FINAL REPORT
Of Project # 2058.

Enhancement of Fracture Healing By A Newly Discovered Peptide (SHMSP)

Prof. Mir Sadat Ali,
MBBS, MS, PhD,FRCS, D Orth. FICS

Dr. Ibrahim Al-Habdan
MBBS, FRCSI

Department of Orthopaedic Surgery,
College of Medicine, King Faisal University,
Dammam

Summary:

Fifty skeletal mature three-month-old rabbits were studies for the effect of SHMSP (Sadat-Habdan Mesenchymal Stimulating Peptide) on fracture healing.

The animals were divided into 5 groups 4 study and 4 control groups (group A-E). Groups A-D were study group and group E was control. In all the groups, under anesthesia, a fracture was created in the right ulna. From day three the study groups received 5 mg/kg body weight of the peptide 1-4 in group A-D. Control group did not receive any peptide nor placebo.

Each week two rabbits of the study group and two of the control group were radiographs. Then sacrificed and the limbs were dissected and stored in formalin was sent for histopathology.

Results:

Radiographs of the animals indicated early union in the study groups as compared to the control groups. Histopathological studies showed that the peptide had stimulated more osteoid in the control group. In the control group the osteotomy sites had no osteoid and more cartilage from the first week onwards.

Conclusions:

The study indicates that SHMSP is a potential peptide which stimulates production of osteoid which is the requirement of every fracture healing. This peptide could prove to be one of the major breakthrough in the treatment of fractures and impaired healing of fractures, as this polypeptide is the smallest chain reported in the literature.

2.1 LITERATURE REVIEW

Introduction:

Fracture healing is a very complex process which involves local and general factors. The reported incidence of impaired healing ranges between 5-20%[1-5] . Both factors are equally important and complement each other. Delayed healing is usually due to failure of the local cellular structures to react to the stimulation of the growth factors which are released at the site of the fractures. In the last 40 years surgeons got a boost to heal fractures way by the help of rigid internal fixation[6, 7] , but only realized later that with adequate fixations fractures also failed to unite. The second method developed to heal fractures was mechanical stimulation was given up due to inconsistencies in the results achieved [8-10].

Biological factors were discovered to enhance fracture healing and became a subject of intense ongoing investigations. Senn (1889)[11] in Chicago showed that decalcified bone had some power of stimulating bone growth. Before the discovery of Urist in (1965)[12] that bone matrix can induce new bone formation, Leriche and Policard (1927)[13], Orell (1934)[14] and Levander (1938)[15] made important contributions in the present day understanding and development of the Bone Morphogenetic Proteins (BMPs).

Since then many growth factors have been isolated which are claimed to enhance fracture healing[16]. Under normal circumstances the mesenchymal stem cells are released from the bone marrow, periosteum and surrounding soft tissues. These mesenchymal cells initially proceed in the form of chondrogenesis, osteogenesis,

callus formation and remodeling. Growth factors such as transforming growth factor-β (TGF–β), (BMPs), Insulin like growth factor-I (IGF-1) and platelet derived growth factor (PDGF), have been found to have some effect on fracture healing[17-21] .

BMP belong to the TGFß family, a group of growth factors[22-24]. BMPs are described as dimeric molecules with two chains held together by one disulphide bond and each monomer consists of about 120 amino acids with seven canonical cysteine residues[25]. BMPs act at cellular level enhancing chemotaxis, mitosis, differentiation and stimulation of extracellular matrix synthesis and binding to matrix components [25].

Bone morphogenic proteins were reported to be factors that can induce transformation of mesenchymal cells into chondroblasts and osteoblast[26-27]. BMP is the only osteoinductive protein that can transform connective tissue cells into osteoprogenitor cells[28]. Various studies in the experimental animals were conducted with varying results. In the last four years Prof. Sadat Mir Ali and Dr. Ibrahim Al-Habdan at the Department Of Orthopedic Surgery, College of Medicine, King Faisal University, Dammam, have isolated a peptide which enhance fracture healing. During this period the peptide was extracted, purified, sequenced, synthesized and tested in two different animal models with extremely promising results.

