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Fracture healing : the effects of local insulin delivery via calcium sulfate and tricalcium phosphate

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ABSTRACT

FRACTURE HEALING: THE EFFECTS OF LOCAL INSULIN DELIVERY VIA CALCIUM SULFATE AND TRICALCIUM PHOSPHATE

by
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Previous studies have documented the major role played by insulin in osseous healing. This is the first study to examine local intramedullary insulin delivery to the fracture site and its effect upon the normal fracture healing process. Preceding results show that when administered at the fracture site of the impaired fracture healing model of the diabetic BB Wistar rat, insulin will regulate early cellular proliferation and chondrogenesis and late mineralized tissue, cartilage content and mechanical strength.

In this study, two novel delivery vehicles have been evaluated for sustained insulin release in the normal fracture model of the BB Wistar rat. Calcium sulfate and tri-calcium phosphate have osteoconductive properties that support bone growth. These materials served as carriers to provide continuous insulin release at the fracture site. The vehicles were evaluated for insulin content at days 2, 4 and 7 and normalized to the total systemic protein content of the animal. Calcium sulfate shows an early burst of insulin release and sustained amounts of insulin throughout the 7 day study. The local treatment of insulin does not affect the animals' systemic insulin levels. Histomorphometric analysis shows a significant difference in new bone content in the fracture calluses that received insulin treatment as compared to control groups. The study concludes that calcium sulfate could be a promising vehicle for local insulin delivery and improvement of the fracture healing process in healthy patients.

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by
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In time of test, family is best.
-Burmese Proverb

Family: A social unit where the father is concerned with parking space, the children with outer space, and the mother with closet space.
-Evan Esar

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INTRODUCTION

1.1 Objective

The following study is an initial attempt to examine the efficacy of local insulin treatment and its effects upon normal fracture healing parameters. Two novel biocompatible vehicles will be used for the intramedullary delivery of insulin to a fracture site. Studies have shown the critical role that insulin plays in fracture healing. This study will examine the ability of both calcium sulfate and tricalcium phosphate to serve as vehicles for continuous release of insulin to the fracture site from day 0-7, the inflammatory and early proliferative phase. We hypothesize that these osteoinductive biomaterials will sustain a continuous release of insulin.

1.2 Fracture Healing

Vascularization and intricate cellular architecture enables bone to remodel and regenerate throughout an individual's lifetime. The complex process of fracture healing occurs in several phases. The early phase of cellular proliferation is initiated by bleeding at the fracture site. Activated platelets trigger an influx of inflammatory cells. The platelets secrete signaling factors which lead fibroblasts, endothelial cells and osteoblasts into the fracture gap. This is the onset of the proliferative phase during which osteoprogenitor and undifferentiated mesenchymal cells migrate to the fracture site. These cells then proliferate and ultimately differentiate through either osteogenic or chondrogenic pathways. The proliferative phase is critical to successful fracture callus formation. The

osteoblasts at the site produce intramembranous bone while chondroblasts temporarily stabilize the fracture through a cartilaginous intermediate. Endochondral ossification then mineralizes the intermediate cartilage into woven boned. The final phase of fracture healing is remodeling. Once remodeling is complete, the new tissue has similar mechanical and structural properties as the original bone. (Gebauer, 2002)

