I expression was present in the distraction-induced bone regenerate and the osteotomy callus.[12,14,30,51] In the present study, distraction osteogenesis did not result in modulation of IGF-I plasma concentrations during lengthening or the level of IGF-I expression in the consolidation phase. Whether

IGF-I up-regulation in the distraction zone plays an important role during active distraction remains unclear.

IGF-II is a major constituent of both local and systemic growth factors.[3] Knowledge about the role of IGF-II in bone healing and distraction osteogenesis is very limited. IGF-I and IGF-II were reported to be important as local growth factors for osteoblast survival and apoptosis and to modulate osteoblast-osteoclast interactions critical in bone remodeling.[20,21] In the present study, IGF-II plasma concentrations and the minimal expression of IGF-II in the bone regenerate during consolidation did not supply us with clues on the role of IGF-II.

As IGFBP-3 and IGFBP-5 are considered to stimulate osteoblast function, these IGFBPs are of major interest during distraction osteogenesis. Unfortunately, it has not been possible to determine IGFBP-3 and IGFBP-5 levels in canine plasma. On the other hand, IGFBP-4 is capable of modulating IGF actions in bone.[26] In the present study, IGFBP-4 concentrations were fairly constant and comparable with plasma levels in human individuals.[48] IGFBP-6 preferentially binds and modulates IGF-II.[46,49,53] In the present study, canine plasma IGFBP-6 levels tended to be twice as high as in their human counterparts.[49] The decrease in IGFBP-6 plasma concentrations possibly mediated through TGF-β1 could enhance local availability of IGF-II and thus stimulate bone formation.[16] Due to the lack of proper sequences of canine IGFBP-4 and -6 mRNA's we were not yet able to amplify IGFBP mRNA in canine tissue samples. The systemic and local role of IGFBP-4 and IGFBP-6 remains unclear.

BMP-2, -4, and -6 expression has been demonstrated in distraction osteogenesis in rats.[43] In a rabbit model, BMP-2, -4, and -7 was specifically expressed during active lengthening, whereas BMP expression gradually disappeared in the consolidation phase of the bone regenerate.[40] In the present study, the relatively lower expression of BMP-2 in the distraction-induced bone regenerate during consolidation in comparison with the expression in the osteotomy callus could indicate a more advanced stage of bone formation in the first.

The present study was limited by only evaluating the expression of GH, GHR, IGF-I, IGF-II, and BMP-2 in the consolidation phase of distraction osteogenesis. Determination of gene expression at more than one time point would be ideal to elucidate the role of these factors during active lengthening. In addition, pursuing other osteotropic and angiogenic factors will be essential to further evaluate osteogenesis. Micro-array techniques, in which gene expression profiles of large groups of these factors can be determined simultaneously, look very promising to achieve this goal.

Summarily, up-regulation of GHR expression seems to play an important role in the consolidation phase of distraction osteogenesis. Increased sensitivity to

endogenous systemic GH may promote bone formation and bone maturation mediated through a direct effect of GH.

References

1. Aronson J. Experimental and clinical experience with distraction osteogenesis. *Cleft Palate Craniofac J* 1994;31:473-481.
2. Bail HJ, Kolbeck S, Lindner T, Dahne M, Weiler A, Windhagen HJ, Raun K, Skjaerbaek C, Flyvbjerg A, Orskov H, Haas NP, Raschke MJ. The effect of growth hormone on insulin-like growth factor I and bone metabolism in distraction osteogenesis. *Growth Horm IGF Res* 2001;11:314-323.
3. Bautista CM, Mohan S, Baylink DJ. Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. *Metabolism* 1990;39:96-100.
4. Canalis E, Pash J, Gabbitas B, Rydziel S, Varghese S. Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. *Endocrinology* 1993;133:33-38.
5. Carvalho RS, Einhorn TA, Lehmann W, Edgar C, Al Yamani A, Apazidis A, Pacicca D, Clemens TL, Gerstenfeld LC. The role of angiogenesis in a murine tibial model of distraction osteogenesis. *Bone* 2004;34:849-861.
6. Cillo JE, Jr., Gassner R, Koepsel RR, Buckley MJ. Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: implications for distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000;90:147-154.
7. Conzemius MG, Brown DC, Brabec M, Smith GK, Washabau R, LaFond E, Chakraborty PK. Correlation between longitudinal bone growth, growth hormone, and insulin-like growth factor-I in prepubertal dogs. *Am J Vet Res* 1998;59:1608-1612.
8. Cruickshank J, Grossman DI, Peng RK, Famula TR, Oberbauer AM. Spatial distribution of growth hormone receptor, insulin-like growth factor-I receptor and apoptotic chondrocytes during growth plate development. *J Endocrinol* 2005;184:543-553.
9. Delloye C, Delefortrie G, Coutelier L, Vincent A. Bone regenerate formation in cortical bone during distraction lengthening. An experimental study. *Clin Orthop Relat Res* 1990;250:34-42.
10. Eckardt H, Bundgaard KG, Christensen KS, Lind M, Hansen ES, Hvid I. Effects of locally applied vascular endothelial growth factor (VEGF) and VEGF-inhibitor to the rabbit tibia during distraction osteogenesis. *J Orthop Res* 2003;21:335-340.
11. Eigenmann JE, Eigenmann RY. Radioimmunoassay of canine growth hormone. *Acta Endocrinol (Copenh)* 1981;98:514-520.
12. Eingartner C, Coerper S, Fritz J, Gaissmaier C, Koveker G, Weise K. Growth factors in distraction osteogenesis. Immuno-histological pattern of TGF-beta1 and IGF-I in human callus induced by distraction osteogenesis. *Int Orthop* 1999;23:253-259.
13. Fang TD, Salim A, Xia W, Nacamuli RP, Guccione S, Song HM, Carano RA, Filvaroff EH, Bednarski MD, Giaccia AJ, Longaker MT. Angiogenesis is required for successful bone induction during distraction osteogenesis. *J Bone Miner Res* 2005;20:1114-1124.
14. Farhadieh RD, Dickinson R, Yu Y, Gianoutsos MP, Walsh WR. The role of transforming growth factor-beta, insulin-like growth factor I, and basic fibroblast growth factor in distraction osteogenesis of the mandible. *J Craniofac Surg* 1999;10:80-86.
15. Favier RP, Mol JA, Kooistra HS, Rijnberk A. Large body size in the dog is associated with transient GH excess at a young age. *J Endocrinol* 2001;170:479-484.
16. Gabbitas B, Canalis E. Growth factor regulation of insulin-like growth factor binding protein-6 expression in osteoblasts. *J Cell Biochem* 1997;66:77-86.
17. Gevers EF, van der Eerden BC, Karperien M, Raap AK, Robinson IC, Wit JM. Localization and regulation of the growth hormone receptor and growth hormone-binding protein in the rat growth plate. *J Bone Miner Res* 2002;17:1408-1419.

18.   Hansen-Algenstaedt N, Algenstaedt P, Bottcher A, Joscheck C, Schwarzloh B, Schaefer C, Muller I, Koike C, Ruther W, Fink B. Bilaterally increased VEGF-levels in muscles during experimental unilateral callus distraction. *J Orthop Res* 2003;21:805-812.

19.   Hedner E, Linde A, Nilsson A. Systemically and locally administered growth hormone stimulates bone healing in combination with osteopromotive membranes: an experimental study in rats. *J Bone Miner Res* 1996;11:1952-1960.

20.   Hill PA, Reynolds JJ, Meikle MC. Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. *Endocrinology* 1995;136:124-131.

21.   Hill PA, Tumber A, Meikle MC. Multiple extracellular signals promote osteoblast survival and apoptosis. *Endocrinology* 1997;138:3849-3858.

22.   Holbein O, Neidlinger-Wilke C, Suger G, Kinzl L, Claes L. Ilizarov callus distraction produces systemic bone cell mitogens. *J Orthop Res* 1995;13:629-638.

23.   Ilizarov GA. The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation. *Clin Orthop Relat Res* 1989;238:249-281.

24.   Ilizarov GA. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. *Clin Orthop Relat Res* 1989;239:263-285.

25.   Kaspar D, Neidlinger-Wilke C, Holbein O, Claes L, Ignatius A. Mitogens are increased in the systemic circulation during bone callus healing. *J Orthop Res* 2003;21:320-325.

26.   Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM, Rosenfeld RG. Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. *Int J Biochem Cell Biol* 1996;28:619-637.

27.   Kelly PA, Finidori J, Moulin S, Kedzia C, Binart N. Growth hormone receptor signalling and actions in bone growth. *Horm Res* 2001;55 Suppl 2:14-17.

28.   Kim IS, Park JW, Kwon IC, Baik BS, Cho BC. Role of BMP, betaig-h3, and chitosan in early bony consolidation in distraction osteogenesis in a dog model. *Plast Reconstr Surg* 2002;109:1966-1977.

29.   Kirpensteijn J, Timmermans-Sprang EP, van Garderen E, Rutteman GR, Lantinga-van Leeuwen IS, Mol JA. Growth hormone gene expression in canine normal growth plates and spontaneous osteosarcoma. *Mol Cell Endocrinol* 2002;197:179-185.

30.   Lammens J, Liu Z, Aerssens J, Dequeker J, Fabry G. Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. *J Bone Miner Res* 1998;13:279-286.

31.   Liu Z, Luyten FP, Lammens J, Dequeker J. Molecular signaling in bone fracture healing and distraction osteogenesis. *Histol Histopathol* 1999;14:587-595.

32.   Merriam GR, Wachter KW. Algorithms for the study of episodic hormone secretion. *Am J Physiol* 1982;243:310-318.

33.   Meyer U, Meyer T, Schlegel W, Scholz H, Joos U. Tissue differentiation and cytokine synthesis during strain-related bone formation in distraction osteogenesis. *Br J Oral Maxillofac Surg* 2001;39:22-29.

34.   Mohan S. Insulin-like growth factor binding proteins in bone cell regulation. *Growth Regul* 1993;3:67-70.

35.   Mohan S, Richman C, Guo R, Amaar Y, Donahue LR, Wergedal J, Baylink DJ. Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms. *Endocrinology* 2003;144:929-936.

36.   Nap RC, Mol JA, Hazewinkel HA. Age-related plasma concentrations of growth hormone (GH) and insulin- like growth factor I(IGF-I) in Great Dane pups fed different dietary levels of protein. *Domest Anim Endocrinol* 1993;10:237-247.

37. Okazaki H, Kurokawa T, Nakamura K, Matsushita T, Mamada K, Kawaguchi H. Stimulation of bone formation by recombinant fibroblast growth factor-2 in callotasis bone lengthening of rabbits. *Calcif Tissue Int* 1999;64:542-546.
38. Orbay JL, Frankel VH, Finkle JE, Kummer FJ. Canine leg lengthening by the Ilizarov technique. A biomechanical, radiologic, and morphologic study. *Clin Orthop Relat Res* 1992;278:265-273.
39. Raschke MJ, Bail H, Windhagen HJ, Kolbeck SF, Weiler A, Raun K, Kappelgard A, Skiaerbaek C, Haas NP. Recombinant growth hormone accelerates bone regenerate consolidation in distraction osteogenesis. *Bone* 1999;24:81-88.
40. Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R. Temporal and spatial expression of bone morphogenetic protein-2, -4, and -7 during distraction osteogenesis in rabbits. *Bone* 2000;27:453-459.
41. Rauch F, Lauzier D, Travers R, Glorieux F, Hamdy R. Effects of locally applied transforming growth factor-beta1 on distraction osteogenesis in a rabbit limb-lengthening model. *Bone* 2000;26:619-624.
42. Richards M, Goulet JA, Weiss JA, Waanders NA, Schaffler MB, Goldstein SA. Bone regeneration and fracture healing. Experience with distraction osteogenesis model. *Clin Orthop Relat Res* 1998;355:191-204.
43. Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. *J Bone Miner Res* 1999;14:1084-1095.
44. Sjogren K, Jansson JO, Isaksson OG, Ohlsson C. A model for tissue-specific inducible insulin-like growth factor-I (IGF-I) inactivation to determine the physiological role of liver-derived IGF-I. *Endocrine* 2002;19:249-256.
45. Sota de la RL, Simmen FA, Diaz T, Thatcher WW. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. *Biol Reprod* 1996;55:803-812.
46. Srinivasan N, Edwall D, Linkhart TA, Baylink DJ, Mohan S. Insulin-like growth factor-binding protein-6 produced by human PC-3 prostate cancer cells: isolation, characterization and its biological action. *J Endocrinol* 1996;149:297-303.
47. Stewart KJ, Weyand B, van't Hof RJ, White SA, Lvoff GO, Maffulli N, Poole MD. A quantitative analysis of the effect of insulin-like growth factor-1 infusion during mandibular distraction osteogenesis in rabbits. *Br J Plast Surg* 1999;52:343-350.
48. van Doorn J, Cornelissen AJ, Buul-Offers SC. Plasma levels of insulin-like growth factor binding protein-4 (IGFBP-4) under normal and pathological conditions. *Clin Endocrinol (Oxf)* 2001;54:655-664.
49. van Doorn J, Ringeling AM, Shmueli SS, Kuijpers MC, Hokken-Koelega AC, Buul-Offers SC, Jansen M. Circulating levels of human insulin-like growth factor binding protein- 6 (IGFBP-6) in health and disease as determined by radioimmunoassay. *Clin Endocrinol (Oxf)* 1999;50:601-609.
50. Wang J, Zhou J, Cheng CM, Kopchick JJ, Bondy CA. Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. *J Endocrinol* 2004;180:247-255.
51. Weiss S, Baumgart R, Jochum M, Strasburger CJ, Bidlingmaier M. Systemic regulation of distraction osteogenesis: a cascade of biochemical factors. *J Bone Miner Res* 2002;17:1280-1289.
52. Yeung HY, Lee SK, Fung KP, Leung KS. Expression of basic fibroblast growth factor during distraction osteogenesis. *Clin Orthop Relat Res* 2001;385:219-229.

53.   Yu H, Mistry J, Nicar MJ, Khosravi MJ, Diamandis A, van Doorn J, Juul A. Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin- like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *J Clin Lab Anal* 1999;13:166-172.

Chapter 5

The Efficacy of the Bone Markers Osteocalcin and Carboxyterminal Cross-linked Telopeptide of Type I Collagen in Evaluating Osteogenesis in a Canine Crural Lengthening Model

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Abstract

The aim of the present study was to determine the efficacy of the bone markers osteocalcin (OC) and carboxyterminal cross-linked telopeptide of type I collagen (ICTP) in evaluating new bone formation in the dog, using commercially available immunoassay kits. Dogs were randomly divided into three groups and a circular external skeletal fixation system (CESF) was mounted on the tibia. In the first group a distraction osteogenesis procedure of the crus was performed. The second group received an osteotomy without crural lengthening, whereas the third group served as a sham-operated control. Bone formation was assessed, using densitometric image analysis of crural radiographs. Despite significant differences in the amount of newly formed bone, this finding was not reflected in the plasma levels of OC and ICTP. In conclusion, OC and ICTP were not efficacious as markers of bone formation and resorption during osteogenesis in this canine model.

Introduction

Bone metabolism can be monitored non-invasively using markers of bone formation and resorption. A variety of commercially available bone marker assays has been developed. Although designed initially for use in humans, several of these assays are also validated to monitor bone metabolism in laboratory animals, dogs and horses.[8,9,10,25]

Bone markers can be subdivided into enzymatic markers and metabolic products of bone formation and resorption. The enzymatic markers include bone-specific alkaline phosphatase (BAP), an osteoblast related marker of bone formation, and tartrate-resistant acid phosphatase (TRAP), an osteoclast-related marker of bone resorption.[12,19,22] Serum or plasma markers of metabolic products of bone metabolism in companion animals include osteocalcin (OC), the carboxyterminal propeptide of type-I procollagen (PICP), the aminoterminal propeptide of type-I procollagen (PINP), the cross-linked carboxyterminal telopeptide of type-I collagen (ICTP), and the C-terminal cross-linked telopeptide of type-I collagen (CTX). OC is an osteoblast-related marker of bone formation PICP and PINP are markers of type-I collagen synthesis and hence bone formation.[11,14,38]

ICTP and CTX are markers of type-I collagen breakdown and hence bone resorption.[30] ICTP is released through the actions of matrix metalloproteinases and is therefore also known as CTX-MMP. CTX is released through he actions of cysteine proteinases, including cathepsin K.[20]

Bone markers have been used successfully in humans to monitor metabolic bone disease, including chronic renal disease, osteoporosis, hyperthyroidism, rheumatoid arthritis and growth hormone deficiency.[1,18,30,38,43,46] In the dog, reports concerning bone markers to monitor bone pathology or osteogenesis are limited to OC, ICTP, and BAP.[15,16,26,37]

In both man and dog, distraction osteogenesis is used to treat a variety of skeletal conditions, including bone length deficits, growth deformities, bone loss after trauma or radical resection, and cosmetic craniofacial surgery.[4,31,42,47] This principle allows the production of large quantities of new bone from the osteotomy sites under controlled mechanical distraction. In the dog, there is extensive experience with distraction osteogenesis, both clinically and as an animal model, although insights in the fundamentals of this type of bone formation are still insufficient. Both in research and clinical settings evaluation of the distraction bone regenerate is mainly performed by plain radiography, sonography, and the histomorphometric assessment of bone biopsies.[6,7,13,18,35,40,41] Application of advanced imaging techniques, including magnetic resonance imaging and

computed tomography, is often impeded by the presence of the external skeletal fixation system, used for the distraction procedure.

Bone markers could provide us with another means to evaluate bone formation and resorption during both clinical and experimental lengthening procedures and other types of bone healing. The objective of the present study was to determine using a canine crural lengthening model whether the bone markers OC and ICTP could be of value in evaluating osteogenesis following either a distraction procedure or bone healing after an osteotomy.

Materials and Methods

Animals

The Utrecht University Ethics Committee for Animal Care and Use approved all procedures in this study. The data presented in this manuscript were collected during a study, assessing the fundamentals of distraction osteogenesis, including the local expression of bone growth factors and systemic induction of growth factors.

Twelve mature Labrador retriever dogs were used, with a mean age of 20 months (range, 12 - 31 months), and a mean body weight of 27 kg (range, 21 - 32 kg). The animals were allocated to three groups. Dogs were fed a balanced commercial dog food twice a day at a set time (i.e., 0900 and 1800 h) and water was available ad libitum.

Surgery and Distraction

Circular External Skeletal Fixation (CESF) systems were prepared prior to surgery and steam sterilized (Imex Veterinary Inc., Longview, TX, USA). All frames were identical and consisted of two proximal and two distal full rings with a 100 mm diameter, connected by three treaded rods with a one mm pitch.

The dogs received medetomidine (Domitor, Pfizer Animal Health B.V., Capelle a/d IJssel, The Netherlands) as a pre-anaesthetic sedative and anaesthesia was induced IV with propofol (Rapinovet, Schering-Plough Animal Health N.V., Bruxelles, Belgium). General inhalation anaesthesia was accomplished with nitrous oxide, oxygen, and isoflurane. Amoxicillin with clavulanic acid (Augmentin, SmithKline Beecham Farma B.V., Rijswijk, The Netherlands) was administered IV (20 mg/kg BW) prior to surgery.

The right hind limb was prepared in a standard sterile fashion. The CESF was placed on the right tibia with the distal ring 3 cm proximal of the tarsocrural

joint. The most proximal and distal rings were mounted on the tibia, using two 1.5 mm diameter transosseus wires each. The two inner rings were both mounted to the tibia with one 1.5 mm diameter transosseus wire. All wires were tensioned, using a dynamometric wire tensioner (Hofmann SaS, Monza, Italy), to an equivalent of 60 kg. An anteromedial approach to the tibia and fibula was used. The periosteum of the tibia was preserved by making a longitudinal incision of 3 cm and by carefully elevating the periosteum circumferentially from the bone. In the dogs of the distraction (n=4) and osteotomy (n=4) group the tibia and fibula were osteotomized in the diaphysis, while protecting the periosteum, at two-thirds of the tibial length from the proximal, using an oscillating saw and ample lavage for cooling. In the dogs of the control group (n=4), after placement of the CESF, the periosteum of the tibia was also elevated. In all three groups the periosteum was closed with an absorbable suture material and closure of the subcutis and skin was in a routine fashion.