Johnson and colleagues reported the first clinical study using human BMP in 1992[29] and recently Valentine-Opran and associates (2002)[30] and McKee (2003)[31] reported beneficial results after using BMP-2 and rh BMP7 in human trails of fractured long bones. As the animal studies of SHMSP showed excellent results, it is highly possible that similar results will be obtained in human trials, hence it is our belief and recommendation that to pursue the human trails by using SHMSP.

Epidemiology of fractures:

As early as 1823 Astley Cooper reported the effects of age on human skeleton[(32)]. Alffframm and Bauer (1962)[33] suggested that Bruns in 1882 gave the concept of the influence of age and sex on various type of fractures. Singer et al (1998)[34] indicated that osteoporotic fractures are on the rise in women who are over 45 years of age. In United States of America alone over 6 million traumatic fractures occur yearly and many go into delayed union[35]. Fracture healing itself is a synchronized step-like fashion and in majority of situations it is a predictable sequence and also depends on the mechanical environement. The process of such healing of fractures is known for long at the cellular level but at molecular level things are still hazy. Fractures occur also due to decreased quantity of osteoid (osteoporosis). In 1996, 2,47,000 hip fractures occurred in persons over 45 years[36]. It is expected that figure to rise to 6 million by the year 2050. The expected mortality due to these fractures is predicted to be around 20 percent within the first year[37].

Three common phases of fracture healing has been described[38,39.] Sandberg, ARO, Vuorio (1993)[40] confirmed the phases of fracture healing from mesenchymal cells to differentiated chondrocytes. Even two weeks after a fracture chondrocytes remain a dominant cell. After the calcification of the cartilage the progenitors of the osteoblasts appear in the fracture site. It is logical to believe if the osteocytes and osteoblasts arrive at the fracture scene. These cells synthesize the matrix with high concentrate of type I collagen fibres and calcium is deposited among the fibrils. Earlier the

calcification the shorter the healing period. For long periosteum was believed to be the source of chondrocyte and osteoblast progenitor cells. It is here the growth factors like BMPs, TGF-β, Insulin like growth factors influence fracture healing. Trippel et al (1996)[41] indicated that growth factors stimulate fracture healing by binding to specific receptor molecules on the target cells. Johnson and Vaillancourt (1994)[42] reported that growth factor receptors are linked to intracellular reactions in the cytoplasm. Activating many genes to carry messages for enhancing the healing process. There are four different ways by which the healing process could be interfered. Firstly theres could be increase in the rate of healing without an alteration of the stages of the healing, secondly the rate of healing could be increased by changes in the process of healing, thirdly there could be increased in the occurrence of the healing and lastly peptides could even improve the quality of the healing. Past studies of the extracted peptide SHMSF has confirmed radiologically and histologically that the peptide stimulates early osteoid production from the first week onwards. We believe the peptide under study increases by the healing by way of first and second ways mentioned above.

.

Risk factors:

Fractures occur after trauma to the skeletal system. The causes of fractures in the developed and developing countries is due to road traffic accidents. Males predominate, and most men are between 15–45 years of age[43]. Meisinger et al (2002)[44] found that the peak incidence of fractures in a recent analysis was between

15–40 years. During the years the causes of fractures have changed tremendously and sports have played an additional reason for fractures.

Local and general diseases affect fracture healing. Osteoporosis, diabetes mellitus, hypothyroidism and renal failure are few of the diseases which can affect fracture healing. Genetically influenced conditions like osteogenesis imperfecta, osteopetrosis, Marfan's syndrome affect the healing response to skeletal injury. Many hormones have been known to influence fracture healing, in either way.

Management of delayed and non-union of fractures.

Bone like few tissues in the body can restore to its original functions and morphology. The cells adjacent to the fracture regenerate and the mesenchymal cells participate in the generation of bony tissues. Numerous cytokines are released which exert influence on the healing process. The cells differentiate to form chondrocytes to osteoblast and osteocytes. Initially cartilage is formed which is replaced by lamellar bone. The process can be interrupted at any stage and here growth factors will be of tremendous use to restart the process of healing.

Apposition of fracture fragments, electrical stimulation, fracture stabilization and bone grafts are methods in use for delayed healing. In the last decade the use of growth factors has appeared on the scene in the management of bone healing.