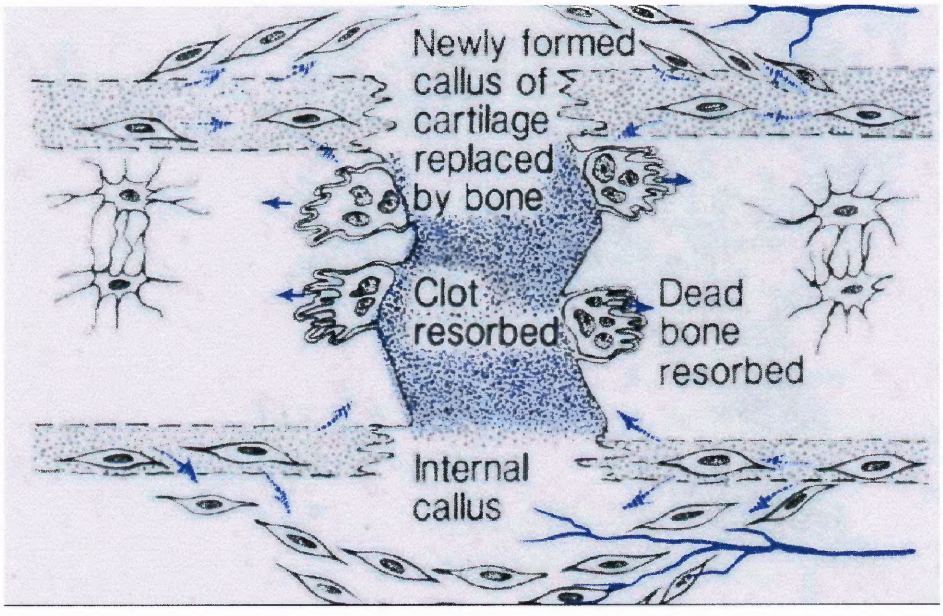

Figure 1.1 Proliferation phase of fracture healing.
(Source: Li et al. 1999)

1.3 Insulin and its Effect on Fracture Healing

Although this study is among the first to examine insulin treatment in normal fracture healing, it is not the first time that insulin has been used to promote bone healing. Insulin has been shown to reverse the complications found between fracture healing and Type I diabetes mellitus (DM). Type I DM) is an autoimmune disease that disrupts the body's ability to produce insulin and therefore regulate glucose levels. DM is associated with a plethora of systemic complications including fracture healing. Clinical and experimental

documentation has shown a relationship between diabetes and impaired osseous healing (Macey, 1989; Loder, 1988; Herbsman, 1968; Funk, 2000). Several clinical studies have documented that the healing time for diabetic patients is approximately twice the length of non-diabetic patients (Cozen, 1972; Loder, 1988). Animal model experimentation suggests that the rate of cellular proliferation during fracture healing is drastically reduced in the spontaneously diabetic BB Wistar rat as compared to the healthy BB Wistar rats (Tyndall, 2003).

A demonstrable reduction in cellular proliferation is seen in the diabetic fracture callus (Macey, 1989), including a decrease in collagen synthesis during the early stage of fracture healing (Spanheimer, 1992; Topping, 1994). Although the cause of these effects remains unknown, insulin has been shown to play a major role in repairing these deleterious results. Insulin receptors have been identified in rat osteoblastic cells (Levy, 1986) an in vitro studies show that insulin stimulates nucleotide synthesis of osteoblasts (Peck, 1970), proliferation of osteoblastic cells (Weiss, 1980) and the production of collagen in fetal rat calvariae (Kream, 1985). Alternatively, Fiorelli et al. (1994) demonstrated delayed cartilage formation and reduced ossification in an in vitro study of an insulin deficient environment.

The UMDNJ Orthopaedic laboratory has used an in vivo model to investigate insulin's effect on fracture healing in diabetes mellitus (DM). The DM BB Wistar rat develops diabetes through a selective, autoimmune destruction of the pancreatic β-cells. These animals have both genetic and immune physiologic factors comparable to type I DM seen in humans (Beam, 2002). Beam et al. (2002) documented improved fracture healing parameters in DM BB Wistar rats that received insulin treatment sufficient to

achieve physiologic blood glucose control. However, that study did not isolate the effects of insulin upon bone healing. Using the same in vivo model, Gandhi et al. (2005) showed that local delivery of insulin at the fracture site reversed DM's effect on fracture healing. The results show that local insulin delivery mediates early and late fracturing healing parameters. In the early phase of fracture healing, days 0-7, the diabetic group treated with intramedullary insulin at the fracture site showed a 60% increase in periosteal and gap callus proliferation when compared to the diabetic group that did not receive treatment. Gandhi et al. (2005) also showed that the local delivery of insulin did not affect the animals' systemic blood glucose levels. This would indicate that intramedullary insulin treatment would not affect the blood glucose levels of a non-diabetic animal.

Studies conducted by Weiss and Reddi (1980) conclude that the timing of incorporation of insulin to the fracture site is also important for proper healing. When insulin is incorporated during proliferation and chondrocyte differentiation (day 0-7), researchers found that cell proliferation in the diabetic animals increased to 81% that of the control group. The data suggests that the absence of insulin during the proliferative phase of healing leads to a decrease in cell proliferation and therefore a decrease in chondrogenesis. When insulin is administered from days 4-11, mineralization measurements in the diabetic animals is comparable to that of the control group. Researchers conclude that insulin is necessary for proliferation, chondrogenesis, osteogenesis and mineralization.