A protective full-leg bandage was applied for 3 days. There after, the CESF was protected only with a bandage. The dogs received analgesics (buprenorphine, 4dd 10 µg/kg BW) for 3 days post operatively. Full loading of the legs was permitted immediately after surgery. Dogs were housed in cages for the duration of the study. After a 4-day latency, distraction was started with a rate of 0.5 mm twice a day for a period of 10 consecutive days in the distraction group. All dogs were euthanatized with an overdose of barbiturates at 6 weeks after surgery.

Collection of plasma samples

Jugular vein blood samples were collected into EDTA tubes in the week prior to surgery (week 0) to obtain preoperative values. The samples were centrifuged for 15 min at 3.4 g and plasma was collected into plain tubes and stored at -20°C until analysis. As we were interested in bone consolidation following the lengthening procedure, plasma samples were then collected once a week, starting at the end of the distraction period (i.e. week 2) for five consecutive weeks. All blood samples were taken at the same time of the day (i.e., 0800 h).

Assay for osteocalcin (OC)

Plasma OC concentrations were determined, using a commercially available intact human osteocalcin ELISA kit validated for the dog and following the manufacturer's instructions (Biomedical Technologies Inc, Stoughton, MA, USA).[16] The assay measures only intact osteocalcin and it eliminates any potential confounding interference by circulating fragments. The assay is a sandwich ELISA, which utilises monoclonal antibodies directed towards the amino- and

carboxy-terminal regions of the protein. The inter- and intra-essay coefficient of variability (CV) were 10.5% and 7%, respectively, with a sensitivity of 0.5 µg/L (unpublished data).

Assay for carboxyterminal cross-linked telopeptide of type-I collagen (ICTP)

Plasma ICTP concentrations were determined using a commercially available UniQ ICTP RIA kit validated for the dog and following the manufacturer's instructions (Orion Corp. Orion Diagnostica, Espoo, Finland). This RIA kit is based on the competitive radioimmunoassay technique. A known amount of labelled ICTP and an unknown amount of unlabelled ICTP in the same sample compete for the limited number of high affinity binding sites of the rabbit anti-ICTP antibody. After separating the free antigen, the amount of labelled ICTP in the sample tube is inversely proportional to the amount of ICTP in the sample. Serial dilutions of canine plasma resulted in a curve parallel to the standard curve of human ICTP. The inter- and intra-assay CVs were 5.7% and 4.9% at 6.1 µg/L, and 4.1% and 4.7% at 32 µg/L, respectively, with a sensitivity of 0.7 µg/L.[44]

Radiography and densitometry

Immediately after surgery standardized radiographs of the right crus in all three groups in a posterior-anterior (PA) and lateromedial (LM) direction were obtained and on a weekly basis thereafter to follow-up bone formation in the distraction and osteotomy zone. The radiographs of the CESF and right tibia included a ruler and an aluminium step-wedge, consisting of a total of ten 2 mm thick aluminium slabs mounted in an overlapping manner. Bone formation was quantified using a densitometric image analysis system at 5 and 6 weeks post-surgery.[16]

Radiographs were recorded with a Sony b/w CCD camera type XC-77CE and digitized for image analysis (frame size 752 x 574 pixels; 256 grey levels) with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the amount of regenerated bone. Each radiograph of the bone was calibrated geometrically and densitometrically, using the image of a ruler and the aluminium step-wedge. The densitometric calibration was performed by measuring the mean optical density of a square area of 50 x 50 pixels in six steps of the aluminium model (0,2,4,6,8, and 10 mm). The measurement was carried out in a median filtered image to reduce the influence of the photographic grains in the film.

The optical density values are a polygonal fit with the aluminium values to produce a transformation table, which enables the amount of newly formed bone to

be express in equivalents of cubic mm of aluminium. The region of interest (ROI) was centred over the distraction or osteotomy zone and included all new bone formation. The ROI was delineated on the digitalized PA and LM images and densitometric analyses for bone area and bone amount were performed. The means of the PA and LM bone area and bone density were determined for statistical analysis.

Statistical analysis

OC and ICTP plasma concentrations were evaluated using an ANOVA for repeated measures and a Tukey HSD post hoc test. Densitometric data, including the bone area and bone amount, and the OC and ICTP concentrations within the groups were compared using a Student's *t* test. Correlations between the bone markers OC and ICTP and the densitometric data were examined, using the Pearson correlation test. *P* values < .05 were considered significant. All data analyses were performed using the SPSS 10.1 statistical package (SPSS Inc, Chicago, IL, USA).

Results

Plasma concentrations of OC

During the postoperative period, OC plasma concentrations tended to be lower than pre surgery in all three groups. In both the distraction and osteotomy group plasma OC concentrations were significantly (*P* < .05) lower than preoperative values from week 2 to 5. Although the decline in OC plasma concentrations was most pronounced in the distraction group, no significant differences were demonstrated between the groups at any time during the study (Fig 1).

Plasma concentrations of ICTP

A significant difference (*P* < .05) in the ICTP plasma concentrations was found between the control group and the osteotomy group only at 2 weeks after surgery. The ICTP plasma concentrations within the groups did not differ from the preoperative levels. In the distraction and osteotomy group ICTP concentrations did not change during the phase of bone formation (Fig 2).
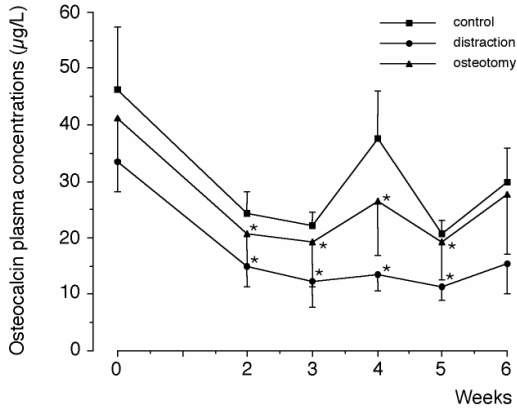
Fig 1. Osteocalcin (OC) plasma concentrations of the control, distraction, and osteotomy group during the 6-week study period. Data are presented as the mean ± SE. * Significant difference in comparison with the preoperative values at week 0 ($P < .05$).
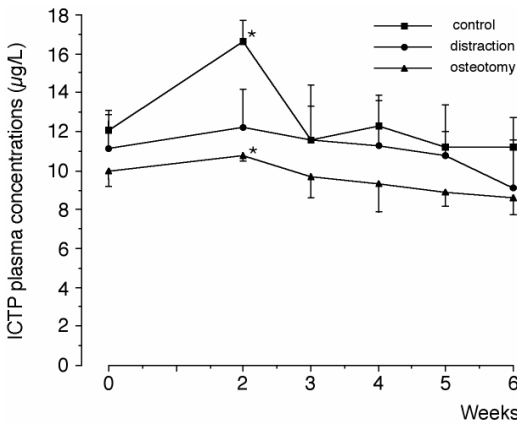

Fig 2. Carboxyterminal cross-linked telopeptide of type I collagen (ICTP) plasma concentrations of the control, distraction, and osteotomy group during the 6-week study period. Data are presented as the mean ± SE. * Significant difference between the groups at that time ($P < .05$).

Radiography and densitometry

The distraction procedure was uneventful and no serious complications regarding the CESF were encountered. Superficial transosseus wire tract infection was the most common complication in all groups, but could be managed successfully with oral antibiotics and local wound care.[33]

Periosteal new bone adjacent to the site of osteotomy was already present as early as 1 week postoperatively in both the distraction and osteotomy group. The amount and density of the periosteal bone increased over time. In the distraction zone new bone formation occurred at 3 weeks postoperative and the periosteal

bone had merged with the bone in the distraction zone at 4 weeks. In the control group no periosteal bone formation was seen at the site of periosteal elevation.

At 5 weeks after surgery, the newly formed callus had mineralised to such an extent that densitometric assessment was possible. The densitometric evaluation revealed a significantly greater bone area and bone amount in the distraction group than in the osteotomy group both at 5 and 6 weeks after surgery. Bone formation as indicated by the bone area and bone amount was larger by more than a factor of 2 larger in the distraction group compared with the osteotomy group. Within the distraction group both the bone area and bone amount had increased significantly from week 5 to 6. In the osteotomy group this increase was only significant for the bone amount (Table 1).

Table 1. Densitometric image analyses of bone formation in the distraction (n=4) and osteotomy (n=4) group.

	Bone area (mm^2)		Bone amount (mm^3 Al x 10^3)	
	Week 5	Week 6	Week 5	Week 6
Distraction	$277.6 \pm 39.7^*$	$366.6 \pm 52.8^{*,\#}$	$278.0 \pm 50.7^*$	$365.6 \pm 67.0^{*,\#}$
Osteotomy	120.6 ± 18.8	149.0 ± 26.7	132.9 ± 24.5	$166.5 \pm 32.7^{\#}$

Data are presented as the mean ± SE. Bone amount is given in equivalents of Aluminium (Al) in mm^3 x 10^3. No new bone formation was found in the control group. * Significant differences between the two groups at the corresponding time ($P < .05$). $^{\#}$ Significant increase within the group between weeks 5 and 6 ($P < .05$).

Correlations between the bone markers OC and ICTP and densitometry

No significant correlations were found between OC and ICTP at any time during the study. The results of the densitometric measurement, including the bone area and bone amount, showed no correlations with the plasma concentrations of either OC or ICTP.

Discussion

The specific aim of this study was to investigate, using a distraction osteogenesis model, whether commercially available OC and ICTP bone marker assays could be of value in monitoring bone formation and resorption during bone healing and whether they might therefore have clinical usefulness in dogs. Ideally, the bone markers should be able to monitor early bone formation, to assess progression of bone consolidation, and to predict the outcome of bone healing.

The discriminatory power of these assays depends on analytic variability and biological variability. The analytic variability is composed of variability within the assay and inter-laboratory variation.[45] Factors affecting the biological variability include age, sex, exercise and systemic disease.[25,27,36,48] In dogs, age has been shown to be an important biological factor of variation.[2,3] In the present study, we used skeletally mature dogs in the same age range which minimized the influence of this factor. ICTP levels have been reported not to differ depending on breed size of the dog.[8] Clear diurnal and seasonal variation has however been demonstrated in various bone markers, specifically OC and ICTP, both in humans and several animal species, including dogs, horses, rats, and mice.[21,25,28,29,34,39]

Standardizing feeding time and the time at which blood was sampled eliminated the diurnal variation of OC and ICTP. Ladlow et al. (2002) reported no significant variation of marker expression over a 12-week period.[25] This was twice the duration of the present study and long-term variation should not be a factor. An issue that cannot be easily resolved is the fact that the direct comparison of results between different studies is impeded by the inter-laboratory variation of bone marker assays.[45]

The radiographic data in this study demonstrated a successful lengthening procedure in analogy with previous models of distraction osteogenesis in both dogs and other species. During distraction osteogenesis bone is primarily produced through intramembranous ossification, whereas endochondral ossification is encountered during healing on an osteotomy.[4] Intramembranous ossification is characterized by direct formation of new bone. During endochondral ossification a cartilaginous callus is formed first, which is secondarily replaced by cancellous bone. Ideally, one would expect earlier and more pronounced alteration of OC plasma concentrations during distraction osteogenesis in comparison with osteotomy bone healing. As remodelling of the primarily cartilaginous callus and thus collagen resorption is essential during endochondral ossification, this could result in more pronounced effects on ICTP plasma concentrations. In addition, the difference in the amount of newly formed bone was expected to be another discriminatory factor. Unfortunately the bone markers OC and ICTP were not efficacious in demonstrating these differences in osteogenesis.

In the present study, OC plasma concentrations decreased and although no significant differences were found between the groups, the OC concentrations were lowest in the distraction group. This is interesting as these dogs showed the highest amount of bone formation. Ducy et al. (1996) demonstrated increased bone formation in OC-deficient mice, indicating a suppressive role of OC on bone formation, possibly through inhibition of osteopontin.[14] In view of this, decreased OC plasma concentrations could be consistent with increased bone formation. The decline in OC plasma concentrations in the control group could be explained by the stimulating effect of the CESF of crural blood flow and thus bone metabolism.[5] In contrast, Lammens et al. (1998) reported increased OC plasma levels during distraction osteogenesis in a dog study.[26] As the assay in the present study only measured intact OC, the difference could be explained by the fact that OC metabolites were included in the earlier study.[23] Whether OC metabolites are biologically active during osteogenesis or this process depends exclusively on intact OC, is not clear. In view of these considerations OC seems to be a questionable marker of bone formation in the dog.

In the present study an osteotomy instead of the technically more demanding corticotomy was performed, as experimental studies comparing both methods revealed no differences in the outcome of the distraction procedure.[13,17,24] Both in experimental and clinical settings, the use of an oscillating saw for the osteotomy in combination with ample lavage has been shown to be effective in dogs.[32] Nevertheless, enhanced ICTP levels were more likely to be expected in the distraction and osteotomy groups than in the control group. An osteotomy will cause some trauma to the bone, which is expected to initially increase bone resorption. In addition, formation of new bone proceeds with both accretion and resorption of collagen and thus should increase ICTP levels. In contrast, we only found a significant difference between the osteotomy and control group at 2 weeks after surgery. The rise in ICTP levels within the control group at 2 weeks post surgery can only be explained as the result of a reaction to the transosseus wires of the CESF. Although there were no radiographic or clinical indications for a local osteomyelitis, enhanced bone resorption at the bone-wire interface could contribute to higher ICTP levels.[37] In the distraction and osteotomy group active bone formation was already present at 2 weeks after surgery with collagen accretion surmounting collagen degradation. As ICTP is released through the actions of MMP, a difference in the expression of MMP in the control group, shifting the equilibrium initially towards collagen degradation, could be responsible for this finding. As we did not measure ICTP levels during the first week after surgery, we could not assess the initial influence of the CESF on ICTP plasma concentrations.

In view of the previous observations, it was not surprising that no correlations were found between OC and ICTP and between these markers and the amount of newly formed bone.

In conclusion, the clinical utility of the commercial assays for the bone markers OC and ICTP in evaluating bone formation related to distraction osteogenesis and osteotomy bone healing is questionable in the dog. Nevertheless, combinations of other bone markers may be efficacious in monitoring new bone formation in dogs and should be explored in more detail.

References

1. Al Awadhi A, Olusi S, Al Zaid N, Prabha K. Serum concentrations of interleukin 6, osteocalcin, intact parathyroid hormone, and markers of bone resorption in patients with rheumatoid arthritis. *J Rheumatol* 1999;26:1250-1256.
2. Allen MJ, Allen LCV, Hoffmann WE, Richardson DC, Breur GJ. Urinary markers of type I collagen degradation in the dog. *Res Vet Sci* 2000;69:123-127.
3. Allen MJ, Hoffmann WE, Richardson DC, Breur GJ. Serum markers of bone metabolism in dogs. *Am J Vet Res* 1998;59:250-254.
4. Aronson J. Experimental and clinical experience with distraction osteogenesis. *Cleft Palate Craniofac J* 1994;31:473-481.
5. Aronson J. Temporal and spatial increases in blood flow during distraction osteogenesis. *Clin Orthop* 1994;301:124-131.
6. Aronson J, Good B, Stewart C, Harrison B, Harp J. Preliminary studies of mineralization during distraction osteogenesis. *Clin Orthop* 1990;250:43-49.
7. Aronson J, Harrison BH, Stewart CL, Harp JH, Jr. The histology of distraction osteogenesis using different external fixators. *Clin Orthop* 1989;241:106-116.
8. Breur GJ, Allen MJ, Carlson SJ, Richardson DC. Markers of bone metabolism in dog breeds of different size. *Res Vet Sci* 2004;76:53-55.
9. Carstanjen B, Hoyle NR, Gabriel A, Hars O, Sandersen C, Amory H, Remy B. Evaluation of plasma carboxy-terminal cross-linking telopeptide of type I collagen concentration in horses. *Am J Vet Res* 2004;65:104-109.
10. Carstanjen B, Sulon J, Banga-Mboko H, Beckers JF, Remy B. Development and validation of a specific radioimmunoassay for equine osteocalcin. *Domest Anim Endocrinol* 2003;24:31-41.
11. Cortet B, Cortet C, Blanckaert F, d'Herbomez M, Marchandise X, Wemeau JL, Decoulx M, Dewailly D. Quantitative ultrasound of bone and markers of bone turnover in Cushing's syndrome. *Osteoporos Int* 2001;12:117-123.
12. de Vernejoul MC. Markers of bone remodelling in metabolic bone disease. *Drugs Aging* 1998;12 Suppl 1:9-14.
13. Delloye C, Delefortrie G, Coutelier L, Vincent A. Bone regenerate formation in cortical bone during distraction lengthening. An experimental study. *Clin Orthop* 1990;250:34-42.
14. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996;382:448-452.
15. Ehrhart N, Dernell WS, Hoffmann WE, Weigel RM, Powers BE, Withrow SJ. Prognostic importance of alkaline phosphatase activity in serum from dogs with appendicular osteosarcoma: 75 cases (1990-1996). *J Am Vet Med Assoc* 1998;213:1002-1006.
16. Fink B, Fox F, Singer J, Skripitz R, Feldkamp J. Monitoring of bone formation during distraction osteogenesis via osteocalcin: a time sequence study in dogs. *J Orthop Sci* 2002;7:557-561.
17. Frierson M, Ibrahim K, Boles M, Bote H, Ganey T. Distraction osteogenesis. A comparison of corticotomy techniques. *Clin Orthop* 1994;301:19-24.
18. Garnero P. Markers of bone turnover for the prediction of fracture risk. *Osteoporos Int* 2000;11 Suppl 6:55-65.
19. Garnero P, Delmas PD. Bone markers. *Baillieres Clin Rheumatol* 1997;11:517-537.
20. Garnero P, Ferreras M, Karsdal MA, Nicamhlaoibh R, Risteli J, Borel O, Qvist P, Delmas PD, Foged NT, Delaisse JM. The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. *J Bone Miner Res* 2003;18:859-867.