Methodology

Fifty skeletally mature three-month-old rabbits were obtained. Under aseptic condition the rabbits were anesthetized with an intramuscular injection with 35 mg/kg of Ketamine mixed with Xylazine 5 mg/kg body weight. The eyes of the rabbits protected with Saline drops to prevent dryness. The right forelimbs of the animals were shaved by using an electric clipper. The area was scrubbed using hebiscrub and draped in a sterile fashion. A 2 centimeter incision was made over the ulna. The soft tissue were retracted and by using a standard point from the wrist joint a osteotomy was created. The wound was irrigated and closed by using 3/0 Dermilon. The limbs were bandaged. Prophylactic dose of intramuscular Zinacef was given at a dose of 25 mg/kg body weight. Analgesics were given as needed. From the third day onwards the study group and the control group were marked in different color codes. Group A received 2 mg/kg body weight of peptide , group B 2.5mg/kg, group C 3mg/kg and group D 3.5mg/kg body weight. The injections were made approximately in the region of the created osteotomy. Every week 2 rabbits of control and 2 from each group were x-rayed, then sacrificed and the forelimbs were reviewed and stored in 2 percent formalin. The procedure was carried out every week.
On the completion of the study the limbs of the animals were sent to Utah, USA for histopathological studies.

Results:

During the study period there were complications of any kind in all the animals. There were no wound infections nor there were any deaths recorded. All radiographs in the study group showed early healing while in the control group there was no indication of the healing process.

The histopathological studies indicated as follows:

VETERINARY PATHOLOGY

VR-02-1299

RABBIT FRACTURE STUDY

RESEARCH

MICROSCOPIC

CONTROL 1ST WEEK: This section of rabbit foreleg demonstrates a section of bone with areas of cartilage and bone proliferation occurring in all areas around

the bone and within the cortex. There are bone fragments in this collection and there are focal areas of bone organization. The bone organization is occurring along the periosteal surface and across the defect. Cartilage is proliferating on the periosteal surface in several areas and, in one or two

sites, is extending into some of the skeletal muscle tissue and connective tissue stroma. Again, the bone proliferation is periosteal and is 1-2 mm of bone on the external surface. There is good cartilage between the two bones at this site. Some evidence of fracture in both bones has occurred at this location. The osteoid proliferation is organized.

GROUP 1 - 1ST WEEK: This section of foreleg bone demonstrates bone fracture with evidence of organization of bone tissue within the cortex and on the periosteal surface. This bone organization at this site supports some evidence of displacement of the bone, but there is organization of bone on the periosteal surface and within the medullary cavity. There is minimal cartilaginous formation within this tissue. The bone formation is well organized, both on the periosteal surface and within the medullary cavity. No evidence of inflammation or any other specific alteration is identified. The bone organization is well demarcated in this tissue and appears to be appropriate at this site. The periosteal surface bone proliferation is within the same limits as described in the control.

GROUP 2 - 1ST WEEK: This section of bone demonstrates bone fracture with a

proliferation of bone on the periosteal surface, but this proliferation is not as extensively prominent over the surface. There is some degeneration around the bone tissue. Cartilage and bone are proliferating within the medullary cavity. The periosteal bone response appears to be less organized at this site than we have seen in other samples or in the control. There is evidence of fibroplasia in the periosteal surface and there are some hair shafts in the

tissue, as well. The hair shafts have been driven into the tissue with fibroplasia and osteoid and cartilage. The osteoid is periosteal in much of the tissue, although there is some medullary cortical bone and cartilage.

GROUP 3 - 1ST WEEK: This section of bone demonstrates bone fracture with cartilage, bone, and periosteal bone reaction. The cartilage and bone are growing within the lumen with organization of the bone tissue and organized bone and cartilage and periosteal tissue. Fibroplasia has occurred in some of the bone reaction. Degenerative change is part of the response. Bone fracture and alteration of the bone tissue can be identified in several sites and there is evidence of slight displacement in this bone tissue. The organization includes cartilage and bone, but with less cartilage than described in the control tissue. The bone appears to be undergoing organization at this site.