In an effort to understand the mechanism by which insulin affects diabetes related impaired bone formation, a marrow ablation model was studied (Lu et al. 2003). The bone ablation model mimics intramembranous bone formation. The effect that diabetes has on bone formation was substantially reversed with insulin treatment. Insulin reversed the inhibitory effects that diabetes was found to have on the expression of bone matrix osteocalcin and collagen type I. The results show that diabetic animals do produce adequate amounts of immature mesenchymal tissue but without the insulin treatment, the genes that regulate osteoblast differentiation are not adequately expressed.

These studies indicate the ability of insulin to normalize the effect of diabetes on fracture healing. They also show the important role that insulin plays in both the early and late parameters of fracture healing. Insulin's therapeutic effect may accelerate both diabetic and non-diabetic bone healing.

1.4 Ceramics as Bone Graft Substitutes

Ceramics are highly crystalline materials formed by heating non-metallic mineral salts to high temperature in a process called sintering. The most commonly utilized ceramics in orthopaedics consist of calcium phosphate and calcium sulfate based ceramics.

The porous nature of these compounds provide an osteoconductive scaffold to which chemotactic factors, circulating proteins (i.e. osteoinductive growth factors) and cells (i.e., mesenchymal stem cells) can migrate and adhere. This scaffold provides critical structure for the progenitor cells to differentiate into functioning osteoblasts. In addition to being biocompatible and bio-resorbable, the crystalline structure of these ceramics can be manufactured so that they are very similar to natural bone. The availability of these

substitutes and the absence of complications that can occur in allograft-induced immunogenic response or disease conveyance, provide an increasing incentive for their use. In addition, the surgical complications of retrieving bone from an autologous donor site are avoided (Arrington, 1996).

1.4.1 General Properties of Bone Substitute Ceramics

Physical properties such as pore size and porosity are critical factors of synthetic bone graft substitutes. In order for new bone to form as the bone graft is resorbed, blood vessels must be able to develop in the graft substitute. The pore size of the bone graft must be large enough to allow this vascular in-growth. Studies have determined that vascular ingrowth does not occur with pore size smaller than 15-40 microns. Osteoid tissue forms on bone graft substitutes with pore sizes greater than 100 microns and it was found that a pore size of 300-500 microns provides ideal vessel and tissue growth. At one time the pore size was considered to be a critical factor for bone formation with in these synthetic bone graft substitutes (Kuhne, 1994), but it is now thought to be porosity, or the interconnectivity of pores, that is critical to the success of the implant (Eggli, 1988). In the absence of adequate interconnectivity, or low porosity, there is low oxygen tension at the pore apex. The relatively poor oxygen tension hampers the differentiation of mesenchymal cells towards osteoblast cell lineage, and the cells rather differentiate into fibrous tissue, cartilage or fat (Nakahara, 1992).

In vivo degradation of these cements has been another area of active research focused on making the degradation rate more predictable and closer to rate of new bone formation. Ideally, a bone graft substitute is expected to resorb as new bone is synthesized and remodeled. If the rate of resorption is faster than the rate at which new bone can be laid down, a collapse of structural integrity will occur. On the other hand if the rate of resorption is slower, impairment of new bone formation will occur (Klein, 1983).

1.4.2 Calcium Sulfate Cement

Calcium sulfate (CaSO4) has been widely used in both medicine and dentistry for bone regeneration as a graft material and as a barrier in guided tissue regeneration. This biocompatible material is completely resorbed following implantation. Calcium sulfate does not evoke a significant immune response. It creates a calcium-rich environment in the area of implantation which is believed to provide stimulation to osteoblasts. The raw source of calcium sulfate is abundant and inexpensive. Despite these advantages, calcium sulfate has never attracted the same degree of research interest as have other biomaterials. Recently, it has been applied to the areas of periodontology, sinus augmentation, and orthopedic surgery. In order for calcium sulfate to become a more widely used biomaterial, research should be directed toward maintaining the biocompatibility of the material while improving its handling characteristics and strength (Thomas et al. 2005).

Peltier did the significant early work on calcium sulfate in bone healing and first described his experience in a preliminary report in 1959. Later, in 1978 Peltier and Jones reported their long term follow up results on 26 unicarmel bone cyst of whom 24 healed without complications.