21.   Gundberg CM, Markowitz ME, Mizruchi M, Rosen JF. Osteocalcin in human serum: a circadian rhythm. *J Clin Endocrinol Metab* 1985;60:736-739.
22.   Halleen JM, Alatalo SL, Suominen H, Cheng S, Janckila AJ, Vaananen HK. Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption. *J Bone Miner Res* 2000;15:1337-1345.
23.   Kaartinen MT, Pirhonen A, Linnala-Kankkunen A, Maenpaa PH. Transglutaminase-catalyzed cross-linking of osteopontin is inhibited by osteocalcin. *J Biol Chem* 1997;272:22736-22741.
24.   Kojimoto H, Yasui N, Goto T, Matsuda S, Shimomura Y. Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. *J Bone Joint Surg Br* 1988;70:543-549.
25.   Ladlow JF, Hoffmann WE, Breur GJ, Richardson DC, Allen MJ. Biological variability in serum and urinary indices of bone formation and resorption in dogs. *Calcif Tissue Int* 2002;70:186-193.
26.   Lammens J, Liu Z, Aerssens J, Dequeker J, Fabry G. Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. *J Bone Miner Res* 1998;13:279-286.
27.   Lepage OM, Carstanjen B, Uebelhart D. Non-invasive assessment of equine bone: an update. *Vet J* 2001;161:10-22.
28.   Lepage OM, DesCoteaux L, Marcoux M, Tremblay A. Circadian rhythms of osteocalcin in equine serum. Correlation with alkaline phosphatase, calcium, phosphate and total protein levels. *Can J Vet Res* 1991;55:5-10.
29.   Liesegang A, Reutter R, Sassi ML, Risteli J, Kraenzlin M, Riond JL, Wanner M. Diurnal variation in concentrations of various markers of bone metabolism in dogs. *Am J Vet Res* 1999;60:949-953.
30.   Loviselli A, Mastinu R, Rizzolo E, Massa GM, Velluzzi F, Sammartano L, Mela Q, Mariotti S. Circulating telopeptide type I is a peripheral marker of thyroid hormone action in hyperthyroidism and during levothyroxine suppressive therapy. *Thyroid* 1997;7:561-566.
31.   Maffulli N, Lombari C, Matarazzo L, Nele U, Pagnotta G, Fixsen JA. A review of 240 patients undergoing distraction osteogenesis for congenital post-traumatic or postinfective lower limb length discrepancy. *J Am Coll Surg* 1996;182:394-402.
32.   Marcellin-Little DJ. Treating bone deformities with circular external skeletal fixation. *Comp Cont Educ Pract Vet* 1999;21:481-491.
33.   Marcellin-Little DJ, Ferretti A, Roe SC, DeYoung DJ. Hinged Ilizarov external fixation for correction of antebrachial deformities. *Vet Surg* 1998;27:231-245.
34.   Muhlbauer RC, Fleisch H. The diurnal rhythm of bone resorption in the rat. Effect of feeding habits and pharmacological inhibitors. *J Clin Invest* 1995;95:1933-1940.
35.   Orbay JL, Frankel VH, Finkle JE, Kummer FJ. Canine leg lengthening by the Ilizarov technique. A biomechanical, radiologic, and morphologic study. *Clin Orthop* 1992;278:265-273.
36.   Panteghini M, Pagani F. Biological variation in bone-derived biochemical markers in serum. *Scand J Clin Lab Invest* 1995;55:609-616.
37.   Philipov JP, Pascalev MD, Aminkov BY, Grosev CD. Changes in serum carboxyterminal telopeptide of type I collagen in an experimental model of canine osteomyelitis. *Calcif Tissue Int* 1995;57:152-154.
38.   Polak-Jonkisz D, Zwolinska D, Bednorz R, Owczarek H, Szymanska A, Nahaczewska W. Procollagen I carboxyterminal propeptide (PICP) as a bone formation marker and carboxyterminal telopeptide of type I collagen (ICTP) as a bone degradation marker in children with chronic renal failure under conservative therapy. *Med Sci Monit* 2003;9:19-23.

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39. Price JS, Jackson BF, Gray JA, Harris PA, Wright IM, Pfeiffer DU, Robins SP, Eastell R, Ricketts SW. Biochemical markers of bone metabolism in growing thoroughbreds: a longitudinal study. *Res Vet Sci* 2001;71:37-44.
40. Richards M, Goulet JA, Weiss JA, Waanders NA, Schaffler MB, Goldstein SA. Bone regeneration and fracture healing. Experience with distraction osteogenesis model. *Clin Orthop* 1998;355:191-204.
41. Rowe NM, Mehrara BJ, Dudziak ME, Steinbreck DS, Mackool RJ, Gittes GK, McCarthy JG, Longaker MT. Rat mandibular distraction osteogenesis: Part I. Histologic and radiographic analysis. *Plast Reconstr Surg* 1998;102:2022-2032.
42. Sabharwal S, Paley D, Bhave A, Herzenberg JE. Growth patterns after lengthening of congenitally short lower limbs in young children. *J Pediatr Orthop* 2000;20:137-145.
43. Sartorio A, Ortolani S, Galbiati E, Conte G, Vangeli V, Arosio M, Porretti S, Faglia G. Effects of 12-month GH treatment on bone metabolism and bone mineral density in adults with adult-onset GH deficiency. *J Endocrinol Invest* 2001;24:224-230.
44. Schoenmakers I, Hazewinkel HAW, Voorhout G, Carlson CS, Richardson D. Effect of diets with different calcium and phosphorus contents on the skeletal development and blood chemistry of growing Great Danes. *Vet Rec* 2000;147:652-660.
45. Seibel MJ, Lang M, Geilenkeuser WJ. Interlaboratory variation of biochemical markers of bone turnover. *Clin Chem* 2001;47:1443-1450.
46. Sneppen SB, Hoeck HC, Kollerup G, Sorensen OH, Laurberg P, Feldt-Rasmussen U. Bone mineral content and bone metabolism during physiological GH treatment in GH-deficient adults--an 18-month randomised, placebo-controlled, double blinded trial. *Eur J Endocrinol* 2002;146:187-195.
47. Stanitski DF, Shahcheraghi H, Nicker DA, Armstrong PF. Results of tibial lengthening with the Ilizarov technique. *J Pediatr Orthop* 1996;16:168-172.
48. Szulc P, Seeman E, Delmas PD. Biochemical measurements of bone turnover in children and adolescents. *Osteoporos Int* 2000;11:281-294.

Chapter 6

Evaluation of Delayed-image Bone Scintigraphy to Assess Bone Formation after Distraction Osteogenesis in Dogs

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Abstract

The aim of our study was to quantitatively assess distraction-induced bone formation in a crural lengthening model in dogs by use of delayed-image bone scintigraphy. Twelve mature Labrador Retrievers were randomly allocated to 3 groups and a circular external skeletal fixation system was mounted on the right crus. Distal osteotomy of the tibia and fibula was performed in the first and second group dogs, followed by a lengthening procedure of 10 mm in the first group only. The third group served as sham-operated controls. Delayed-image bone scintigraphy with technetium-99m hydroxy methylene diphosphonate was performed 2, 4, and 6 weeks after surgery. Delayed image:region-of-interest, delayed image:crural, and delayed image:femoral scintigraphic activity ratios were calculated and evaluated by use of ANOVA for repeated measures. New bone formation was quantified by use of densitometric image analysis, and values for the scintigraphic ratios were compared. In the distraction and osteotomy groups, values of delayed-image:region-of-interest and crural ratios increased significantly. Although densitometric image analysis revealed increased bone formation after distraction, the region-of-interest ratios and crural ratios were similar in both groups. All dogs had increased delayed image:femoral ratios. In summary, delayed-image bone scintigraphy ratios were not effective at differentiating between the amounts of distraction-induced bone and osteotomy-induced bone. Metabolic bone activity in the adjacent femur was increased as a consequence of circular external skeletal fixator placement. Delayed-image bone scintigraphy was not adequately sensitive to quantitatively monitor bone formation, but may be useful as an early predictor of bone healing.

Introduction

Distraction osteogenesis has been used in the management of various skeletal conditions, including bone length deficits, bone deformities, bone loss after traumatic injury or radical resection, and craniofacial reconstruction.[21,33] The principle of distraction osteogenesis describes formation of new bone under conditions of controlled mechanical distraction of an osteotomy site. Distraction osteogenesis is characterized by intramembranous bone formation.[2,4] In contrast, routine healing at an osteotomy site is initiated with callus formation and proceeds via endochondral bone formation. The extent of distraction osteogenesis is determined by many factors, including the site of the osteotomy, latency period, distraction rate, soft tissue condition, local blood supply, and age of the animal. Local blood flow to the area of affected bone is closely associated with osteogenesis.[3]

Although the use of histologic, ultrastructural, and radiologic methods to characterize distraction osteogenesis has been described, those methods are either invasive or only applicable in later phases of the mineralization process.[7,27,30] Delayed-image bone scintigraphy is a non-invasive quantitative method for evaluating changes in bone metabolic activity.[12,19] In contrast to radiography, which reveals the amount of mineralization, delayed-image bone scintigraphy evaluates uptake of technetium-99m tracer by immature bone and thus precedes actual accretion of bone.[26,35] In distraction osteogenesis, delayed-image bone scintigraphy has been used successfully to predict the progression of bone formation in the early stages of the lengthening process and to assess the optimal time of bone consolidation in the later stages of bone maturation.[12,19] We hypothesized that delayed-image bone scintigraphy would be useful in the quantitative assessment of bone regeneration after a distraction osteogenesis procedure. Because distraction osteogenesis is known to increase local and regional blood flow, we also speculated that the lengthening procedure would increase the rate of bone metabolism in the adjacent long bone.[3]

Materials and Methods

Animals

Procedures were approved by the Utrecht University Ethics Committee for Animal Care and Use, and all experimentation was conducted in conformity with ethical and humane principles of research. Twelve mature Labrador Retrievers with a mean age of 19 months (range, 12 to 31 months) and mean body weight of 27 kg

(range, 21 to 32 kg) were allocated to 3 groups (n = 4 each). Dogs were individually housed and fed a standard commercial dog food twice a day and had ad libitum access to water.

Surgery and distraction procedures

The lengthening procedure was performed by application of a CESF system (Imex Veterinary Inc., Longview, TX, USA). All frames were identical and consisted of 2 proximal and 2 distal full rings with a 100-mm diameter, connected by 3 treaded rods with a 1-mm pitch. Frames were assembled prior to surgery and steam sterilized. Dogs received medetomidine (Domitor, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands) as a pre-anesthetic sedative and anesthesia was induced with propofol (Rapinovet, Schering-Plough Animal Health NV, Bruxelles, Belgium) administered IV. After intubation, inhalation anesthesia with nitrous oxide, oxygen, and isoflurane commenced. Amoxicillin with clavulanic acid (Augmentin, SmithKline Beecham Farma BV, Rijswijk, The Netherlands) was administered (20 mg/kg, IV) prior to surgery. The skin of the right hind limb was aseptically prepared in a standard fashion. A CESF was attached to the right tibia by use of two 1.5-mm diameter transosseous wires on both the proximal and distal rings and one 1.5-mm diameter transosseous wire on both central rings. An equivalent of 60 kg of tension was applied to the wires with a dynamometric wire tensioner (Hofmann SaS, Monza, Italy).

A craniomedial surgical approach to the tibia and fibula was used to facilitate circumferential elevation of the soft tissues and periosteum. In dogs in the distraction and osteotomy groups, the tibia and fibula were osteotomized in the diaphysis at the level of two-thirds of the tibial length from its proximal aspect by use of an oscillating saw. Ample volumes of fluids were used for lavage during the osteotomy procedure for thermal protection of bone and periosteum.

Dogs in the control group were sham-operated. In all dogs, the periosteum was closed with an absorbable suture material and closure of the subcutis and skin proceeded routinely. Dogs wore a protective full-leg bandage for 3 days after surgery and received buprenorphine (Temgesic, Schering-Plough, Weesp, The Netherlands) as an analgesic (10 µg/kg, SC, q 6 h) for 3 days after surgery. Full loading of the limbs was permitted immediately after surgery. After 4 days, dogs in the distraction group were subjected to lengthening via adjustment of the CESF at a rate of 0.5 mm twice daily, for 10 consecutive days. The site of bone regeneration was allowed to mature for an additional 4 weeks. Dogs were euthanatized with a barbiturate overdose 6 weeks after the initial surgery.

Delayed image bone scintigraphy

Delayed-image bone scintigraphy was performed 2, 4, and 6 weeks after surgery. Each dog received 550 MBq of ^{99m}Tc-HDP IV, 3 hours before data collection. Scintigraphic imaging was performed with a gamma camera system (Siemens Medical Systems, Den Haag, The Netherlands) equipped with a high-resolution parallel-hole collimator. The gamma camera was connected to a dedicated open workstation computer. Immediately prior to scintigraphic imaging, dogs were premedicated with medetomidine and anesthesia was induced and maintained by a continuous rate infusion of propofol delivered via an infusion pump. Dogs were positioned in dorsal recumbency with both crura placed parallel to the tabletop. The camera was positioned over both hind legs with the collimator centered over the distraction zone, osteotomy zone, or corresponding zone in the control group, perpendicular to the long axis of the right and left crura. Counts were collected during a 5-minute period by use of a 256 X 256 matrix with a pixel size of 1.68 mm. Simultaneously, 1 mL of a dilution of the injection dose (dilution, 1:1000) was counted and used as a standard. Bone metabolic activity was determined (by focusing the camera on the distraction, osteotomy, and control zones) for the region of interest, the entire crus, and the distal third of the femur. The regions of interest were selected to include the area of all mineralized callus that was radiographically visible adjacent to the osteotomy zone 6 weeks after surgery. The region of interest was centered over the osteotomy zone with an equal distribution over the proximal and distal segments of the bone. The dimensions of the region of interest were 20 X 10 pixels (5.64 cm^2). An identical region of interest was used for the opposite limb. The same regions of interest were used in the sham-operated dogs. In the distraction group, the region of interest on the right (experimental) limb was enlarged in a proximodistal direction to 20 X 16 pixels (9.03 cm^2) to include the same amount of original crural bone as was included in dogs in the osteotomy and control groups as well as the area of distraction-induced bone regeneration in the lengthening zone. The region of interest for the left limb remained identical to that used in dogs in the other groups. The precise locations of the regions of interest were chosen with reference to the radiographs of each dog so that the same anatomic area evaluated in the densitometric image analysis was accurately reflected.

Activity in the entire tibia and fibula (i.e., crural activity*)* on the right and left limbs was measured. The distal one-third of the femur *(*i.e., femoral activity*)* was delineated on the scans and activity was measured (Fig 1A and 1B). Activity in the right and left limbs was expressed as a percentage of total dose activity. The dose percentage of activity in the right limb was divided by that in the left limb to yield a ratio that expressed the difference in bone metabolic activity between the 2

limbs. Thus, the delayed image:region-of-interest ratio, delayed image:crural ratio, and delayed image:femoral ratios were determined.
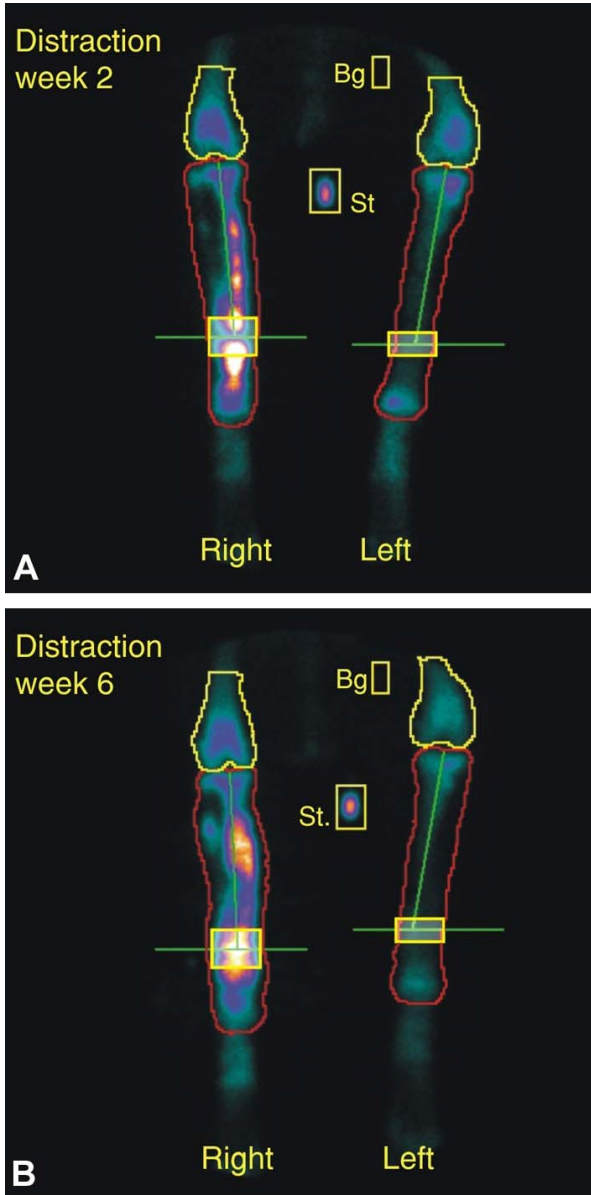

Fig 1. Delayed-image bone scintigraphic images of the right and left crura and distal portion of the femurs in a dog at the end of the active lengthening period 2 weeks postoperatively (A) and during the consolidation phase at 6 weeks after surgery (B). The region of interest (high-lighted), entire crus, and distal portion of the femur are outlined. Notice the area of high uptake of 99mTc-HDP tracer adjacent to and in the distraction zone in the distal portion of the experimental (right) tibia
St = Standard.   Bk = Background.

Densitometric image analysis

Densitometric image analysis of radiographs was used to measure new bone formation.[13] Immediately after surgery and once weekly for 6 weeks thereafter, radiographs of the right crus and CESF were obtained in the caudocranial and lateromedial planes. The radiographic views included a ruler and an aluminum step-wedge consisting of ten 2-mm thick aluminum slabs mounted in an overlapping manner. Bone formation was quantified by use of a densitometric image analysis system. Radiographs were recorded with a high-resolution camera (Sony b/w CCD camera type XC-77CE, Sony Corporation, Tokyo, Japan) and digitized for image analysis (frame size, 752 X 574 pixels; 256 gray levels) with a personal computer-based system equipped with imaging software (KS400 version 3.0 software, Carl Zeiss Vision, Oberkochen, Germany). A program was developed to quantify the amount of mineralized callus observed. Each radiograph was calibrated geometrically and densitometrically by use of the image of the ruler and the aluminum step-wedge. The densitometric calibration was performed by measuring the mean optical density of a square area of 50 X 50 pixels in 6 steps of the aluminum model (i.e., 0, 2, 4, 6, 8, and 10 mm). Measurements were obtained from a median filtered image to reduce the influence of photographic grain in the film. The optical density values were a polygonal fit with the aluminum values to produce a transformation table, which enabled expression of the amount of newly formed bone in units of equivalents of cubic millimeters of aluminum. The region of interest was centered over the distraction or osteotomy zone similarly as in the delayed image bone scintigraphy procedure and included all new bone formation. The regions of interest were delineated on the digitalized caudocranial and lateromedial images and densitometric analyses (including the bone area and bone amount), were performed. For each dog, mean bone area and bone amount in the caudocranial and lateromedial images were determined and used for statistical analysis.

Statistical analysis

Delayed-image ratios were evaluated via ANOVA for repeated measures and a least significant difference post-hoc test. Densitometric data, including values for bone area and bone amount, were compared by use of a Student *t*-test. Correlations between delayed-image ratios and densitometric bone area and bone amount were examined by use of the Pearson correlation test. Power was set at 0.80 and values of $P < .05$ were considered significant. Analyses were performed by use of commercially available statistical software (SPSS 10.1 statistical package, SPSS Inc, Chicago, IL, USA).
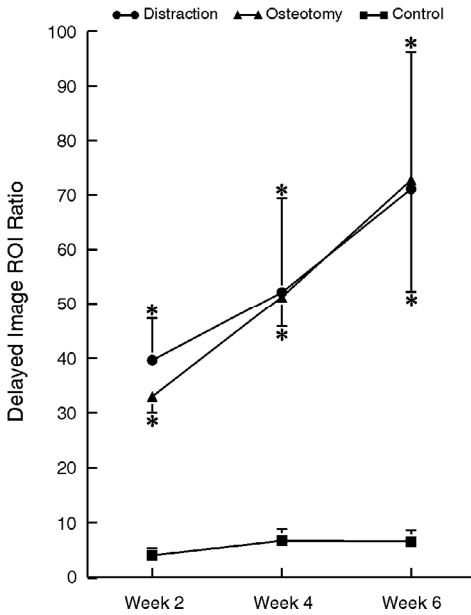
Fig 2. Changes in delayed-image: region-of-interest scintigraphic activity ratios after surgery in 12 dogs that underwent osteotomy alone (n = 4), osteotomy and distraction osteogenesis (4), or a sham procedure (4). Delayed image: region-of-interest ratios were calculated as activity in the region of interest as a percentage of total dose activity. Data are presented as mean ± SEM. *Significant increase in comparison with the controls ($P < .05$).