GROUP 4 - 1ST WEEK: This section of bone demonstrates an area of fracture with slight displacement of bone tissue and some evidence of degeneration in

the bone, but there are areas of cartilage and bone proliferation, both periosteal and within the medullary cavity. Fibrosis and fibrinous exudation are present. This bone repair is similar to what has been described with organization which appears to be appropriate. Very little periosteal bone, however, is identified in this repair. There is fragmentation of the bone tissue at this site. Other significant cellularity, such as infection, cannot be identified, but there is good organization with variations between the first weeks, but they all have some similarity to the organization. Group 1

has less cartilage than the other sites and the control appears to have more cartilage than the other sites.

CONTROL - 2ND WEEK: This section of control fracture site includes extensive cartilage growth between the fracture sites. The cartilage is growing along the medullary cavity and into the periosteal site with prominent cartilage in the periosteal tissue. There is some displacement. The cartilage is then undergoing ossification in irregular patterns, both in the periosteal surface and within the medullary cavity. The periosteal bone is quite well organized around this fracture site.

GROUP 1 - 2ND WEEK: This section of foreleg demonstrates a fracture site that is undergoing organization with bone. This organization includes periosteal bone proliferation with well organized bone within the cortex and medullary cavity. This tissue is almost all bone without cartilage. There is some periosteal fibrosis in the tissue. The organization is supporting good

aggregates of bone tissue in the surrounding periosteal tissue and within the medullary cavity and within the cortex. Inflammation or other significant change is not identified.

GROUP 2 - 2ND WEEK: This section of fracture demonstrates organization of bone within the cortical tissue and within the medullary cavity. There is periosteal bone reaction and some proliferation of bone tissue around this area in the periosteal region. The organization of the bone in this collection is very good, and particularly in the periosteal tissue it is extremely good. Minimal cartilage is present in the tissue in this collection.

GROUP 3 - 2ND WEEK: This section of bone tissue demonstrates a localized area of fibrosis between what appear to be the bone fracture with some amorphous material in the center of the bone tissue. There are heterophils with epithelioid cells in the medullary cavity. There is a periosteal bone reaction around the tissue with evidence of fibrosis and alteration of the bone tissue. This fracture is in a different location than the other bone fractures and difficult to compare. It demonstrates minimal tissue and no evidence of specific cartilaginous development or altered bone reaction. No other significant change is identified in this tissue.

GROUP 4 - 2ND WEEK: This section of bone demonstrates fracture with organization of the fracture site and evidence of periosteal bone proliferation and fibrosis. The periosteal reaction has minimal cartilage and

there is fibroplasia around the periosteal tissue. Alteration of the cortical bone is present. There is some evidence of remodeling, but it appears to be growing outwardly into the periosteal bone with osteoclasts and degenerate debris in those areas. The periosteal bone appears to be being remodeled in this site. Fibroplasia is prominent. Fibrosis is present over the surface. The bone proliferation over the cortical tissue supports minimal cartilage. Fibrosis is occurring in the surrounding tissue. No other significant alteration is identified.

CONTROL - 3RD WEEK: Examination of this site reveals that there is organization of the bone tissue along the fracture. Focal areas of cartilage are present and the bone is growing across the medullary cavity with some fragments into the medullary cavity. There is an extensive periosteal

reaction around the bone tissue with fibrosis at that site. The bone appears to be enlarged at this repair site. It is swollen with multiple aggregates of cartilage in this collection and some early osteoid. The fibrous connective tissue stroma is mainly limited to the periosteal site with bone across the fracture site.

GROUP 1 - 3RD WEEK: This fracture site is well organized with bone on the periosteal surface which is undergoing organization at this site. Minimal cartilage is present. There is some bone growing across the medullary cavity. There is fibrosis around the tissue. Periosteal fibrous connective tissue stromal response is part of the reaction and there are bone fractures in this tissue. Bone fragments are part of the collection. The bone is enlarged, but not as large as the control sample in this instance. The bone in the periosteal surface appears to be undergoing organization.

GROUP 2 - 3RD WEEK: Examination of this fracture site reveals that there is displacement of bone tissue in a rather enlarged nodule of bone with periosteal reaction and cartilage. This cartilage and bone is as extensive as that which we have seen in the control. The cortical tissue, however, appears to be somewhat displaced. There is organization, but there is more cartilage in the tissue at this point. Bone and cartilage are growing in the medullary cavity. The osteoid is extremely extensive over the periosteal bone in this particular site.

GROUP 3 - 3RD WEEK: No tissue submitted.

GROUP 4 - 3RD WEEK: This fracture site demonstrates organization of osteoid both from the periosteal surface and across the medullary cavity. This organization is demonstrating fracture of bone with a spicule growing across the medullary cavity. There is minimal cartilage in the periosteal surface with mild fibrosis. The osteoid proliferation is irregular throughout the tissue. Degenerative change is occurring secondarily. This type of fibrotic process is irregular. The bone proliferation is organized throughout the tissue.

CONTROL - 4TH WEEK: This section of bone tissue demonstrates very good organized bone with evidence of cartilaginous proliferation on the periosteal surface. There is also proliferation of osteoid into the medullary cavity. There is a good periosteal proliferation of bone, but cartilage is quite prominent throughout this collection. Other significant cellularity or change is not identified. The bone organization is somewhat prominent and appropriately organized on one cortical side, but not on the opposite side. There is more cartilage on the opposite side with some reaction to the opposite bone. Degenerative change is occurring secondarily. The cellularity is certainly working towards a normal bone reaction.

GROUP 1 - 4TH WEEK: This section of bone fracture demonstrates cartilage and osteoid which is growing irregularly in the periosteal surface. The cortical tissue is thinned and there is a fragment of cortical tissue within the

medullary cavity. The periosteal proliferation is well organized with

appropriate spicules and bone tissue. There is some inflammation in the surrounding muscular tissue and there is definite evidence of a suppurative process with abscessation and degeneration in the surrounding tissue. This causes concern about the possibility of past inflammation or infection in the surrounding soft tissue. The bone itself, however, appears to be undergoing organization and fracture repair with minimal cartilage.

GROUP 2 - 4TH WEEK: This fracture demonstrates some fibrosis and alteration, but evidence of nonhealing in the fracture tissue that can be evaluated. There appears to be fibrosis between the two fragments of bone with some periosteal response in this area. This junction does not appear to be healing across the bone tissue and there is minimal periosteal reaction in this location. There is little evidence of periosteal bone reaction or cartilaginous response.

There is a small area of fragmentation which also includes organization of bone across the medullary cavity. In this instance, however, there is minimal periosteal tissue and some suggestion of healing in this location. In fact, in this group 2 bone there is good organization of the cortical tissue. This has some fibroplasia around the tissue with a thickening of cortical bone right at the site where the organization has occurred. This organization includes good fibroplasia in the periosteal surface with a reorganization of the cortical bone and minimal evidence of poor organization at this site.

GROUP 3 - 4TH WEEK: This section of bone demonstrates well organized cortical bone with periosteal fibrosis. In one site there is evidence of bone growing into the medullary cavity and it may have been the previous cortex. The opposite cortical bone is well organized. No other significant change or degeneration is identified, but there is evidence of well organized cortical bone in this site. The cortical bone is surrounded by mature granulation tissue and fibrosis. No other significant cellular change is identified.

GROUP 4 - 4TH WEEK: This section of bone tissue demonstrates organization of cortical bone at the fracture with some fibrosis in the periosteal tissue. The opposite cortex appears to be well organized. There is little cartilage in this tissue. The periosteal bone is now undergoing organization to normal cortical bone. There is minimal evidence of organization into the medullary cavity. Significant other change or degenerative process cannot be identified and there is good evidence of healing at this site.

COMMENTS:

In evaluating these healing processes in the bone tissue, it is interesting that all of the control samples have an extensive cartilaginous response with less osteoid than the treatment samples. The only site that has as much cartilage as the control samples is Group 2 - 3rd week. All of the other sites demonstrate much more prominent osteoid in and around the periosteal surface and across the medullary cavity. The bone tissue is well organized in all of the treatment groups and I am having difficulty in identifying variations between the treatment groups, but there is definitely evidence of

more cartilage in the control fracture site as compared to the treatment groups. It was interesting that as we approach the 4th week, it became more and more difficult to identify the fracture site and the healing process. Cortical bone became much more appropriately modeled than we identified in the control or earlier collected samples in the treatment groups. Thus, to compare treatment groups to the control, there is certainly more cartilage in the control sites than in the treatment groups. The bone organization between the various sites was quite similar.