Results

Scintigraphy

Bone metabolic activity increased significantly on the instrumented right side in all dogs in the distraction, osteotomy, and control groups. The higher uptake of ^{99m}Tc-HDP resulted in increased delayed image:region-of-interest ratios, delayed image:crural ratios, and delayed image:femoral ratios at all time points. In the distraction group, the mean region of interest ratio initially had the largest increase, but values were not significantly different from those in the osteotomy group (Fig 2). Both the distraction and osteotomy groups had significantly higher mean delayed image:region-of-interest ratio than the controls at all time points. Although delayed image:crural ratios were highest in the distraction group, the differences between those ratios and ratios in the osteotomy group were not significantly different (Fig 3). The distraction and osteotomy groups had significantly higher delayed image:crural ratios than controls at all time points. Although delayed image:femoral ratios indicated a higher uptake of ^{99m}Tc-HDP in the femurs of instrumented limbs, compared with the contralateral limbs, no differences were observed among groups (Fig 4). Delayed-image ratios of the region of interest, crus, and femur tended to increase during the study period.
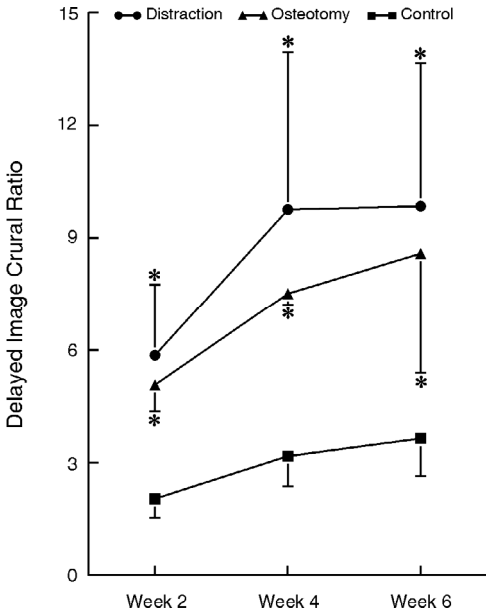
Fig 3. Changes in delayed image: crural scintigraphic activity ratios in the same dogs as in Fig 2. Data are presented as mean ± SEM. *Significant increase in comparison with controls (P < .05).
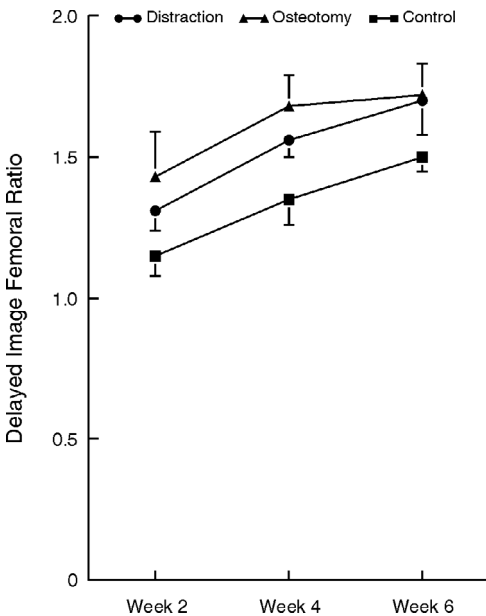

Fig 4. Changes in delayed image: femoral scintigraphic activity ratios in the same dogs as in Fig 2 and 3. Data are presented as mean ± SEM. Notice that there were no significant differences among the groups.

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Densitometric image analysis

The distraction procedures were uneventful in all dogs and no complications were encountered. In dogs in both the distraction and osteotomy groups, periosteal new bone adjacent to the osteotomy site was observed radiographically as early as 1 week after surgery. The amount and density of periosteal bone increased over time. In the distraction zone, new bone formation was observed 3 weeks after surgery and periosteal bone had merged with bone in the distraction zone at 4 weeks. In the control dogs, no periosteal bone formation was detected at the site of periosteal elevation. Mineralization in the distraction and osteotomy groups had progressed to an extent that densitometric evaluation was possible beginning in week 5. In the distraction group, measurements of bone area and bone amount were significantly larger 5 and 6 weeks after surgery, compared with measurements in the osteotomy group. In dogs in the distraction group, bone area and bone amount increased during that period (Table 1).

Table 1. Summary of densitometric image data for new bone formation in dogs that underwent an osteotomy procedure alone (n = 4 dogs) or osteotomy and distraction osteogenesis (4). Measurements were taken 5 and 6 weeks after surgery.

Group	Bone area (mm^2)		Bone amount (mm^3 Al X 10^3)	
	Week 5	Week 6	Week 5	Week 6
Distraction	$277.6 \pm 39.7^*$	$366.6 \pm 52.8^{*,\dagger}$	$278.0 \pm 50.7^*$	$365.6 \pm 67.0^{*,\dagger}$
Osteotomy	120.6 ± 18.8	149.0 ± 26.7	132.9 ± 24.5	$166.5 \pm 32.7^{\dagger}$

Data are given as mean ± SEM. Values for bone area are given in units of square millimeters. Values for bone amount are given in equivalents of aluminum (Al) in mm^3 X 10^3.
[*] Significant differences between the 2 groups at the corresponding time (*P* < .05).
[†] Significant increase in time within the group (*P* < .05).

The mean delayed image:region-of-interest ratio in the distraction group was positively correlated with densitometric bone area, but not with the densitometric bone amount, at 6 weeks (R = 0.95; *P* = .02 and R = 0.84; *P* = .08, respectively). In the osteotomy group, no significant correlations were detected

between the delayed image:region of interest ratio versus bone area and bone amount (R= 0.61, *P* = .20 and R=0.65, *P* = .18, respectively).

Discussion

We hypothesized that delayed-image bone scintigraphy would be useful in quantitatively assessing bone regeneration after a distraction osteogenesis procedure. We also speculated that a lengthening procedure would increase bone metabolic activity in the adjacent long bone. Delayed-image bone scintigraphy ratios were not effective in quantitatively differentiating between distraction-induced bone formation and osteotomy-induced bone formation. Metabolic bone activity in the adjacent femur was increased as a consequence of placement of the CESF device.

In our study, dogs had no prior bone disease or injury and all healed without complication, but results in a clinical setting could be different. Radiographic data revealed the lengthening procedure to be successful via analogy with previous models of distraction osteogenesis in dogs and other species.[4,14] In the present study, an oscillating saw and high-volume lavage were used during the osteotomy, a technique that has been effective in experimental and clinical settings.[7,20,22,23] Densitometric image analysis revealed a greater amount of bone formation in association with the distraction procedure, compared with bone formation after osteotomy alone.

Three-phase bone scintigraphy is a noninvasive method for semi-quantitative assessment of blood flow, blood distribution, and bone metabolic activity.[26] Angiographic imaging during 3-phase scintigraphy reflects local perfusion. Hyperemia of bone and soft tissues is revealed on the blood-pool image. Accumulation of ^{99m}Tc-HDP in the delayed image was presumed to reflect osteoblast activity and new bone formation. Many studies have revealed that blood flow increases during the processes of bone healing and distraction osteogenesis.[1,3,19,24,25,32] Although blood supply is considered to be closely related to rate of osteogenesis, blood flow, as indicated by the perfusion index, appears to be of questionable value as a predictor of new bone formation.[5,12,19] In contrast to the first 2 phases of bone scintigraphy, the value of delayed-image bone scintigraphy in predicting the outcome of bone consolidation after a distraction procedure in humans has been reported.[19] Other recent studies have revealed the role of angiogenic factors, including vascular endothelial growth factor and basic fibroblast growth factor, in distraction-induced bone regeneration.[6,9,17,28] Expression of those factors is maximal during active bone lengthening and decreased during the consolidation phase.

In the present study, we used delayed-image bone scintigraphy to determine the activity of bone metabolism and new bone formation during a lengthening procedure. Initially, the delayed image:region of interest ratio was increased most notably in dogs in the distraction group. This point in time coincided with the end of active lengthening, which is characterized by the highest expression of angiogenic and osteotropic growth factors (i.e., bone morphogenetic proteins, insulin-like growth factor-I, and transforming growth factor ß) and thus the highest rate of bone formation.[8,10,11,28,29,34] Because densitometric image analysis revealed the mean area of new bone formation in the distraction group to be more than twice the size of that in the osteotomy group 6 weeks after surgery, we expected that the delayed image:region-of-interest ratio would reveal this difference. Whether the lack of increase in the region-of-interest ratio can be attributed to a different rate of uptake of ^{99m}Tc-HDP during intramembranous bone formation is uncertain.[2,4] Another explanation is that there could have been altered distribution of tracer as a result of increased blood flow in the lengthened crus. Nevertheless, observation of increasing delayed image:region-of-interest ratios from week 2 onwards in both the distraction and osteotomy groups was consistent with the radiographic evidence of advancing bone formation.[12]

The strong correlation between the delayed image:region-of-interest ratio and densitometric bone area, but not bone amount, in the distraction group at 6 weeks was consistent with the assumption that delayed-image bone scintigraphy is not a measure of mineralization. Although delayed image:region-of-interest ratios were similar in the distraction and osteotomy groups, crural ratios tended to be higher after distraction throughout the study period. Upregulation of bone metabolism outside the lengthening zone, mediated via production of osteotropic and angiogenic growth factors during the distraction procedure, may play a role in this finding.[8,10,11,15,28,29,34] Potential significant differences between the regions of interest may have been obscured by the sample size in the present study, but differences would be less relevant, compared with results of densitometric image analysis.

In the control group, the effect of the CESF system on bone response in the right limbs suggested that the metabolic response of bone to a minimally invasive external fixator can be substantial. Whether enhanced bone metabolic activity was the result of production of angiogenic and osteotropic growth factors as a reaction to the transosseous wires is unclear. Although the local response, characterized by the delayed image:region-of-interest ratios and crural ratios, differed significantly in both the distraction and osteotomy groups, compared with controls, the distant effect on the femur was similar in magnitude in all 3 groups. In actively growing patients, the increased bone metabolism in the adjacent long bone during

distraction osteogenesis or fracture healing could be responsible for the phenomenon of longitudinal bone growth stimulation.[16,18,31]

In summary, delayed-image bone scintigraphy ratios were not effective in quantitatively differentiating between the amount of distraction-induced and osteotomy-induced bone formation. Delayed image:region-of-interest and crural ratios revealed bone formation in the areas of distraction-induced activity and bone callus at the osteotomy site. In the clinical setting, delayed-image bone scintigraphy ratios may be valuable as early predictors of bone healing. Nevertheless, quantification of newly formed bone in individual patients does not appear to be feasible. Instrumentation with a CESF system appears to stimulate bone metabolism not only in the instrumented bone, but also in adjacent long bones.

References

1.   Aalto K, Slatis P. Blood flow in rabbit osteotomies studied with radioactive microspheres. *Acta Orthop Scand* 1984;55:637-639.
2.   Aronson J. Experimental and clinical experience with distraction osteogenesis. *Cleft Palate Craniofac J* 1994;31:473-481.
3.   Aronson J. Temporal and spatial increases in blood flow during distraction osteogenesis. *Clin Orthop* 1994;301:124-131.
4.   Aronson J, Good B, Stewart C, Harrison B, Harp J. Preliminary studies of mineralization during distraction osteogenesis. *Clin Orthop* 1990;250:43-49.
5.   Averill SM, Johnson AL, Chambers M, Smith CW, Kneller S, Twardock AR, Schaeffer DJ. Qualitative and quantitative scintigraphic imaging to predict fracture healing. *Vet Comp Orthop Traumatol* 1999;12:142-150.
6.   Carvalho RS, Einhorn TA, Lehmann W, Edgar C, Al Yamani A, Apazidis A, Pacicca D, Clemens TL, Gerstenfeld LC. The role of angiogenesis in a murine tibial model of distraction osteogenesis. *Bone* 2004;34:849-861.
7.   Delloye C, Delefortrie G, Coutelier L, Vincent A. Bone regenerate formation in cortical bone during distraction lengthening. An experimental study. *Clin Orthop* 1990;250:34-42.
8.   Eingartner C, Coerper S, Fritz J, Gaissmaier C, Koveker G, Weise K. Growth factors in distraction osteogenesis. Immuno-histological pattern of TGF-beta1 and IGF-I in human callus induced by distraction osteogenesis. *Int Orthop* 1999;23:253-259.
9.   Fang TD, Salim A, Xia W, Nacamuli RP, Guccione S, Song HM, Carano RA, Filvaroff EH, Bednarski MD, Giaccia AJ, Longaker MT. Angiogenesis is required for successful bone induction during distraction osteogenesis. *J Bone Miner Res* 2005;20:1114-1124.
10. Farhadieh RD, Dickinson R, Yu Y, Gianoutsos MP, Walsh WR. The role of transforming growth factor-beta, insulin-like growth factor I, and basic fibroblast growth factor in distraction osteogenesis of the mandible. *J Craniofac Surg* 1999;10:80-86.
11. Farhadieh RD, Gianoutsos MP, Yu Y, Walsh WR. The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. *J Craniofac Surg* 2004;15:714-718.
12. Felemovicius J, Ortiz-Monasterio F, Gomez-Radillo LS, Serna A. Determining the optimal time for consolidation after distraction osteogenesis. *J Craniofac Surg* 2000;11:430-436.
13. Fink B, Fox F, Singer J, Skripitz R, Feldkamp J. Monitoring of bone formation during distraction osteogenesis via osteocalcin: a time sequence study in dogs. *J Orthop Sci* 2002;7:557-561.
14. Frierson M, Ibrahim K, Boles M, Bote H, Ganey T. Distraction osteogenesis. A comparison of corticotomy techniques. *Clin Orthop* 1994;301:19-24.
15. Hansen-Algenstaedt N, Algensaedt P, Bottcher A, Joscheck C, Schwarzloh B, Schaefer C, Muller I, Koike C, Ruther W, Fink B. Bilaterally increased VEGF-levels in muscles during experimental unilateral callus distraction. *J Orthop Res* 2003;21:805-812.
16. Holbein O, Neidlinger-Wilke C, Suger G, Kinzl L, Claes L. Ilizarov callus distraction produces systemic bone cell mitogens. *J Orthop Res* 1995;13:629-638.
17. Hu J, Zou S, Li J, Chen Y, Wang D, Gao Z. Temporospatial expression of vascular endothelial growth factor and basic fibroblast growth factor during mandibular distraction osteogenesis. *J Craniomaxillofac Surg* 2003;31:238-243.
18. Kaspar D, Neidlinger-Wilke C, Holbein O, Claes L, Ignatius A. Mitogens are increased in the systemic circulation during bone callus healing. *J Orthop Res* 2003;21:320-325.

19. Kawano M, Taki J, Tsuchiya H, Tomita K, Tonami N. Predicting the outcome of distraction osteogenesis by 3-phase bone scintigraphy. *J Nucl Med* 2003;44:369-374.

20. Latte Y. 75 applications of the Ilizarov method (part 2). *Eur J Comp Anim Pract* 1998;8:64-81.

21. Maffulli N, Lombari C, Matarazzo L, Nele U, Pagnotta G, Fixsen JA. A review of 240 patients undergoing distraction osteogenesis for congenital post-traumatic or postinfective lower limb length discrepancy. *J Am Coll Surg* 1996;182:394-402.

22. Marcellin-Little DJ. Treating bone deformities with circular external skeletal fixation. *Compend Contin Educ Pract Vet* 1999;21:481-491.

23. Marcellin-Little DJ, Ferretti A, Roe SC, DeYoung DJ. Hinged Ilizarov external fixation for correction of antebrachial deformities. *Vet Surg* 1998;27:231-245.

24. Minematsu K, Tsuchiya H, Taki J, Tomita K. Blood flow measurement during distraction osteogenesis. *Clin Orthop* 1998;347:229-235.

25. Mueller M, Schilling T, Minne HW, Ziegler R. A systemic acceleratory phenomenon (SAP) accompanies the regional acceleratory phenomenon (RAP) during healing of a bone defect in the rat. *J Bone Miner Res* 1991;6:401-410.

26. Nutton RW, Fitzgerald R-HJ, Kelly PJ. Early dynamic bone-imaging as an indicator of osseous blood flow and factors affecting the uptake of 99mTc hydroxymethylene diphosphonate in healing bone. *J Bone Joint Surg Am* 1985;67:763-770.

27. Orbay JL, Frankel VH, Finkle JE, Kummer FJ. Canine leg lengthening by the Ilizarov technique. A biomechanical, radiologic, and morphologic study. *Clin Orthop* 1992;278:265-273.

28. Pacicca DM, Patel N, Lee C, Salisbury K, Lehmann W, Carvalho R, Gerstenfeld LC, Einhorn TA. Expression of angiogenic factors during distraction osteogenesis. *Bone* 2003;33:889-898.

29. Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R. Temporal and spatial expression of bone morphogenetic protein-2, -4, and -7 during distraction osteogenesis in rabbits. *Bone* 2000;27:453-459.

30. Richards M, Goulet JA, Weiss JA, Waanders NA, Schaffler MB, Goldstein SA. Bone regeneration and fracture healing. Experience with distraction osteogenesis model. *Clin Orthop* 1998;355:191-204.

31. Sabharwal S, Paley D, Bhave A, Herzenberg JE. Growth patterns after lengthening of congenitally short lower limbs in young children. *J Pediatr Orthop* 2000;20:137-145.

32. Smith SR, Bronk JT, Kelly PJ. Effect of fracture fixation on cortical bone blood flow. *J Orthop Res* 1990;8:471-478.

33. Stanitski DF, Shahcheraghi H, Nicker DA, Armstrong PF. Results of tibial lengthening with the Ilizarov technique. *J Pediatr Orthop* 1996;16:168-172.

34. Tavakoli K, Yu Y, Shahidi S, Bonar F, Walsh WR, Poole MD. Expression of growth factors in the mandibular distraction zone: a sheep study. *Br J Plast Surg* 1999;52:434-439.

35. van Roermund PM, Hoekstra A, Haar-Romeny BM, Renooij W. Bone healing during lower limb lengthening by distraction epiphysiolysis. *J Nucl Med* 1988;29:1259-1263.

Chapter 7

Growth Hormone Stimulates Bone Healing in a Critical-sized Bone Defect Model

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Abstract

Growth hormone plays an important role in bone metabolism. Treating bone deficits is a major topic in orthopedic surgery. Our hypothesis was that local continuous growth hormone administration stimulates bone healing in a canine critical sized bone defect model. Bone formation in the defects was quantified using densitometric image analysis and histomorphometry. After growth hormone treatment expression levels of insulin-like growth factors-I and -II, and growth hormone receptor were determined in the bone regenerate of the original defects. Circulating plasma concentrations of insulin-like growth factors-I and -II, and insulin-like growth factor binding proteins-4, and -6 were measured during treatment. Growth hormone administration resulted in healing of bone defects but without an additional effect of local infusion. Expression of insulin-like growth factor-I in the bone regenerate was lower in the growth hormone treated dogs, whereas insulin-like growth factor-II and growth hormone receptor expression were not increased. Growth hormone increased circulating insulin-like growth factors-I and -II plasma concentrations. Continuous infusion of growth hormone stimulated bone healing in a canine critical-sized bone defect model. Local delivery of growth hormone did not additionally enhance bone healing. Increased circulating plasma concentrations of insulin-like growth factors-I and -II most likely induced bone formation.

Introduction

Bone healing is mediated through complex interactions of growth factors, hormones, cytokines, and matrix-associated proteins. Bone is under endocrine control by several hormones, including growth hormone, parathyroid hormone, calcitonin, estrogens, and androgens.[6,14-16,40] Growth hormone (GH) and insulin-like growth factors-I and -II (IGF-I and IGF-II) play a critical role during bone growth and bone accretion.[31] The actions of IGF-I and -II are modulated through their six high-affinity binding proteins (IGFBPs).[26,30] In general, IGFBP-1, -2, -4, and -6 inhibit and IGFBP-3 and -5 stimulate osteoblast function. .

Stimulating bone formation, using GH, growth factors, and to a lesser extent cytokines, is of major interest in dealing with compound fractures, bone deficits, and arthrodeses. Various studies have shown the applicability of these factors as osteoinductive agents and as accelerators of bone healing.[7,20,29,34,43] The actions of GH on bone are twofold. First, GH exerts a direct stimulatory effect on longitudinal bone growth and on bone healing.[6,21] Second, GH has an indirect effect, which is supposed to be mainly mediated through the induction of IGF-I.[6,8,12,14,24,35] Systemic application of GH-stimulated bone healing has been shown in several fracture models.[9,21,34,44] In a porcine distraction osteogenesis model, systemic treatment with GH accelerated bone healing after lengthening.[2,3,33] Although GH enhanced bone formation in these models, they did not use critical-sized bone defects. As bone loss and defects are major concerns in orthopedic surgery, stimulation of bone healing is these cases is critical.

We hypothesized that continuous GH administration induces bone healing in a canine critical-sized bone defect model. We speculated that direct infusion of GH into the defect is most effective in enhancing bone healing.[21] In addition, we hypothesized that GH administration stimulated bone healing by increasing the expression of IGF-I, IGF-II, and GH receptor within the bone regenerate of the original defect.[4,11,19,21,27]

Materials and Methods

Animals

Eight mature Labrador retrievers were allocated with an even gender distribution to one treatment (n = 4) and one control group (n = 4). Four dogs were treated with GH and four dogs in the control group received a placebo solution. Sample size was determined assuming a difference between means of 50% with a common standard deviation for both groups of 20%, a significance level of .05, and

a power of 0.80. The equal sample size for both groups was calculated to be 2.97. Four females and four males were included, with an age of 32 ± 5 months (mean $\pm$ SEM), and a body weight of 31 ± 1 kg (mean $\pm$ SEM). Bilateral ulnar critical-sized bone defect were created.[7] A critical sized bone defect is designed not to heal spontaneously and thus will develop into a nonunion. In the four GH-treated dogs, the bone defect in the right ulna was continuously infused with GH, using an osmotic pump with a catheter placed inside the defect. In the control group, the defects in the right ulna were infused with placebo solution. The formation of new bone was evaluated, using densitometric image analysis and histomorphometry. To assess bone healing in the defects, bone formation was monitored until 6 weeks after starting GH treatment. Bone healing in the defects was compared between the GH-treated group (eight defects) and control group (eight defects). In addition, differences in bone healing within the GH-treated dogs between the right-sided and left-sided defects (four each) were determined. Circulating plasma concentrations of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 were determined to assess the systemic effect of GH treatment. Only IGFBP-4 and –6 were included because determining additional IGF-binding proteins in dog plasma was not yet feasible. After 6 weeks, segmental bone specimens were collected to measure the expression levels of IGF-I, IGF-II, and GH receptor within the original bone defects. Because of the lack of proper sequences of canine IGFBP-4 and -6, mRNAs we were not yet able to amplify IGFBP mRNA in canine tissue samples. Enhanced bone healing was expected to coincide with increased circulating plasma concentrations of IGF-I and IGF-II and lower concentrations of inhibiting binding proteins IGFBP-4 and IGFBP-6.[3,9,21,33,34]

Surgery

After sedation with medetomidine (Domitor®, Pfizer Animal Health B.V., Capelle a/d IJssel, The Netherlands), anesthesia was induced intravenously with propofol (Rapinovet®, Schering-Plough Animal Health N.V., Bruxelles, Belgium), and maintained by inhalation anesthesia with isoflurane, nitrous oxide, and oxygen. Amoxicillin with clavulanic acid (Augmentin®, SmithKline Beecham Farma B.V., Rijswijk, The Netherlands) were administered intravenously (20 mg/kg body weight) before surgery. Both front limbs were prepared in a sterile fashion and positioned for surgery. A craniolateral approach to the ulna was used, and a longitudinal skin incision was made, ending 4 cm proximal of the ulnar styloid process. The periosteum was preserved, using a 3-cm longitudinal incision and careful periosteal elevation. A 20-mm bone segment was removed 4 cm proximal of the ulnar styloid process, using an oscillating saw and ample lavage. In this way

a critical-sized bone defect, or a defect that will not heal spontaneously, was created.[7]

As canine and porcine GH are identical at the protein level, recombinant porcine GH provided by Dr. A.F. Parlow of the National Hormone and Pituitary Program (NHPP, Torrance, CA), was used in this study.[1] Growth hormone was dissolved in 0.03 mol/L sodium bicarbonate in 0.15 mol/L NaCl, and after adjusting the pH to 9.5, the sterile solution was diluted with 0.15 mol/L NaCl to the desired concentration of 30 mg/mL. Four 2ML4 Alzet® osmotic pumps (Alzet® Osmotic pumps, Alza Corporation, Pala Alto, CA) capable of continuous drug delivery for 28 days were filled with 2 mL rpGH solution and four pumps with the vehicle of the solution (i.e., 0.03 mol/L sodium bicarbonate in 0.15 mol/L NaCl, adjusted to pH 9.5). With this GH solution, the pumps delivered 1.8 mg GH per day for 4 weeks. The pumps were mounted with a 4-cm long Teflon catheter and implanted subcutaneous distally from the critical sized bone defect, on the right side only. The tip of the catheter was placed within the critical-sized bone defect. The periosteal cylinder was closed with absorbable suture material, while fixing the catheter to the periosteum. In this way a concentration gradient was established, with the highest amount of GH in the right-sided defect.[18,39] Closure of the subcutaneous tissues and skin was in a routine fashion. A protective bandage was applied for 3 days. The dogs received analgesics (buprenorphine, 4 times per day 10 μg/kg/ bodyweight subcutaneously) for 3 days postoperatively. Full loading of the legs was permitted immediately after surgery. En bloc biopsies, incorporating a 4-cm segment of the right and left ulnas, including the critical-sized defect, were harvested with the dogs under general anesthesia at 6 weeks. These 16 biopsy specimens were split longitudinally and ½ was processed for total RNA isolation, whereas the other ½ was fixed in 70% alcohol for histomorphometric analysis. The dogs were not euthanatized, but nursed until completely recovered from surgery. Before the study, new owners were found to adopt the dogs as pets. After recovery, all dogs were united with their owners. Throughout the entire study, the dogs were fed a standard commercial dog food twice a day and water was available ad libitum.

Densitometric image analysis

Standardized mediolateral radiographs of the right and left antebrachium were obtained before surgery, immediately after surgery, and once every 2 weeks thereafter. The radiographs included a ruler and an aluminum step-wedge, consisting of 10, 2-mm thick aluminum slabs mounted in an overlapping manner. Early bone formation and bridging of the critical sized bone defects were assessed visually, and bone formation was quantified using a densitometric image analysis

system. Radiographs were recorded with a black and white CCD camera (Sony Corporation, Tokyo, Japan), Type XC-77CE, and digitized for image analysis (frame size 752 x 574 pixels; 256 grey levels) with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the amount of regenerated bone. Each radiograph was calibrated geometrically and densitometrically, using the image of the ruler and the aluminum step-wedge. The densitometric calibration was performed by measuring the mean optical density of a square area of 50 x 50 pixels in six steps of the aluminum step-wedge (0,2,4,6,8, and 10 mm). The measurements were carried out in a median-filtered image to reduce the influence of the photographic grains in the film. The optical density values are a polygonal fit with the aluminum values to produce a transformation table, which enables expression of the amount of newly formed bone in equivalents of cubic mm of aluminum. The region of interest (ROI) included the critical sized bone defect and all new bone formation within10 mm proximal and distal of the defect. The ROI was delineated on the digitalized mediolateral images, and densitometric analyses for bone area and bone amount were performed. Densitometric image analysis was performed using the 4-week and 6-week radiographs, as the 2-week data only showed limited mineralization of the newly formed bone.

Histomorphometric analysis

The segmental bone biopsy specimens were fixed in 70% ethanol. After dehydration, using increasing concentrations of up to 100% ethanol, the bone biopsy specimens were embedded in methylmethacrylate, which was allowed to polymerize (polymethylmethacrylate [PMMA]). Longitudinal sections of the PMMA-embedded bone specimens were made, using a Leica SP1600 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The PMMA-embedded bone was mounted on the microtome, the surface was corroded with 1% hydrochloric acid in ethanol, and stained with a 1% methylene blue solution and a 0.3% basic fuchsine solution. Basic fuchsine preferentially binds to bone, whereas fibrous tissue and cartilage is indicated by methylene blue. A cover slip was fixed to the PMMA-bone specimen, using light-curing epoxy resin glue. Sections with a thickness of 20 μm were sawn. Four longitudinal sections from the center of each critical-sized bone defect including two ulnar cortices on either side of the original defect were selected and used for histomorphometric analysis. To measure the area of trabecular bone in the microscopic sections of the critical-sized defect, a PC-based image analysis system, equipped with the KS400 version 3.0 software package (Carl Zeiss Vision, Oberkochen, Germany), was used. Sections of each defect were scanned macroscopically with a black and white CCD camera type

XC-77CE (frame size 752 x 574 pixels; 256 grey levels; Sony Corporation). Before measurement, the system was calibrated geometrically, and shading correction was done. The complete area containing the trabecular bone was outlined manually by one observer (LT), followed by a computerized dynamic discrimination (i.e., a threshold procedure accounting for the local background values) of the trabecular differential staining and morphometric properties. With this technique a distinction was established, between trabecular bone opposed to fibrous and cartilaginous tissue. The measured areas were expressed in square millimeters.

Gene expression

The bone biopsy specimens were frozen immediately in liquid nitrogen and stored at $-70°$ C until required for RNA isolation. Frozen bone tissue was ground in liquid nitrogen prefrozen cups of a micro-dismembrator (Micro-Dismembrator U, B. Braun Biotech International GmbH, Melsungen, Germany). One to five hundred milligrams of milled bone tissue was resuspended in Qiagen lysis buffer (Qiagen GmbH, Hilden, Germany) and centrifuged for 10 minutes at 5 *g*. The supernatant was applied to a Qiagen midi-column (Qiagen GmbH, Hilden, Germany), and total RNA was isolated according to the manufacturer's protocol. After DNase I treatment (DNAfree^{TM} kit, Ambion, TX), RNA was ethanol precipitated and resuspended in 5 µL of RNase-free water. As much as 1 µg of total RNA was used in a 20 µL complementary deoxyribonucleic acid (cDNA) synthesis reaction (Reverse Transcription System, Promega Corp, Madison, WI) according to the manufacturer's instructions. The cDNA solution was diluted to an equivalent of 10 ng RNA/µL.

Real-time polymerase chain reaction (PCR), based on the high-affinity double-stranded DNA-binding dye SYBR green I, was performed in triplicate in a spectrofluorometric thermal cycler (iCycler, BioRad, Hercules, CA). Data were collected and analyzed with the provided application software. For each real-time PCR reaction, 5 µL of the cDNA dilution was used in a reaction volume of 50 µL, containing 1x PCR buffer, 3 mmol/L MgCl$_2$, 1:100,000 dilution of SYBR$^®$ green I (BMA, Rockland, ME), 10 nmol/L fluorescein calibration dye (BioRad, Hercules, CA), 200 µmol/L dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, Branchburg, NJ). Cycling conditions were 5 minutes at 95° C, followed by 45 cycles of 15 seconds at 95° C, 30 seconds at 55° C, and 40 seconds at 72° C. Primer pairs were designed, using PrimerSelect software (DNASTAR, Inc, Madison, WI) (Table 1).

Table 1. Primer Pairs Used in PCR Amplification

Gene	Primer (5' – 3')	Exon	Amplicon Length (bp)
β-actin	F: TGGCACCACACCTTCTACAACGAG	3	180
	R: AGAGGCATACAGGGACAGGACAGC	4	
IGF-I	F: ATGTCCTCCTCGCATCTCTT	3	355
	R: TCCCTCTACTTGCGTTCTTC	5	
IGF-II	F: CGCAGCCGTGGCATCGTTGAGGAG	5	200
	R: CTGCGCAGGCGCTGGGTGGACT	6	
GH receptor	F: ACCCATCGGCCTCAACTG	6	175
	R: AGGGTCCATCATTTTCCACTG	6,7	

Melt curves (iCycler, BioRad, Hercules, CA) and agarose gel electrophoresis were used to examine each sample for purity, and standard sequencing procedures (ABI PRISM^{TM} 310 Genetic Analyzer, Applied Biosystems, Roche) were used to verify the analytical specificity of the PCR products.

Standard curves constructed by plotting the log of starting amount versus the threshold cycle were generated using serial 10-fold dilutions of known amounts of PCR products (from a conventional PCR). The amplification efficiency, E (%) = $(10^{(1/-s)} -1)$ x 100 (s = slope), of each standard curve was determined and seemed to be greater than 90% over a large dynamic range (6-8 orders of magnitude). Serial dilutions of cDNA were also tested resulting in similar E values over 3-4 orders of magnitude.

For each experimental sample, the amount of target (IGF-I, IGF-II, and GHR) and β-actin as endogenous reference was determined from the appropriate standard curve. To confirm that β-actin levels were not influenced by the experimental manipulation and to further rule out interference of β-actin pseudogenes, β-actin, using a primer pair that distinguishes cDNA from pseudogene DNA, was also tested as an internal standard. The amount of target was divided by the amount of endogenous reference to obtain a normalized target value.

Biochemical analysis

During the week before surgery, two jugular vein blood samples were collected with a 1-hour interval in ice-chilled ethylenediaminetetraacetic acid (EDTA) tubes and kept on ice before centrifugation for 15 minutes at 3.000 rpm and 4°C, within 60 minutes after collection. Plasma samples were stored at -20°C

until analysis of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6. During the 6-week study, two blood samples, with a 1-hour interval, were collected once a week and processed accordingly. To rule out diurnal variations, all blood samples were taken at the same time of the day (i.e., 0800 and 0900 hours).

Plasma GH concentrations were measured in a homologous radioimmunoassay as previously described.[13] Total plasma IGF-I and IGF-II concentrations were measured after acid-ethanol extraction to remove interfering IGFBPs.[14] In addition, the absence of interfering IGFBPs in the plasma extracts was investigated.[36] Insulin-like growth factor-I concentrations were measured in a heterologous radioimmunoassay validated for the dog.[32] Insulin-like growth factor-I antiserum (UBK 487) was the gift of Doctors Underwood and Van Wyk from the University of North Carolina, via the hormone distribution program of The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Insulin-like growth factor-II concentrations were determined with a heterologous radioimmunoassay validated for the dog, using monoclonal antibodies against rat IGF-II (Amano Enzyme U.S.A. Co., Lombard, IL), as described previously.[14]

Plasma IGFBP-4 and IGFBP-6 concentrations were determined by specific heterologous radioimmunoassays, using polyclonal antiserum WKZ8209 and WKZ6278, respectively.[41,42]

Statistical analysis

All statistical analyses were performed using the SPSS 10.1 statistical package (SPSS Inc, Chicago, IL). Levene's statistic was used to test homogeneity of variance between groups. The bone area and bone amount in the densitometric image analysis and the histomorphometric data were evaluated using a Student's t test and a paired samples t test. The gene expression data were evaluated using a Student's t test for statistical analysis of the log-transformed normalized target values. The GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 data were evaluated using a general linear model (GLM) for repeated measures, with the preoperative levels of Week 0 as a covariant, and a paired samples t test. Differences were considered significant when $P < .05$.

Results

All dogs had the surgical procedures without complications, and soft tissue wound healing was uneventful. The dogs were fully loading both front legs the day after surgery. After 2 weeks, all dogs showed normal locomotion without signs of lameness.

Growth hormone treatment induced bone healing in the critical-sized defects. New bone formation was visible radiographically in the defects as soon as 2 weeks after surgery in both the GH-treated group and the controls. Bone formation started adjacent to the proximal and distal osteotomy sites. In the GH group, bone formation progressed, and at 6 weeks, three of the dogs showed advanced bilateral bridging of the bone defect, whereas one dog showed extensive new bone formation, but without complete bridging of the right-sided and left-sided defects (Fig 1A). In the control group, dogs showed very little progression of new bone formation and no bridging of defects (Fig 1B). Densitometric image analysis demonstrated an increased bone amount (p = .03 and p = .01) in the GH group at 4 and 6 weeks after surgery. The bone area was increased (p = 0.01) at 6 weeks, but not at 4 weeks (p = .05)(Table 2). Within the GH group, bone amount and bone area increased (p = .02 and p = .002) between Week 4 and Week 6. In the histologic sections of the GH-treated dogs, bridging of the defects with trabecular bone was seen (Fig 2A). The bone defects in the controls showed very little new bone formation, and the defects were filled mainly with fibrous tissue (Fig 2B). In the GH-treated bone defects, the amount of trabecular bone was greater (p = .008) than in the controls with 70.9 ± 15.3 mm^2 and 22.4 ± 4.0 mm^2, respectively.
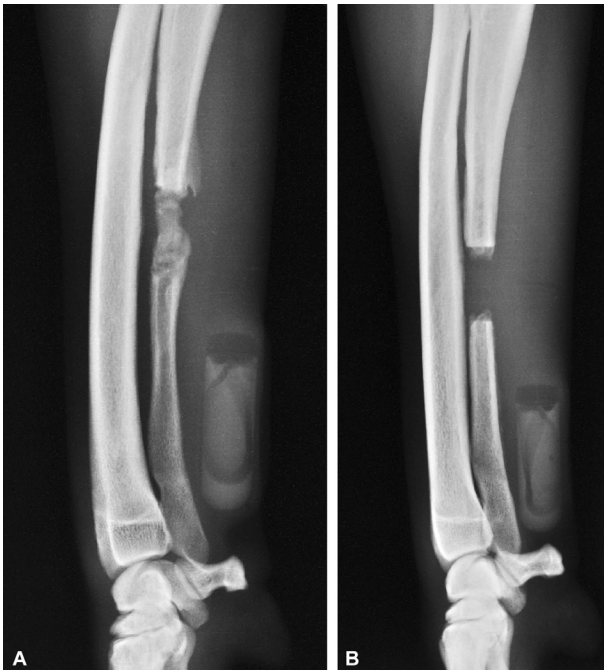

Fig 1A-B. (A) A radiograph was taken of the critical-sized bone defect in the ulna after continuous infusion with GH. The osmotic micropump is located distal to the bone defect. Growth hormone treatment stimulated bone healing with extensive new bone formation and bridging of the defect. (B) A radiograph was taken of the critical-sized bone defect in the ulna after continuous infusion with vehicle solution in the control group. The osmotic micropump is located distal to the defect. The defect shows minimal new bone formation.

Local infusion of GH directly into the defects did not additionally enhance bone formation in the GH group. The densitometric image analysis and histomorphometric analysis demonstrated similar bone healing between the contralateral bone defects.
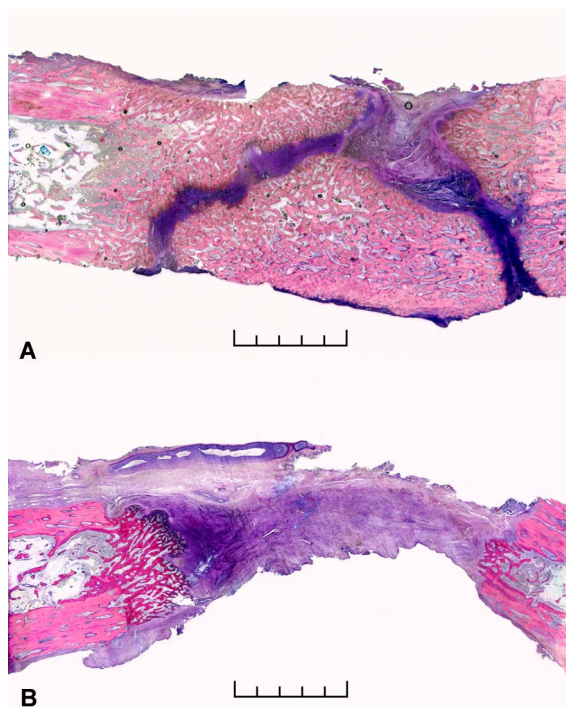

Fig 2A-B. (A) A microscopic section of the critical-sized bone defect, obtained after continuous infusion with growth hormone, shows the bone regenerate in the original defect. Growth hormone treatment has induced bone healing, filling the defect almost completely with trabecular bone. The bar represents 5 mm. (B) A microscopic section of the critical-sized bone defect, obtained after continuous infusion with vehicle solution in the control group, shows the defect is filled with fibrous tissue and there is only minimal formation of trabecular bone. The bar represents 5 mm.