06/25/02

(LDM/jae) Verified by: L. D. McGill, D.V.M., Ph.D., DACVP
Veterinary Pathologist,
Animal Reference Pathology,
500 Chipeta Way, Salt Lake City,
Utah- 84108. USA

Discussion:

The present study demonstrates the effect of the SHMSP on rapid healing of the osteotomy site in rabbit's ulna. SHMSP is a 13- aminoacid polypeptide discovered and synthesized by standard Fmoc chemistry. The fractures sites in the study group healed faster than the control group. Moreover the histopathological studies indicate abundant osteoid as compared to the control group at the same period of time. This indicates that the peptide has taken the direct bone formation pathway (Intramembranous), the other being the intermediate cartilage pathway (enchondral). In normal situation the fracture healing takes the second pathway. One can envisage that by the first pathway time is saved as from the beginning the fracture heals osteoid rather cartilage. Reddi [45,46,47] confirmed that BMPs induces differentiation of mesenchymal cells to firstly to cartilage and then to osteoid. Experimental studies have shown three phases of BMP induction of fracture healing; chemotaxis and mitosis of mesenchymal cells, differentiation of mesenchymal cells first into cartilage which continues for 7-9 days and later the phase progresses to osteogenesis[48]. SHMSP is different from BMP in two ways; structurally it is quite different from BMP as it contains 13- aminoacids with molecular weight of less than 2000n where as BMP has more than 200 aminoacids and secondly SHMSP skips the induction of cartilage and progresses to osteoid production . It is speculated that the peptide has stimulated the mesenchymal cells at the fractured tissue which in this situation is bone by producing enormous amount of osteoid. In the control group the healing proceeded in a normal way of the mesenchymal cells stimulating the chondrocytes . BMPs as described is a family of proteins which has osteogenic properties.

Fourteen such proteins have been identified BMP2 - BMP15, some has been purified, cloned and sequenced. Out of these fourteen only two have been proven as capable of bone induction. BMPs are known to function differently from each other, but principally they all induce chondrogenesis, this is in contrast to SHMSP where from the start osteogenesis is observed. SHMSP also has been purified, sequenced and synthetically manufactured for use in animal models and human trials are awaited. Growth factors such as TGF-β was used in the healing of fractures in experimental animals. Joyce et al (1990)[49] injected the TGF-β in the subperiosteal region of the fractured femur in the rats but it ended in the production of chondrogenesis rather than osteogenesis. There is a view that a combination of the growth factors used will have more potential affect than the individual affects[50]. Whether the combination of the growth factors or single out factors, they play a definite role in the fracture healing. The therapeutic implications of the growth factors need to be clearly defined. Their dosage, complications and immunological reactions need to be further clarified. In the present economic times the cost of production of the growth factors and the rationale of their use is to be ascertained.

The dosage of BMPs which needed the effect of osteogenesis is reported to be different in different studies. Recently Govender et al (2002)[51] used a total of 12mg per patient in case of fracture tibia in human trials. In this study in an arbitrary manner the dose of peptide was started from 2.mg/kg body weight was given which amounts to 18 mg for the four week period. Valentin-Opran et al[30] believes that the dose of growth factors based on weight or surface area is not suitable but the release of the

factor through a matrix is a better option. As there is no generalized reaction to the injections, local release with supplementation with local injections may be appropriate In badly comminuted fractures.

Eventhough there were no complications nor any deaths in this study, toxicology studies needed to be completed before any human trials are envisaged. Once such studies are completed human trials need to be undertaken. In conclusion, this study demonstrated that the SHMSP accelerated fracture healing by the production of the osteoid. Further tests are needed in the human trails to show the efficacy of the peptide and secondly to develop a single dose application of the peptide using absorbable collagen sponge.

Acknowledgement

The authors sincerely thank the Deanship of the Scientific Research, King Faisal University, Al-Hufoof for the grant, without which this study could not have been carried out.

REFERENCES:

1. Karladarie AH, Grandhed H, Edshage B, Jerre R, Styf J, Displaced tibial shaft fractures: A prospective randomized study of closed intermedullary nailing versus cast treatment in 53 patients. Acta Orthop Sacn 2000; 71: 160-67.

2. Young BT, Rayan GM. Outcome following non-operative treatment of displaced radius fractures in low-demand patients older than 60 years. J Hand Surg (AM) 2000; 25: 19-28.