Table 2. Densitometric Image Analysis of Bilateral Ulnar Critical-sized Bone Defects

Densitometric image analysis	Week	rpGH	Control
Bone area (mm^2)	4	163.8 ± 27.7	86.5 ± 23.9
Bone amount (mm^3 Al x 10^3)	4	$117.9 \pm 20.8^*$	57.6 ± 16.4
Bone area (mm^2)	6	$226.8 \pm 35.1^{*,\#}$	103.7 ± 28.3
Bone amount (mm^3 Al x 10^3)	6	$164.9 \pm 28.8^{*,\#}$	$66.1 \pm 17.7^{\#}$

Data are presented as mean ± SEM. The bone amount is presented as equivalents of aluminium (Al) in mm^3 x 10^3; * significant difference in comparison with the control group ($P < .05$); # significant increase in comparison with Week 4 ($P < .01$)

Six weeks after creation of the bone defects, the expression level of IGF-I in the bone regenerate was lower (p = .03) in the GH group than in the controls with 2.9 ± 0.6 (mean ± SEM) and 7.3 ± 1.9, respectively. No increase was seen for the expression levels of IGF-II in the GH-treated animals compared to the controls, with 5.3 ± 1.1 and 2.5 ± 0.7, although the trend was greater for expression of IGF-II. The expression level of GH receptor was similar in both groups with 4.4 ± 0.8 and 4.4 ± 0.7, respectively. In the GH group, the expression levels of IGF-I, IGF-II, and GH receptor in the bone regenerate of the right and left defects were similar in magnitude.
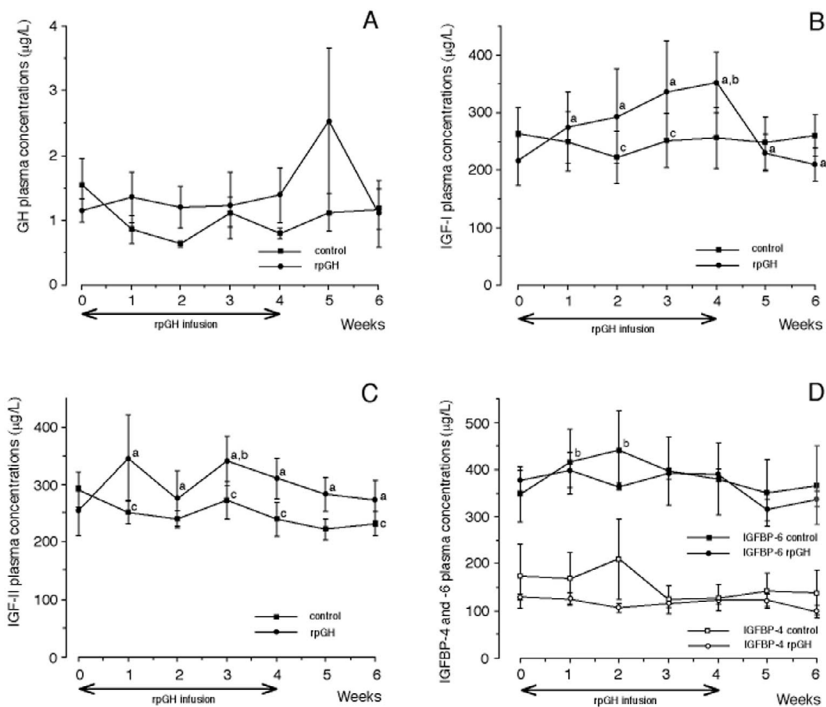

Fig 3A-D. Plasma concentrations are shown for (A) growth hormone (GH), (B) IGF-I, (C) IGF-II, and (D) IGFBP-4, and IGFBP-6 in a canine critical sized bone defect model during continuous infusion with recombinant porcine GH (rpGH). Data are presented as means ± SEM, before surgery (0) and in weeks after surgery. Growth hormone treatment increased IGF-I and IGF-II plasma concentrations, but did not decrease the inhibitory binding proteins IGFBP-4 and IGFBP-6. [a] Significant increase in the GH group in comparison with the controls (p < .05). [b] Significant increase in comparison with the level before surgery (p < .05). [c] Significant decrease in comparison with the level before surgery (p < .05).

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Treatment with recombinant porcine GH at this dosage did not result in an increase of GH plasma concentrations (Fig 3A). The IGF-I and IGF-II plasma concentrations increased (p = .01 and p = .01) during GH treatment and were greater (p = .02 and p = .008) than in the control group (Fig 3B-C). In the GH-treated group, IGF-I plasma concentrations peaked at 4 weeks (p = .01) to rapidly return to pretreatment levels after the GH infusion had stopped. In contrast, IGF-II levels continued to be greater (p = .008) than in the control group for the remainder of the study. In the control group, IGF-II (p = .02) and to a lesser extent IGF-I (p = .03) plasma concentrations tended to be lower than the preoperative levels. Plasma IGFBP-4 and IGFBP-6 concentrations were not decreased in the GH-treated dogs, but were similar to the controls. Increases (p = .02) in IGFBP-6 levels were seen in the controls at Weeks 1 and 2 (Fig 3D).

Discussion

Dealing with bone deficits is a major concern in orthopedic surgery. We hypothesized that continuous GH administration induces bone healing in a critical-sized bone defect model. In addition, we speculated that local administration of GH was most effective in enhancing bone healing. The effect of GH was expected to be mediated twofold, first by stimulating the expression of IGF-I, IGF-II, and GH receptor in the bone regenerate of the original defects and second by altering circulating plasma concentrations of IGF-I, IGF-II, IGFBP-4 and IGFBP-6. Results of our study indicate that continuous infusion with GH stimulates bone formation and bone healing in a critical-sized bone defect. The model reproduced a nonunion in all nontreated dogs.[7] Local delivery of growth hormone did not additionally enhance bone healing. Growth hormone treatment did not increase the expression of IGF-I, IGF-II, and GH receptor in the bone regenerate at 6 weeks after creation of the defect. Increased circulating plasma concentrations of IGF-I and IGF-II most likely induced bone formation during GH treatment.

The local continuous administration of GH into the right-sided bone defect using an osmotic pump and a catheter was limited by the redistribution of GH to the circulation. Nevertheless, our interest was to determine whether this route of infusion, which is feasible in a clinical setting, could effectively enhance local bone regeneration. Another approach could be delivery of GH with a slow-releasing carrier, but this route of administration was not readily available and technically more demanding.

Exploring gene-expression levels, including IGF-I, IGF-II, and GH receptor, is limited by the fact that mRNA levels can change in a short time. Determining transcription levels at more than one time can overcome this problem,

but requires substantially more experimental animals as biopsies of the bone regenerate will interfere with normal bone healing. Therefore, we decided to limit our study to the expression levels during the consolidation phase of the bone regenerate at 6 weeks after the creation of the defects. Another restriction is the fact that we only determined the expression of IGF-I, IGF-II, and GH receptor. Pursuing additional bone growth factors, including bone morphogenetic proteins, transforming growth factor-β, fibroblast growth factors, vascular endothelial growth factor, platelet derived growth factor, and stimulatory IGF binding proteins is essential to elucidate the interactions between bone growth factors.[5,7,18,20,29,43] Simultaneous determination of these factors is hampered by the amount of bone needed for mRNA isolation.

        Although stimulation of bone healing with GH has been reported, our study was the first to show the effectiveness of GH on bone regeneration in a critical-sized bone defect. [2,3,9,21,33,34,44] Hypophyseal GH represents the major constituent of GH in the circulation, but GH also is expressed in numerous other tissues, including bone.[6,28] Expression of growth hormone in healing bone has not been reported and local production of GH in the bone regenerate is not expected to play an important role.

        A direct effect of GH on bone healing was seen in a rat model.[21] This direct effect of GH should involve the GH receptor, and although the role of the GH receptor in bone growth is slowly being elucidated, little is known regarding its part in osteogenesis in the mature animal.[27,28] The presence and hormonal control of GH receptor were reported in germinal and proliferative cells in the growth plate and in maturing chondrocytes.[17] In our study, no additional direct effect of local GH application was seen. As GH receptor expression levels were equal in GH-treated dogs and controls, there was no indication that modulation of GH receptor expression was responsible for enhanced bone formation. Nevertheless, GH receptor expression was consistent with the concept of a potential direct effect of GH on bone healing. We found that this direct effect of GH was less important during bone regeneration than the indirect effects of GH infusion. Growth hormone plasma concentrations were not greater in the recombinant porcine GH-treated dogs, most likely due to the pulsatile release of endogenous GH.[14]

        The GH-induced increases in IGF-I and IGF-II plasma concentrations in the dogs in our study were consistent with the finding of increased circulating IGF-I and IGF-II concentrations during GH treatment in postmenopausal women.[25] The stimulation of bone healing through GH-induced IGF-I was shown in several models.[3,9,21,33,34] In a mouse model, the major part of circulating IGF-I was liver-derived.[35] In our study, we used continuous GH infusion because this was most effective in increasing IGF-I plasma concentrations in humans.[24] In addition to liver-derived IGF-I production, IGF-I also is known to be expressed locally during

bone healing.[5,11,19] Local IGF-I application stimulated bone healing in a rat model.[34] In our study, IGF-I expression levels were lower during the consolidation phase of the GH-stimulated bone regenerate. In theory, this suggests that local IGF-I production was not responsible for the progression of bone healing at this stage. The decreased expression of IGF-I also can be related to the temporal aspect of ceasing GH infusion after 4 weeks, at which time IGF-I plasma concentrations peaked, and in determining local IGF-I expression after 6 weeks. Bone accretion progressed despite the fact that IGF-I plasma concentrations returned to preoperative levels after cessation of GH infusion. These findings could be consistent with a role of IGF-I during the early stages of callus formation.[5]

Insulin-like growth factor-II is considered to be a major constituent of local and systemic growth factors in bone.[4] Knowledge about the role of IGF-II in bone healing, however, is limited. Insulin-like growth factor -I and IGF-II were shown to be important for osteoblast survival and apoptosis.[23] In addition, IGF-I and IGF-II were proposed to modulate osteoblast-osteoclast interactions, which are critical in bone remodeling.[22] In our study, IGF-II plasma concentrations remained elevated even after cessation of GH infusion. As IGF-II expression did not differ between the GH treated defects and the controls, we theorize that IGF-II production by skeletal osteoblasts outside the defects is responsible for the enhanced bone regeneration. Elevated IGF-II plasma concentrations could account for the increase in densitometric bone amount and bone area between Weeks 4 and 6. The increase in bone amount, but not bone area, in the controls could be attributed to mineralization of the small amount of bone formed at the osteotomy sites.

Insulin-like growth factor binding protein-4 and IGFBP-6 are reported to play an important role in modulating IGF actions in bone, mainly by inhibiting osteoblast function.[10,26] In our study, IGFBP-4 plasma concentrations were comparable with plasma concentrations in humans.[41] Continuous infusion with rpGH did not result in an increase of IGFBP-4 levels as seen in previous studies during GH replacement therapy in humans.[25,38] Canine IGFBP-6 plasma concentrations tended to be twice as high as in their human counterparts.[42] Because IGFBP-6 preferentially binds IGF-II, IGFBP-6 could be important in modulating IGF-II actions.[37,42,45] In our study, IGFBP-4 and IGFBP-6 plasma concentrations were not affected by continuous GH infusion and did not seem to play a role during enhanced bone regeneration.

Continuous GH infusion stimulated bone healing in this canine critical-sized bone defect model. Local delivery of GH did not additionally enhance bone healing. Increased plasma concentrations of IGF-I and IGF-II most likely induced bone regeneration. Growth hormone could play an important role in stimulating bone formation during the surgical treatment of bone defects.

Acknowledgements

References

1. Ascacio-Martinez JA, Barrera-Saldana HA. A dog growth hormone cDNA codes for a mature protein identical to pig growth hormone. *Gene* 1994;143:277-280.
2. Bail HJ, Kolbeck S, Krummrey G, Schmidmaier G, Haas NP, Raschke MJ. Systemic application of growth hormone for enhancement of secondary and intramembranous fracture healing. *Horm Res* 2002;58(suppl 3):39-42.
3. Bail HJ, Kolbeck S, Lindner T, Dahne M, Weiler A, Windhagen HJ, Raun K, Skjaerbaek C, Flyvbjerg A, Orskov H, Haas NP, Raschke MJ. The effect of growth hormone on insulin-like growth factor I and bone metabolism in distraction osteogenesis. *Growth Horm IGF Res* 2001;11:314-323.
4. Bautista CM, Mohan S, Baylink DJ. Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. *Metabolism* 1990;39:96-100.
5. Bourque WT, Gross M, Hall BK. Expression of four growth factors during fracture repair. *Int J Dev Biol* 1993;37:573-579.
6. Conzemius MG, Brown DC, Brabec M, Smith GK, Washabau R, LaFond E, Chakraborty PK. Correlation between longitudinal bone growth, growth hormone, and insulin-like growth factor-I in prepubertal dogs. *Am J Vet Res* 1998;59:1608-1612.
7. Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC. Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. *Clin Orthop* 1994;301:302-312.
8. Drake WM, Carroll PV, Savage MO, Monson JP. Skeletal consequences of discontinuation of growth hormone at final height. *J Pediatr Endocrinol Metab* 2002;15 (suppl 5):1351-1354.
9. Dubreuil P, Abribat T, Broxup B, Brazeau P. Long-term growth hormone-releasing factor administration on growth hormone, insulin-like growth factor-I concentrations, and bone healing in the Beagle. *Can J Vet Res* 1996;60:7-13.
10. Durham SK, Riggs BL, Conover CA. The insulin-like growth factor-binding protein-4 (IGFBP-4)-IGFBP-4 protease system in normal human osteoblast-like cells: regulation by transforming growth factor-beta. *J Clin Endocrinol Metab* 1994;79:1752-1758.
11. Edwall D, Prisell PT, Levinovitz A, Jennische E, Norstedt G. Expression of insulin-like growth factor I messenger ribonucleic acid in regenerating bone after fracture: influence of indomethacin. *J Bone Miner Res* 1992;7:207-213.
12. Ehrnborg C, Lange KH, Dall R, Christiansen JS, Lundberg PA, Baxter RC, Boroujerdi MA, Bengtsson BA, Healey ML, Pentecost C, Longobardi S, Napoli R, Rosen T. The growth hormone/insulin-like growth factor-I axis hormones and bone markers in elite athletes in response to a maximum exercise test. *J Clin Endocrinol Metab* 2003;88:394-401.
13. Eigenmann JE, Eigenmann RY. Radioimmunoassay of canine growth hormone. *Acta Endocrinol (Copenh)* 1981;98:514-520.
14. Favier RP, Mol JA, Kooistra HS, Rijnberk A. Large body size in the dog is associated with transient GH excess at a young age. *J Endocrinol* 2001;170:479-484.
15. Fukuda S, Iida H. Effects of orchidectomy on bone metabolism in beagle dogs. *J Vet Med Sci* 2000;62:69-73.
16. Geusens P, Schot LP, Nijs J, Dequeker J. Calcium-deficient diet in ovariectomized dogs limits the effects of 17 beta-estradiol and nandrolone decanoate on bone. *J Bone Miner Res* 1991;6:791-797.
17. Gevers EF, van der Eerden BC, Karperien M, Raap AK, Robinson IC, Wit JM. Localization and regulation of the growth hormone receptor and growth hormone-binding protein in the rat growth plate. *J Bone Miner Res* 2002;17:1408-1419.

18.   Goodman SB, Song Y, Yoo JY, Fox N, Trindade MC, Kajiyama G, Ma T, Regula D, Brown J, Smith RL. Local infusion of FGF-2 enhances bone ingrowth in rabbit chambers in the presence of polyethylene particles. *J Biomed Mater Res A* 2003;65:454-461.

19.   Hayden JM, Mohan S, Baylink DJ. The insulin-like growth factor system and the coupling of formation to resorption. *Bone* 1995;17:93-98.

20.   Heckman JD, Boyan BD, Aufdemorte TB, Abbott JT. The use of bone morphogenetic protein in the treatment of non-union in a canine model. *J Bone Joint Surg Am* 1991;73:750-764.

21.   Hedner E, Linde A, Nilsson A. Systemically and locally administered growth hormone stimulates bone healing in combination with osteopromotive membranes: an experimental study in rats. *J Bone Miner Res* 1996;11:1952-1960.

22.   Hill PA, Reynolds JJ, Meikle MC. Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. *Endocrinology* 1995;136:124-131.

23.   Hill PA, Tumber A, Meikle MC. Multiple extracellular signals promote osteoblast survival and apoptosis. *Endocrinology* 1997;138:3849-3858.

24.   Jaffe CA, Turgeon DK, Lown K, Demott-Friberg R, Watkins PB. Growth hormone secretion pattern is an independent regulator of growth hormone actions in humans. *Am J Physiol Endocrinol Metab* 2002;283:1008-1015.

25.   Kassem M, Brixen K, Mosekilde L, Blum WF, Flyvbjerg A. Effects of growth hormone treatment on serum levels of insulin-like growth factors (IGFs) and IGF binding proteins 1-4 in postmenopausal women. *Clin Endocrinol (Oxf)* 1998;49:747-756.

26.   Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM, Rosenfeld RG. Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. *Int J Biochem Cell Biol* 1996;28:619-637.

27.   Kelly PA, Finidori J, Moulin S, Kedzia C, Binart N. Growth hormone receptor signalling and actions in bone growth. *Horm Res* 2001;55(suppl 2):14-17.

28.   Kirpensteijn J, Timmermans-Sprang EP, van Garderen E, Rutteman GR, Lantinga-van Leeuwen IS, Mol JA. Growth hormone gene expression in canine normal growth plates and spontaneous osteosarcoma. *Mol Cell Endocrinol* 2002;197:179-185.

29.   Linkhart TA, Mohan S, Baylink DJ. Growth factors for bone growth and repair: IGF, TGF beta and BMP. *Bone* 1996;19:1-12.

30.   Mohan S. Insulin-like growth factor binding proteins in bone cell regulation. *Growth Regul* 1993;3:67-70.

31.   Mohan S, Richman C, Guo R, Amaar Y, Donahue LR, Wergedal J, Baylink DJ. Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms. *Endocrinology* 2003;144:929-936.

32.   Nap RC, Mol JA, Hazewinkel HA. Age-related plasma concentrations of growth hormone (GH) and insulin- like growth factor I(IGF-I) in Great Dane pups fed different dietary levels of protein. *Domest Anim Endocrinol* 1993;10:237-247.

33.   Raschke MJ, Bail H, Windhagen HJ, Kolbeck SF, Weiler A, Raun K, Kappelgard A, Skiaerbaek C, Haas NP. Recombinant growth hormone accelerates bone regenerate consolidation in distraction osteogenesis. *Bone* 1999;24:81-88.

34.   Schmidmaier G, Wildemann B, Heeger J, Gabelein T, Flyvbjerg A, Bail HJ, Raschke M. Improvement of fracture healing by systemic administration of growth hormone and local application of insulin-like growth factor-1 and transforming growth factor-beta1. *Bone* 2002;31:165-172.

35.   Sjogren K, Jansson JO, Isaksson OG, Ohlsson C. A model for tissue-specific inducible insulin-like growth factor-I (IGF-I) inactivation to determine the physiological role of liver-derived IGF-I. *Endocrine* 2002;19:249-256.

36.   Sota de la RL, Simmen FA, Diaz T, Thatcher WW. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. *Biol Reprod* 1996;55:803-812.
37.   Srinivasan N, Edwall D, Linkhart TA, Baylink DJ, Mohan S. Insulin-like growth factor-binding protein-6 produced by human PC-3 prostate cancer cells: isolation, characterization and its biological action. *J Endocrinol* 1996;149:297-303.
38.   Thoren M, Hilding A, Brismar T, Magnusson P, Degerblad M, Larsson L, Saaf M, Baylink DJ, Mohan S. Serum levels of insulin-like growth factor binding proteins (IGFBP)-4 and -5 correlate with bone mineral density in growth hormone (GH)- deficient adults and increase with GH replacement therapy. *J Bone Miner Res* 1998;13:891-899.
39.   Tiangco DA, Papakonstantinou KC, Mullinax KA, Terzis JK. IGF-I and end-to-side nerve repair: a dose-response study. *J Reconstr Microsurg* 2001;17:247-256.
40.   Tryfonidou MA, Holl MS, Vastenburg M, Oosterlaken-Dijksterhuis MA, Birkenhager-Frenkel DH, van den Brom WE, Hazewinkel HA. Hormonal regulation of calcium homeostasis in two breeds of dogs during growth at different rates. *J Anim Sci* 2003;81:1568-1580.
41.   van Doorn J, Cornelissen AJ, Buul-Offers SC. Plasma levels of insulin-like growth factor binding protein-4 (IGFBP-4) under normal and pathological conditions. *Clin Endocrinol (Oxf)* 2001;54:655-664.
42.   van Doorn J, Ringeling AM, Shmueli SS, Kuijpers MC, Hokken-Koelega AC, Buul-Offers SC, Jansen M. Circulating levels of human insulin-like growth factor binding protein- 6 (IGFBP-6) in health and disease as determined by radioimmunoassay. *Clin Endocrinol (Oxf)* 1999;50:601-609.
43.   Welch RD, Jones AL, Bucholz RW, Reinert CM, Tjia JS, Pierce WA, Wozney JM, Li XJ. Effect of recombinant human bone morphogenetic protein-2 on fracture healing in a goat tibial fracture model. *J Bone Miner Res* 1998;13:1483-1490.
44.   Wilkens BE, Millis DL, Daniel GB, Munson L, Patel KR, Buonomo FC. Metabolic and histologic effects of recombinant canine somatotropin on bone healing in dogs, using an unstable ostectomy gap model. *Am J Vet Res* 1996;57:1395-1401.
45.   Yu H, Mistry J, Nicar MJ, Khosravi MJ, Diamandis A, van Doorn J, Juul A. Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin- like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *J Clin Lab Anal* 1999;13:166-172.

Chapter 8

General Discussion

Bone is capable of complete regeneration after destruction of its architecture. This ability of bone to repair itself is the basis of distraction osteogenesis in which a bone regenerate is formed under gradual distraction of two bone segments.[30,31] The mechanisms orchestrating bone regeneration are of great interest not only in understanding the process of bone repair, but also in gaining insight into the regeneration of non-skeletal tissues. Distraction osteogenesis can be used as an orthopaedic treatment option, but also as a model to investigate the basics of bone regeneration experimentally.

Antebrachial growth deformities (AGD) are the most common limb malformation in dogs.[32] These growth deformities are characterized by a combination of antebrachial length deficit, angular and rotational malalignment, elbow incongruity (EI), and carpal subluxation. Clinically, AGD results in compromised limb function and altered cosmetic appearance. In these dogs, lameness is the result of a combination of antebrachial length deficit, limb malalignment, and joint pain. The treatment of AGD is aimed at correcting length deficits and limb malalignment, at restoring joint function and at preventing secondary degenerative changes in the joints. The concept of distraction osteogenesis, using a circular external skeletal fixation (CESF) system, proved to be very effective in veterinary orthopaedics.[38,39,44] Our experience with distraction osteogenesis in correcting canine AGD dates back to 1994. The aim of the study presented in **Chapter 3** was to evaluate distraction osteogenesis in correcting AGD and to determine prognostic factors in treating these deformities. At presentation, dogs with AGDs are typically less than 7 months of age, which implies functional growth plates and thus growth potential in the contralateral antebrachium. The growth potential of the contralateral limb should be taken into account during the distraction procedure to compensate for the remaining growth. Realignment of the mechanical axis of joint movement and reduction of EI and carpal subluxation should be achieved as soon as possible. Incongruity of the elbow joint will lead to malformation, which is not amenable to correction. By analogy, carpal subluxation results in malformation of the antebrachiocarpal joint. Established OA is a major factor in the outcome of AGD treatment. Preventing OA is therefore critical in these dogs. Carpal OA after distraction of the antebrachium could be attributed to compression of the antebrachiocarpal joint.[24,51] Careful monitoring of imminent antebrachiocarpal flexor contracture is recommended. In many cases distraction was ended prematurely for this reason.

Summarily, AGDs can be treated successfully with a CESF lengthening procedure despite small remaining length deficits. Treatment limitations are mainly determined by the pre-existing OA and malformation in the elbow and carpal joints. Initial elbow OA and initial limb function are prognostic factors in predicting functional outcome. The cosmetic appearance after treatment is

determined by the magnitude of the initial radial and ulnar length deficits. This study determined the medium-term function after AGD treatment. Progression of elbow and carpal OA may have a negative effect on the long-term functional outcome.

Knowledge about the role of osteotropic growth factors in relation to distraction osteogenesis remains limited. In **Chapter 4** we hypothesized that distraction osteogenesis differs from osteotomy bone healing in the expression of growth factors in the bone regenerate and also differs in the circulating levels of these factors.[18,20,21,37,41,49] In order to gain insight into the regeneration of bone we determined the expression of GH, GHR, IGF-I, IGF-II, and BMP-2 in distraction-induced and osteotomy-induced bone regenerate. In addition, plasma GH profiles and plasma concentrations of IGF-I, IGF-II, IGFBP-4, and IGFBP-6 were determined to assess their potential systemic role during bone formation. The role of GHR in the growth plate has been addressed recently.[12,26] Our study is the first to demonstrate enhanced expression of GHR in distraction-induced bone regenerate. This finding is in agreement with the first part of our hypothesis. Up-regulation of GHR expression in distraction osteogenesis may enhance sensitivity to endogenous systemic GH and thus promote consolidation of the bone regenerate. Our study supports the concept of a direct effect of GH on bone.[29,54] Treatment with GH was effective in stimulating bone formation after distraction osteogenesis, which is consistent with up-regulation of GHR.[7,8,48] Our study was limited by the fact that the expression of GH, GHR, IGF-I, IGF-II, and BMP-2 was determined in the consolidation phase of distraction osteogenesis only. Gene expression should ideally be evaluated continuously to elucidate the role of these factors during active lengthening and during maturation of the bone regenerate. In addition, pursuing other osteotropic and angiogenic factors will be essential to a further understanding of osteogenesis. We reject the second part of our hypothesis in this chapter as changes in the circulating levels of the osteotropic growth factors GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 do not seem to play an important role during distraction osteogenesis.

In **chapter 5** we hypothesized that the bone markers OC and ICTP are effective in monitoring bone formation. Commercially available immunoassay kits for OC and ICTP were used to determine bone formation and bone resorption, respectively, during distraction osteogenesis. Ideally, these bone markers should be able to determine early bone formation, to assess progression of bone consolidation, and to predict the outcome of bone healing. Although OC is considered to be an osteoblast-related marker of bone formation, its precise function is unknown.[3,11] In contrast, osteocalcin-deficient mice demonstrated increased bone formation.[14] Matrix metalloproteinases are responsible for type-I collagen breakdown thus releasing ICTP.[25] Age is an important biological factor of

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bone marker variation in dogs.[2,3] Clear circadian rhythms were demonstrated for OC and ICTP in dogs.[36,40] Reports concerning bone markers to monitor osteogenesis in dogs are limited to OC, ICTP, and BAP.[17,23,37,47] The hypothesis stated in this chapter was discarded as plasma concentrations of OC and ICTP did not correlate with the amount of bone regenerate induced after distraction osteogenesis. The markers OC and ICTP were not effective in monitoring bone formation in this canine model. The marker BAP was effective in predicting the progression of osteosarcoma in dogs.[17] This marker looks promising as a candidate to assess bone formation and the possibilities of BAP should be explored in more detail.

Delayed-image bone scintigraphy is a non-invasive quantitative method for evaluating changes in bone metabolic activity.[22,33] In contrast to radiography, which reveals the amount of mineralization, delayed-image bone scintigraphy evaluates uptake of technetium-99m tracer by newly formed bone and thus precedes actual accretion of bone.[45,53] Delayed-image bone scintigraphy has been used successfully during distraction osteogenesis to predict the progression of bone formation in the early stages of the lengthening process and to assess the optimal time of bone consolidation in the later stages of bone maturation in human patients.[22,33] In **Chapter 6** we hypothesized that delayed-image bone scintigraphy is effective in quantitatively monitoring bone formation after distraction osteogenesis. In addition, we speculated that distraction osteogenesis, which is known to increase local and regional blood flow, increases bone metabolism in the adjacent long bone.[4] Although blood supply is considered closely related to rate of osteogenesis, blood flow, as indicated by the perfusion index, appears to be an unreliable predictor of new bone formation.[6,22,33] In our study, delayed-image bone scintigraphy was not effective in quantitatively differentiating between distraction-induced bone formation and osteotomy-induced bone formation, thus rejecting the first hypothesis of this chapter. Nevertheless, increasing delayed-image bone scintigraphy ratios were consistent with the radiographic evidence of advancing bone formation.[22] Increased metabolic bone activity in the adjacent femur was demonstrated not only after distraction osteogenesis, but also during osteotomy-induced bone healing. Placement of a CESF on the crus resulted in a similar increase of bone metabolism in the femur as induced after distraction or osteotomy. In view of this, the second hypothesis in this chapter was accepted. Whether enhanced bone metabolic activity was the result of production of angiogenic and osteotropic growth factors is unclear. Although delayed-image bone scintigraphy may be clinically valuable as an early predictor of bone healing, quantification of bone regenerate in individual patients does not appear to be feasible.

Dealing with bone deficits is a major concern in orthopaedic surgery. In **Chapter 7** we hypothesized that continuous GH infusion is effective in stimulating

bone healing in a critical-sized bone defect model. In addition, we speculated that local administration of GH by its direct effect on the GHR is most effective in enhancing bone healing. We expected GH to stimulate the expression of IGF-I, IGF-II, and GHR within the original bone defect and to alter circulating plasma concentrations of IGF-I, IGF-II, IGFBP-4 and IGFBP-6. Our study demonstrates that continuous infusion with GH stimulates bone formation and bone healing in a critical sized bone defect, thus confirming the first part of the hypothesis in this chapter. In contrast to our second hypothesis, local delivery of GH with an infusion pump does not additionally enhance bone healing. Growth hormone treatment did not increase the expression of IGF-I, IGF-II, and GHR in the bone regenerate during the consolidation phase. Plasma concentrations of IGF-I and IGF-II were increased during GH treatment. Although the stimulation of bone healing with GH has been reported previously, our study was the first to show the effectiveness of GH on bone regeneration in a critical-sized bone defect.[7,8,13,48,55]

Expression of GHR was similar in GH-treated dogs and controls and is consistent with the concept of a direct effect of GH on bone regeneration. The fact that local infusion with GH into the defect had no additional effect on bone healing may be attributed to redistribution of GH into the circulation. In our study, IGF-I expression levels were lower during the consolidation phase of the GH-stimulated bone regenerate. In theory, this suggests that IGF-I production in the bone regenerate was not responsible for the progression of bone healing at this stage. Bone accretion progressed despite the fact that IGF-I plasma concentrations returned to preoperative levels after cessation of GH infusion. These findings are consistent with a role of IGF-I during the early stages of callus formation.[10] Whether IGF-I production in the bone regenerate during GH treatment plays an important role in comparison with circulating liver-derived IGF-I remains unclear. In contrast to IGF-I, IGF-II plasma concentrations remained elevated even after cessation of GH infusion. As IGF-II expression did not differ between the GH treated defects and the controls, sustained IGF-II production in skeletal or even non-skeletal tissues outside the defects could be partly responsible for enhanced bone regeneration. Although systemic treatment with GH has proved effective, local routes of GH application focusing on the direct effect of GH merit further research.

Recently, several reports have demonstrated the crucial role of GH and GHR in non-skeletal tissues. The stimulation of liver tissue regeneration with GH in particular has received substantial attention.[34,43] Transgenic rats with GH deficiency demonstrated a decreased reparative response after administration of an hepatotoxic drug.[52] In transgenic mice with blocked GH action, regeneration of liver tissue was dramatically reduced, whereas mice with blocked IGF-I action demonstrated a normal regenerative potential.[46] Stimulation of liver regeneration

with GH was more effective than treatment with IGF-I and a direct effect of GH was proposed in rats.[5] Proliferation of old-age liver after GH stimulation was mediated through forkhead box m1b.[35] Treatment with GH was effective in stimulating liver regeneration after hepatectomy in human patients with hepatocellular carcinoma.[42] In epidermal tissues GH was used to stimulate regeneration in skin wounds and in burn patients.[16,27] The action of GH treatment in skin tissue was mediated directly with local production of IGF-I in the epidermal tissue contributing to the healing process.[15] Growth hormone and GHR were demonstrated to play an important role in the regeneration of several tissues of the digestive tract, including gastric and colonic mucosa.[50,56] Treatment with GH stimulated healing of gastric ulcers in rats.[9] The role of GH was also convincingly demonstrated in nerve and muscle tissue.[1,28] Treatment with GH resulted in reversal of thymic involution in a human patient.[19]

Summarily, GH plays an important role in modulating bone metabolism. Part of the effect of GH on bone metabolism and bone regeneration is exerted directly without the intervention of IGF-I. This underscores the crucial role of its receptor GHR in bone. There is increasing evidence to support the role of GH and GHR in both bone tissue regeneration and non-skeletal tissue regeneration. The full potential of GH as a universal stimulating factor of regeneration has yet to be explored.

References

1. Aberg ND, Brywe KG, Isgaard J. Aspects of growth hormone and insulin-like growth factor-I related to neuroprotection, regeneration, and functional plasticity in the adult brain. *Sci World J* 2006;6:53-80.
2. Allen MJ, Allen LCV, Hoffmann WE, Richardson DC, Breur GJ. Urinary markers of type I collagen degradation in the dog. *Res Vet Science* 2000;69:123-127.
3. Allen MJ, Hoffmann WE, Richardson DC, Breur GJ. Serum markers of bone metabolism in dogs. *Am J Vet Res* 1998;59:250-254.
4. Aronson J. Temporal and spatial increases in blood flow during distraction osteogenesis. *Clin Orthop* 1994;301:124-131.
5. Asakawa K, Hizuka N, Takano K, Horikawa R, Sukegawa I, Toyoda C, Shizume K. Human growth hormone stimulates liver regeneration in rats. *J Endocrinol Invest* 1989;12:343-347.
6. Averill SM, Johnson AL, Chambers M, Smith CW, Kneller S, Twardock AR, Schaeffer DJ. Qualitative and quantitative scintigraphic imaging to predict fracture healing. *Vet Comp Orthop Traumatol* 1999;12:142-150.
7. Bail HJ, Kolbeck S, Krummrey G, Schmidmaier G, Haas NP, Raschke MJ. Systemic application of growth hormone for enhancement of secondary and intramembranous fracture healing. *Horm Res* 2002;58 Suppl 3:39-42.
8. Bail HJ, Kolbeck S, Lindner T, Dahne M, Weiler A, Windhagen HJ, Raun K, Skjaerbaek C, Flyvbjerg A, Orskov H, Haas NP, Raschke MJ. The effect of growth hormone on insulin-like growth factor I and bone metabolism in distraction osteogenesis. *Growth Horm IGF Res* 2001;11:314-323.
9. Beckert S, Class N, Farrahi F, Coerper S. Growth hormone enhances gastric ulcer healing in rats. *Med Sci Monit* 2004;10:255-258.
10. Bourque WT, Gross M, Hall BK. Expression of four growth factors during fracture repair. *Int J Dev Biol* 1993;37:573-579.
11. Breur GJ, Allen MJ, Carlson SJ, Richardson DC. Markers of bone metabolism in dog breeds of different size. *Res Vet Sci* 2004;76:53-55.
12. Cruickshank J, Grossman DI, Peng RK, Famula TR, Oberbauer AM. Spatial distribution of growth hormone receptor, insulin-like growth factor-I receptor and apoptotic chondrocytes during growth plate development. *J Endocrinol* 2005;184:543-553.
13. Dubreuil P, Abribat T, Broxup B, Brazeau P. Long-term growth hormone-releasing factor administration on growth hormone, insulin-like growth factor-I concentrations, and bone healing in the Beagle. *Can J Vet Res* 1996;60:7-13.
14. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996;382:448-452.
15. Dunaiski V, Belford DA. Contribution of circulating IGF-I to wound repair in GH-treated rats. *Growth Horm IGF Res* 2002;12:381-387.
16. Edmondson SR, Thumiger SP, Werther GA, Wraight CJ. Epidermal homeostasis: the role of the growth hormone and insulin-like growth factor systems. *Endocr Rev* 2003;24:737-764.
17. Ehrhart N, Dernell WS, Hoffmann WE, Weigel RM, Powers BE, Withrow SJ. Prognostic importance of alkaline phosphatase activity in serum from dogs with appendicular osteosarcoma: 75 cases (1990-1996). *J Am Vet Med Assoc* 1998;213:1002-1006.
18. Eingartner C, Coerper S, Fritz J, Gaissmaier C, Koveker G, Weise K. Growth factors in distraction osteogenesis. Immuno-histological pattern of TGF-beta1 and IGF-I in human callus induced by distraction osteogenesis. *Int Orthop* 1999;23:253-259.

19.   Fahy GM. Apparent induction of partial thymic regeneration in a normal human subject: a case report. *J Anti Aging Med* 2003;6:219-227.
20.   Farhadieh RD, Dickinson R, Yu Y, Gianoutsos MP, Walsh WR. The role of transforming growth factor-beta, insulin-like growth factor I, and basic fibroblast growth factor in distraction osteogenesis of the mandible. *J Craniofac Surg* 1999;10:80-86.
21.   Farhadieh RD, Gianoutsos MP, Yu Y, Walsh WR. The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. *J Craniofac Surg* 2004;15:714-718.
22.   Felemovicius J, Ortiz-Monasterio F, Gomez-Radillo LS, Serna A. Determining the optimal time for consolidation after distraction osteogenesis. *J Craniofac Surg* 2000;11:430-436.
23.   Fink B, Fox F, Singer J, Skripitz R, Feldkamp J. Monitoring of bone formation during distraction osteogenesis via osteocalcin: a time sequence study in dogs. *J Orthop Sci* 2002;7:557-561.
24.   Fink B, Schwinger G, Singer J, Sager M, Wilke C, Braunstein S. The effect of tibial lengthening using the Ilizarov method on the cartilage and the menisci of the knee joint. *J Orthop Res* 2001;19:665-670.
25.   Garnero P, Ferreras M, Karsdal MA, Nicamhlaoibh R, Risteli J, Borel O, Qvist P, Delmas PD, Foged NT, Delaisse JM. The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. *J Bone Miner Res* 2003;18:859-867.
26.   Gevers EF, van der Eerden BC, Karperien M, Raap AK, Robinson IC, Wit JM. Localization and regulation of the growth hormone receptor and growth hormone-binding protein in the rat growth plate. *J Bone Miner Res* 2002;17:1408-1419.
27.   Ghofrani A, Holler D, Schuhmann K, Saldern S, Messmer BJ. The influence of systemic growth hormone administration on the healing time of skin graft donor sites in a pig model. *Plast Reconstr Surg* 1999;104:470-475.
28.   Gorio A, Citterio C, Muller EE, Di Giulio AM. Glycosaminoglycan-promoted muscle reinnervation and insulin-like growth factor-I levels are affected by anti-growth hormone-releasing hormone exposure. *J Neurosci Res* 2001;66:1112-1117.
29.   Hedner E, Linde A, Nilsson A. Systemically and locally administered growth hormone stimulates bone healing in combination with osteopromotive membranes: an experimental study in rats. *J Bone Miner Res* 1996;11:1952-1960.
30.   Ilizarov GA. The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation. *Clin Orthop* 1989;238:249-281.
31.   Ilizarov GA. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. *Clin Orthop* 1989;239 :263-285.
32.   Johnson JA, Austin C, Breur GJ. Incidence of canine appendicular musculoskeletal disorders in 16 veterinary teaching hospitals from 1980 through 1989. *Vet Comp Orthop Traumatol* 1994;7:56-69.
33.   Kawano M, Taki J, Tsuchiya H, Tomita K, Tonami N. Predicting the outcome of distraction osteogenesis by 3-phase bone scintigraphy. *J Nucl Med* 2003;44:369-374.
34.   Koniaris LG, McKillop IH, Schwartz SI, Zimmers TA. Liver regeneration. *J Am Coll Surg* 2003;197:634-659.
35.   Krupczak-Hollis K, Wang X, Dennewitz MB, Costa RH. Growth hormone stimulates proliferation of old-aged regenerating liver through forkhead box m1b. *Hepatology* 2003;38:1552-1562.
36.   Ladlow JF, Hoffmann WE, Breur GJ, Richardson DC, Allen MJ. Biological variability in serum and urinary indices of bone formation and resorption in dogs. *Calcif Tissue Int* 2002;70:186-193.