3. Einhorn TA. Enhancement of fracture-healing. J Bone Joint Surg 1995; 77: 940-56.

4. Heckman JD, Sarasohn-Kahn J. The economics of treating tibia fractures. The cast of delayed unions. Bull Hosp joint Dis 1997 56: 63-72.

5. Marsh D. Concepts of fracture union, delayed union and non-union. Clin Orthop. 1998; (355 Suppl): S 22-30.

6. Allen WC, Pitrowski G, Burstein AH, Frankel VH. Biomechanical principles of intramedullary fixation. Clin Orthop 1968; 60:13-20.

7. Allgower M, Speigel P: Internal fixation of fractures: Evolution of concepts. Clin Orthop 1979; 138: 26-29.

8. Ryaby JT. Clinical effects of electromagnetic and electric fields on fracture healing. Clin Orthop 1998; (335 Suppl): S 205-15.

9. Claes LE, Helgele CA, Neidlinger-Wilke C, Kasper D, Seidl W, Margevicius KJ, Augat P. Effect of mechanical factors on the fracture healing process. Clin Orthop 1998; (335 Suppl) S 132-47.

10. Hadjiargyron M, McLeod K, Ryaby JP, Rubin C. Enhancement of fracture healing by low intensity ultrasound. Clin Orthop 1998; (355 Suppl) S 216-29.

11. Senn N: On the healing of aseptic bone cavities by implantation of antiseptic decalcified bone. Am J Med Sci 1889; 98: 219-40.

12. Urist MR. Bone: Formation by autoinduction. Science 1965; 150:893-99.

13. Leriche R, Policard R: The normal and pathological physiology of bone. London, Klimpton 1927.

14. Orell S : Studien uber knoctenimplantation und knochenneubildung. Acta Chir Scan 1934; 74 (Suppl): 1-31.

15.Lavender G. A study of bone regeneration. Surg Gynecol Obstet 1938; 67: 705-48

16.Bolander ME. Regulation of fracture repair by growth factors.
Proc Soc Exp β10l Med 1992; 200: 165-70.

17.Assorian RK. Purification of type-β. Transforming growth factor from human platelets. Methods Enzymol 1987; 146; 153-63.

18.Barnard JA, Lyons RM, Moses HL. The cell biology of transforming growth factor-β.. Biochem Brophys. Acta 1990; 1032: 79-87.

19.Centrella M, McCarthy TL, Canalis E. Transforming factor-β and remodeling of bone. J Bone Joint Surg 1991; 73-A: 1418-28.

20. Cook SD, Boffes GC, Wolf 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: 302-12.

21. Hock JM, Canalis E. Platelet-derived growth factor enhances bone cell replication but dot differentiated function of osteoblasts. Endocrinology 1994; 134: 1423-28.

22. Einhorn TA. The cell and molecular biology of fracture healing..
Clin Orthop 1998; 355(Suppl): 7-21.

23. Wozeny JM. The bone morphogenetic protein family. Multifunactional cellular regulators in the embryo and adult. Eur J Oral Sci 1998; 106 (Suppl 1): 160-66.

24. Wozney JM, Rosen V, Byrne M et al. Growth factors influencing bone development. J Cell Sci 1990; 13 (Suppl): 149-56.

25. Reddi AH. Initiation of fracture repair by bone morphogenetic proteins.
Clin Orthop 1998; 355(Suppl): 66-72.

26.Wang EA, Rosen V, Cordes P et al: Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci USA 1988; 85: 9484-88.

27.Yabu M, Takaoka K, Hashimoto J et al. Ultramicroscopic aspect of the conversion of fibroblasts to chondrocytes in the mouse dorsal subfusion induced by bone morphogenetic protein (BMP). Arch Histol Cyto 1991; 54: 95-102.

28. Rengachary SS: Bone morphogenetic proteins: basic concepts.
Neurosurg Focus 2002; 13(6):1-6.

29. Johnson EE, Urist MR, Finerman GA. Resistant non-unions and partial and complete segmental defects of long bones. Treatment with implants of a composite human bone morphogenetic proteins (BMP).
Clin Orthop 1992; 277: 229-37.