37.   Lammens J, Liu Z, Aerssens J, Dequeker J, Fabry G. Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. *J Bone Miner Res* 1998;13:279-286.
38.   Latte Y. Application of the Ilizarov method in veterinary orthopaedic surgery (part 1). *Eur J Comp Anim Pract* 1997;7:26-50.
39.   Latte Y. 75 applications of the Ilizarov method (part 2). *Eur J Comp Anim Pract* 1998;8:64-81.
40.   Liesegang A, Reutter R, Sassi ML, Risteli J, Kraenzlin M, Riond JL, Wanner M. Diurnal variation in concentrations of various markers of bone metabolism in dogs. *Am J Vet Res* 1999;60:949-953.
41.   Liu Z, Luyten FP, Lammens J, Dequeker J. Molecular signaling in bone fracture healing and distraction osteogenesis. *Histol Histopathol* 1999;14:587-595.
42.   Luo SM, Liang LJ, Lai JM. Effects of recombinant human growth hormone on remnant liver after hepatectomy in hepatocellular carcinoma with cirrhosis. *World J Gastroenterol* 2004;10:1292-1296.
43.   Mackey S, Singh P, Darlington GJ. Making the liver young again. *Hepatology* 2003;38:1349-1352.
44.   Marcellin-Little DJ, Ferretti A, Roe SC, DeYoung DJ. Hinged Ilizarov external fixation for correction of antebrachial deformities. *Vet Surg* 1998;27:231-245.
45.   Nutton RW, Fitzgerald R-HJ, Kelly PJ. Early dynamic bone-imaging as an indicator of osseous blood flow and factors affecting the uptake of 99mTc hydroxymethylene diphosphonate in healing bone. *J Bone Joint Surg Am* 1985;67:763-770.
46.   Pennisi PA, Kopchick JJ, Thorgeirsson S, LeRoith D, Yakar S. Role of growth hormone (GH) in liver regeneration. *Endocrinology* 2004;145:4748-4755.
47.   Philipov JP, Pascalev MD, Aminkov BY, Grosev CD. Changes in serum carboxyterminal telopeptide of type I collagen in an experimental model of canine osteomyelitis. *Calcif Tissue Int* 1995;57:152-154.
48.   Raschke MJ, Bail H, Windhagen HJ, Kolbeck SF, Weiler A, Raun K, Kappelgard A, Skiaerbaek C, Haas NP. Recombinant growth hormone accelerates bone regenerate consolidation in distraction osteogenesis. *Bone* 1999;24:81-88.
49.   Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R. Temporal and spatial expression of bone morphogenetic protein-2, -4, and -7 during distraction osteogenesis in rabbits. *Bone* 2000;27:453-459.
50.   Si JM, Cao Q, Gao M. Expression of growth hormone and its receptor in chronic atrophic gastritis and its clinical significance. *World J Gastroenterol* 2004;10:2908-2910.
51.   Stanitski DF, Rossman K, Torosian M. The effect of femoral lengthening on knee articular cartilage: the role of apparatus extension across the joint. *J Pediatr Orthop* 1996;16:151-154.
52.   Tani Y, Kamai Y, Yamate K, Mizuno H, Yamamoto H, Hosokawa T, Doi K. Restitutive response of Mini rat liver to injury induced by a single oral administration of thioacetamide. *J Toxicol Sci* 2001;26:25-37.
53.   van Roermund PM, Hoekstra A, Haar-Romeny BM, Renooij W. Bone healing during lower limb lengthening by distraction epiphysiolysis. *J Nucl Med* 1988;29:1259-1263.
54.   Wang J, Zhou J, Cheng CM, Kopchick JJ, Bondy CA. Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. *J Endocrinol* 2004;180:247-255.
55.   Wilkens BE, Millis DL, Daniel GB, Munson L, Patel KR, Buonomo FC. Metabolic and histologic effects of recombinant canine somatotropin on bone healing in dogs, using an unstable ostectomy gap model. *Am J Vet Res* 1996;57:1395-1401.

56.   Williams KL, Fuller CR, Dieleman LA, DaCosta CM, Haldeman KM, Sartor RB, Lund PK. Enhanced survival and mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth hormone. *Gastroenterology* 2001;120:925-937.

Chapter 9

Summary

Bone is one of the few tissues capable of complete regeneration. The mechanisms behind this phenomenon are of great interest not only in understanding the processes of bone repair and bone metabolism, but also in gaining insight into the regeneration of non-skeletal tissues. Growth factors are now generally accepted to play a crucial role in regulating bone formation and bone resorption. Nevertheless, the cascade of growth factors dictating bone regeneration still needs to be elucidated. Distraction osteogenesis, in which bone formation is induced under gradual distraction of two bone surfaces, can be used both as an orthopaedic treatment option and as a model to investigate the basics of bone regeneration experimentally.

In **Chapter 1** the aims of this thesis are presented. First aim of this thesis was to evaluate the clinical results of treatment of antebrachial growth deformities with a distraction osteogenesis procedure and to identify prognostic factors to predict the functional outcome in dogs with these growth deformities. Second goal was to investigate the role of local and systemic growth factors during distraction-induced bone regeneration. Third aim was to stimulate bone healing in a critical-sized bone defect model.

In **Chapter 2** a review of literature is provided covering relevant knowledge of bone histology and histiogenesis, growth plate injuries, healing of bone fractures, distraction osteogenesis, circular external skeletal fixation, hormonal regulation of bone formation, skeletal growth factors, and bone markers.

The results in **Chapter 3** demonstrate that incongruity of the elbow joint and osteoarthritis of the elbow and antebrachiocarpal joint are major complicating factors in treating antebrachial growth deformities in dogs. Treatment with a circular external skeletal fixation system was effective in correcting angular and rotational growth deformities. Nevertheless, it was not possible to completely restore all antebrachial length deficits in these patients with the distraction procedure. Limb alignment and function improved in all dogs. Elbow and antebrachiocarpal osteoarthritis progressed despite surgical correction of the growth deformity. Initial elbow osteoarthritis, initial function, radial length deficit, and ulnar length deficit were identified as prognostic factors in dogs with antebrachial growth deformities. These factors should be addressed to predict the functional outcome of treatment with a distraction osteogenesis procedure.

In **Chapter 4** the expression is reported for growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), and bone morphogenetic protein-2 (BMP-2) in distraction-induced bone regenerate. Expression of these factors was assessed during the consolidation phase, comparing distraction osteogenesis with new bone formation induced by an osteotomy. In addition, plasma GH profiles and plasma concentrations were determined for IGF-I, IGF-II, and insulin-like growth factor

binding protein- 4 (IGFBP-4) and IGFBP-6. Expression of GHR in the distraction-induced bone regenerate was significantly higher than in osteotomy-induced new bone. Expression of GHR, IGF-I, and BMP-2 in the distraction-induced bone regenerate and in the newly formed bone induced by osteotomy had increased compared with the expression of these factors in mature bone. Circulating levels of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 did not change during the distraction osteogenesis procedure. We conclude that up-regulation of GHR expression may enhance sensitivity to endogenous systemic GH and thus promote consolidation of the bone regenerate after distraction osteogenesis.

In **Chapter 5** the efficacy of the bone markers osteocalcin (OC) and carboxyterminal cross-linked telopeptide of type I collagen (ICTP) is assessed in monitoring bone formation during distraction osteogenesis in dogs. Commercially available immunoassay kits for OC and ICTP were used to monitor bone formation and bone resorption, respectively, during a distraction osteogenesis procedure and during bone healing of an osteotomy. The radiographic amount of newly formed bone was determined using densitometric image analysis. Plasma levels of OC and ICTP did not reflect the differences in the amount of newly formed bone. We concluded that the bone markers OC and ICTP are not effective in monitoring bone formation and bone resorption, respectively, in this canine model of distraction osteogenesis.

In **Chapter 6** delayed-image bone scintigraphy is evaluated to quantitatively assess distraction-induced bone formation. Delayed-image bone scintigraphy is a non-invasive method to monitor changes in bone metabolic activity. Bone scintigraphy relies on the uptake of technetium-99m tracer by newly formed bone. Delayed-image bone scintigraphy was conducted during a distraction osteogenesis procedure and compared with healing of an osteotomy. Scintigraphic ratios were calculated and compared with the amount of bone formation as determined with densitometric image analysis. Distraction osteogenesis and bone healing of an osteotomy resulted in increased delayed-image scintigraphy ratios not only in the affected crus, but also in the adjacent femur. Delayed-image bone scintigraphy was not effective at differentiating between the amounts of distraction-induced bone and osteotomy-induced bone. We conclude that delayed-image bone scintigraphy is not adequately sensitive to quantitatively monitor bone formation, but may be useful as an early predictor of bone healing.

Growth hormone plays an important role in bone metabolism. Treating bone defects is a major topic in orthopedic surgery. In **Chapter 7** we hypothesize that local continuous GH administration stimulates bone healing in a canine critical-sized bone defect model. Bone formation in the defects was quantified with densitometric image analysis and histomorphometry. Treatment with GH resulted in healing of bone defects, but without an additional effect of local infusion.

Expression of IGF-I was lower in the bone regenerate of GH treated dogs, whereas IGF-II and GHR expression were not increased. Growth hormone administration increased circulating levels of IGF-I and IGF-II. We conclude that continuous infusion of GH stimulates bone healing in this critical-sized bone defect model. Local application of GH did not additionally enhance bone healing in this model. Increased plasma concentrations of IGF-I and IGF-II most likely induce bone formation.

In **Chapter 8** the results of the presented studies are discussed in the context of their hypotheses postulated in **Chapter 2**.

Chapter 10

Samenvatting in het Nederlands

Bot is als één van de weinige weefsels in staat om volledig te regenereren. De mechanismen achter dit fenomeen zijn van groot belang, niet alleen om de processen van botherstel en botmetabolisme te begrijpen, maar ook om inzicht te krijgen in de regeneratie van andere niet-skelet gerelateerde weefsels. De cruciale rol van groeifactoren bij de regulatie van botaanmaak en botresorptie wordt nu algemeen onderschreven. Desalniettemin behoeft de cascade van groeifactoren, die de regeneratie van bot dicteert, nog steeds opheldering. Distractie osteogenesis, waarbij botvorming wordt geïnduceerd onder geleidelijke distractie van twee botoppervlaktes, kan zowel als een orthopedische behandelingsmogelijkheid worden toegepast als experimenteel worden gebruikt om de basisbeginselen van botregeneratie te onderzoeken.

In **Hoofdstuk 1** worden de doelstellingen van het proefschrift uiteengezet. Het eerste doel van dit proefschrift was om de klinische resultaten te evalueren van de behandeling van groeideformiteiten van het antebrachium met een distractie osteogenesis procedure en om prognostische factoren, die het functionele herstel kunnen voorspellen, te identificeren bij honden het deze groeideformiteiten. Het tweede streven was om de rol van lokale groeifactoren en groeifactoren in de circulatie te onderzoeken tijdens de regeneratie van bot, geïnduceerd door distractie. Het derde doel was om botgenezing te stimuleren in een model met een botdefect van kritische grootte.

In **Hoofdstuk 2** wordt een overzicht gegeven van de literatuur, waarbij de relevante kennis over bothistologie, histiogese van bot, groeischijffracturen, fractuurgenezing, distractie osteogenesis, circulaire externe skelet fixatie, hormonale regulatie van botvorming, groeifactoren van het skelet en markers van botmetabolisme aan de orde komen.

De resultaten in **Hoofdstuk 3** tonen dat incongruentie van het ellebooggewricht en osteoarthritis van het ellebooggewricht en antebrachiocarpale gewricht belangrijke complicerende factoren zijn bij de behandeling van groeideformiteiten bij de hond. Behandeling met een circulair extern skelet fixatiesysteem was effectief bij het corrigeren van de hoek- en torsie-afwijkingen tengevolge van de groeideformiteit. Desalniettemin bleek het niet mogelijk te zijn om elk lengtedeficit van radius en ulna bij deze patiënten volledig te herstellen met een distractie procedure. De stand en functie van de betreffende extremiteit verbeterden bij alle honden. De mate van osteoarthrose van de elleboog en het antebrachiocarpale gewricht nam toe ondanks de chirurgische correctie van de groeideformiteit. De initiële osteoarthrose in de elleboog, de initiële functie, en het lengtedeficit van de radius en de ulna werden geïdentificeerd als prognostische factoren bij honden met groeideformiteiten van het antebrachium. Deze factoren moeten in beschouwing worden genomen om het functionele resultaat van de behandeling met een distractie osteogenesis procedure te voorspellen.

In **Hoofdstuk 4** wordt de expressie van groeihormoon (GH), groeihormoon receptor (GHR), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) en bone morphogenetic protein-2 (BMP-2) beschreven in het door distractie geïnduceerde botregeneraat. Expressie van deze factoren werd beoordeeld in de consolidatiefase, waarbij distractie osteogenesis werd vergeleken met de vorming van nieuw bot geïnduceerd na een osteotomie. Daarnaast werden de plasma GH profielen en plasmaconcentraties bepaald van IGF-I, IGF-II, en insulin-like growth factor binding protein- 4 (IGFBP-4) en IGFBP-6. De expressie van GHR in het door distractie geïnduceerde botregeneraat was significant hoger dan in het nieuwgevormde bot geïnduceerd na een osteotomie. De expressie van GHR, IGF-I en BMP-2 in het door distractie geïnduceerde botregeneraat en in het nieuwgevormde bot geïnduceerd na osteotomie was verhoogd in vergelijking met de expressie van deze factoren in volgroeid bot. De hoeveelheid GH, IGF-I, IGF-II, IGFBP-4 en IGFBP-6 in de circulatie veranderde niet tijdens de distractie osteogenesis procedure. Wij concluderen dat een verhoogde expressie van GHR de gevoeligheid voor endogeen GH in de circulatie mogelijk verhoogt en daarmee de consolidatie van het botregeneraat na distractie osteogenesis bevordert.

In **Hoofdstuk 5** wordt de bruikbaarheid van de botmarkers osteocalcine (OC) en carboxyterminal cross-linked telopeptide van type I collageen (ICTP) bepaald om de mate van botvorming tijdens distractie osteogenesis bij de hond vast te stellen. Commercieel verkrijgbare immunoassay kits voor OC en ICTP werden gebruikt om respectievelijk botvorming en botresorptie tijdens een distractie osteogenesis procedure en gedurende botgenezing van een osteotomie te bepalen. De röntgenologisch waarneembare hoeveelheid nieuwgevormd bot werd bepaald met een densitometrische beeldanalyse. De plasmaconcentraties van OC en ICTP gaven de verschillen in de hoeveelheid nieuwgevormd bot niet weer. Wij concluderen dat de botmarkers OC en ICTP niet bruikbaar zijn bij het bepalen van respectievelijk botvorming en botresorptie in dit model van distractie osteogenesis bij de hond.

In **Hoofdstuk 6** wordt het gebruik van *delayed-image bone scintigraphy* (= de botfase van skeletscintigrafie) geëvalueerd om daarmee kwantitatief de mate van botvorming geïnduceerd door distractie te bepalen. *Delayed-image bone scintigraphy* is een niet-invasieve methode om veranderingen in de activiteit van het botmetabolisme vast te stellen. Botscintigrafie berust op de opname van een radioactieve technetium-99m tracer door nieuwgevormd bot. *Delayed-image bone scintigraphy* werd uitgevoerd gedurende een distractie osteogenesis procedure en vergeleken met botgenezing na een osteotomie. Scintigrafie ratios werden berekend en vergeleken met de hoeveelheid botvorming, zoals bepaald met densitometrische beeldanalyse. Distractie osteogenesis en botheling van de osteotomie resulteerden in verhoogde *delayed-image bone scintigraphy* ratios niet alleen in de betreffende

onderpoot, maar ook in het belendende dijbeen. *Delayed-image bone scintigraphy* was niet effectief om te differentiëren tussen de hoeveelheid door distractie en door osteotomie geïnduceerd bot. Wij concluderen dat delayed-image bone scintigraphy niet voldoende gevoelig is om botvorming kwantitatief te bepalen, maar is mogelijk wel bruikbaar om botgenezing in een vroegtijdig stadium te voorspellen.

Groeihormoon speelt een belangrijke rol in het botmetabolisme. Het behandelen van botdefecten is een belangrijk onderwerp binnen de orthopedische chirurgie. In **Hoofdstuk 7** poneren we de hypothese dat lokale continue GH toediening botgenezing in een botdefect van kritische grootte zal stimuleren. Botvorming in de defecten werd gekwantificeerd middels densitometrische beeldanalyse en histomorfometrie. Behandeling met GH resulteerde in het genezen van de botdefecten, maar een additioneel effect van lokale toediening werd niet aangetoond. Expressie van IGF-I was lager in het botregeneraat van de met GH behandelde honden, terwijl de expressie van IGF-II en GHR niet toenam. Groeihormoon toediening verhoogde de concentraties van IGF-I en IGF-II in de circulatie. Wij concluderen dat continue toediening van GH botgenezing bevordert in dit model van een botdefect met kritische grootte. Lokale toediening van GH gaf geen additionele verbetering van de botgenezing in dit model. Verhoogde plasma concentraties van IGF-I en IGF-II induceren hoogstwaarschijnlijk botvorming.

In **Hoofdstuk 8** worden de bevindingen, die zijn verkregen uit dit onderzoek, in samenhang met de hypotheses uit **Hoofdstuk 2** besproken.

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 10 februari 1963 te Nijmegen. Hij doorliep de lagere en middelbare school in Ede. Na het behalen van zijn Gymnasium ß diploma startte hij in 1981 de studie Diergeneeskunde aan de Faculteit der Diergeneeskunde, Universiteit Utrecht te Utrecht. Na het behalen van het diploma Dierenarts vervulde hij zijn militaire dienstplicht bij de cavalerie te Soesterberg (1989-1990). In juni 1990 vervolgde hij zijn opleiding als roulant bij het Departement Geneeskunde van Gezelschapsdieren, Faculteit der Diergeneeskunde en vervolgens doorliep hij de opleiding tot specialist in de chirurgie van gezelschapsdieren (1991-1994). Na het afronden van zijn opleiding tot specialist werd hij aangesteld als junior staflid bij de divisie Orthopedie van het Departement Geneeskunde van Gezelschapsdieren. In 1999 behaalde hij zijn Europese specialisten erkenning met de titel "diplomate of the European College of Veterinary Surgeons (ECVS) in small animal surgery". In hetzelfde jaar startte hij het onderzoek dat gepresenteerd wordt in dit proefschrift. Vanaf 2002 tot heden is hij staflid bij de divisie Orthopedie.

The author of this thesis was born the 10th of February 1963 in Nijmegen, the Netherlands. He attended primary and secondary school in Ede. In 1981 he graduated from the Gymnasium ß and in the same year started his education at the Faculty of Veterinary Medicine, Utrecht University, in Utrecht. After graduation in Veterinary Medicine he was called for army duty and served his term in the cavalry division at Soesterberg (1989-1990). In June 1990 he started as an intern at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, followed by a residency in Companion Animals Surgery (1991-1994). After completing his residency, he was appointed junior staff member at the Division of Orthopaedic Surgery of the Department of Clinical Sciences of Companion Animals. In 1999 he was accepted as diplomate of the European College of Veterinary Surgeons (ECVS) in small animal surgery. In the same year he started his research efforts presented in this thesis. He was appointed full staff member in 2002 and holds this position up till now.