30. Valentin-Opran A, Wozney J, Csimma C, Riedel GE: Clinical evaluation of recombinant human morphogenetic protein-2.
Clin Orthop 2002; 395:110-20.

31. McKee MD. The effect of human recombinant bone morphogeneic protein (rhBMP-7) on the healing of open tibial shaft fractures: Results of a multi-centre, prospective, randomized clinical trial. Presnted at the AAOS Meeting at New Orleans, 2003.

32.Cooper AR. Treatise on dislocations and on fractures of the joints: Fracture of the neck of the thigh bone. Sir Astley Cooper, Bart-FRCS, Surgeon to the King.
Clin Orthop 1973; 92: 3-5.

33.Alffframm PA, Bauer GCH. Epidemiology of fractures of the forearm: A Biochemical investigation of bone strength.
J Bone Joint Surg 1962; 44-A: 105-14.

34.Singer BR, McLauchlan GJ, Robinson CM, Christie J. Epedemiology of fractures in 15 000 adults. The influence of age and gender.
J Bone Joint Surg 1998; 80-B: 243-48.

35.Bouxsein ML, Turek TJ, Blake BS, D' Auguta, Stevens M, Seeherman HJ, Wozney JM. Recombenant Human Bone Morhogenetic Protein-2 Accelerates Healing in a Rabbit Ulnar Osteotomy Model.
J Bone Joint Surg. 2001; 83-A: 1219-1230.

36.National Center for Health Statistics 1985. Summary: National hospital discharge Survey: Advance data from vital and health statistics No 127, DHDH Pub. No (DHS) 86-1250, Public Health Services, Hyateville, MD. September 25th 1986.

37. Cummings SR: Epidemiology of osteoporotic fractures. In osteoporosis update 1987. Ed. Genant 1987; 7-12.

38. Ashhurst DE: The influence of mechanical conditions on the healing of experimental fractures in the rabbit. A microscopical study. Phil Trans R Soc London 1986; 313: 271.
39. McKibbin B: The biology of fracture healing in long bones.
J Bone Joint Surg 1978; 60B: 150.

40. Sandberg MM, ARO HT, Vuorio EI. Gene expression during bone repair.
Clin Orthop 1993; 289: 292-312.

41.Trippel SB, Coutts RD, Einhorn TA, Mundy GR, Rosenfeld RG. Growth factors as therapeutic agents. J Bone Joint Surg 1996; 78-A: 1272-86.

42. Jonhson GL, Vaillancourt RR. Sequential protein kinase reactions controlling cell growth and differentiation. Curr opin. Cell boil, 1994; 6: 230-38.

43.Hedlung R, Lindgren U. Epidemiology of diaphyseal femoral fractures.
Acta Orthop Scan 1986; 57: 423-27.

44. Meisinger C, Wildner M, Stieber J, Heies M, Sangha O, Doring A. Epidemiology of limb fractures. Orthopade 2002; 31 (1): 92-9.

45. Reddi AH: Cartilage morphogenesis : Role of bone and cartilage morphogenetic proteins, homeobox genes and extracellular matrix.
Matrix Biol 1994; 14: 599-606, 1994.

46.Reddi AH. BMP-1: Resurrection of procollagen C- Proteinase.
Science 1996; 271-463.

47. Reddi AH. Bone morphogenetic proteins: An unconventional approach to isolation of first mammalian morphogens.
Cytokine Growth Factor Rev 1997; 8: 11-20.

48. Reddi AH, Anderson WA. Collagenous bone matrix-induced enchondral ossification hemopoiesis. J Cell Biol 1976; 69: 557-72.

49. Joyce ME, Roberts AB, Sporn MB, Bolander ME. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur.
J Cell Biol 1990a; 110: 2195-207.

50. Pfeilschifter J, Oechsner M, Naumann A, Gronwald RG, Maine HW, Ziegler R. Stimulation of bone matrix apposition in vitro by local growth factors: A comparison between insulin-like growth factor I, platelet-derived growth factor and transforming growth factor beta. Endocrinology 1990; 127: 69-75.

51. Govender S, Csimma C, Genant HK, Valentin-Opran A. Reombinant Human Bone Morphogenetic Protein-2 for the treatment of open tibial fractures.
J Bone and Joint Surg 2002; 84-A(12): 2123-34.