Calcium sulfate as bone graft substitute is available in two chemical forms: Calcium sulfate hemihydrate (plaster of paris) and calcium sulfate dihydrate (gypsum). The latter form, produced after hydration of the hemihydrate form, is chemically stable and is available in solid shapes like pellets and blocks. The hemihydrate formulation when mixed with diluent (water, saline or other liquids) undergoes hydration reaction and forms a putty/paste thereby getting converted into dihydrate form. In this putty form the calcium sulfate is injectable till it sets in as solid calcium dihyrate (gypsum). The dihydrate form can be converted back to hemihydrate form by heating it to temperatures above 100°C, a process known as calcination. Depending upon the rate of heating, use of pressure while heating and addition of chemical additives during calcination the resulting hemihydrate forms have different crystal structure and dissolution properties. Presence of impurities like fluorine in the lattice of crystals can change the crystalline structure and alter the biological properties of the calcium sulfate. Special care must be maintained when processing calcium sulfate material in order to produce surgical grade calcium sulfate with a predictable resorption rate and optimal crystalline structure to provide osteoconductive medium for new bone growth.

Calcium sulfate resorption mechanism is not well understood but it appears that resorption occurs by dissolution into surrounding body fluids rather than active degradation by cellular mechanism (Bucholz, 2002; Pietrzak, 2000). Recently it has

been suggested the calcium sulfate may not actually be osteoconductive and that new bone formation occurs as the cement dissolves, acting perhaps just as a bone void filler (Damien, 1991). The resorption of calcium sulfate in vivo is rapid and thus it is not suitable for clinical situations where cement is required to provide structural support. If used alone, it is useful for contained nonstructural defects or as an adjunct to internal fixation devices to improve their holding strength in bone. Calcium sulfate can also be used as a carrier for growth factors and bone morphogenic proteins in appropriate clinical applications. Calcium sulfate's use as a delivery vehicle for growth factors and antibiotics has not been extensively explored in the clinical setting.

1.4.3 Tricalcium Phosphate Cement

Tricalcium phosphate (TCP) is a porous, ceramic biomaterial that supports bone growth. Its use as a bone substitute has been well documented. In a study conducted by Rejda et al. (1977), TCP was used as a ceramic implant. The implant caused a minimal foreign body reaction, while osteoclastic activity was shown to be involved in biodegradation. Newly formed bone mineral was deposited directly onto the surface of the ceramic implant.

Chouteau et al. (2003) examined the osteoconductive effects of porous calcium phosphates, including TCP. Their work assessed the in vitro interaction of osteoblast and fibroblast cultures on macroporous calcium-phosphate bone substitutes to analyze the interaction between cells and bone substitute. Both osteoblast and fibroblast cellular growth was exponential on the bone substitute. Cells spread on the surface of the material covering macropores and colonizing the depth of the discs. The study concluded that

even though the bone substitute was osteoconductive, the results could be improved if osteoinductive compounds, such as growth factors or osteogenic cells, were incorporated into the ceramic.

Calcium phosphate exists in three basic ionic combinations with phosphate- Tribasic (Tricalcium Phosphate), Dibasic (Secondary calcium Phosphate) and Monobasic. Of these three forms the tri-calcium phosphate (TCP) form is most commonly used in manufacturing calcium phosphate cements. The chemical composition of tricalcium phosphate (TCP) is $(Ca_3(PO_4)_2$. TCP is an amorphous, odorless, tasteless powder which is insoluble in water, alcohol or acetic acid and soluble in dilute HCl or HNO_3. The calcium to phosphate (Ca:P) molar ratio of TCP is 1.5. TCP is available in two forms- alpha and beta TCP. Both are high temperature TCPs with a chemical composition similar to amorphous TCP but are more crystalline than the latter (Termine et al. 1970). Alpha TCP is more soluble than beta TCP and is a major component of calcium phosphate cements.

The injectable preparation of calcium phosphate cement is prepared by mixing a range of calcium phosphates with an aqueous solution. The resulting paste hardens to form calcium phosphate apatite of low crystalline order and small crystal size similar to the mineral phase of natural bone.

Brown and Chow (1983) prepared the first calcium phosphate cement that could be constituted at room temperature by using equimolar mixture of tetracalcium phosphate and calcium hydrogen phosphate. Initially, dicalcium phosphate dihydrate is formed with a plate-like morphology which later yields calcium deficient hydroxyapatite. The reaction is isothermal and avoids cell and tissue damage that would normally occur from

the heat of the reaction. All modern formulations of cements are set by endothermic reactions rather than exothermic reactions thereby limiting the potential for local tissue damage.

Hardening of the cement occurs mostly within the first six hours, yielding an 80% conversion to hydroxyapatite and a compressive strength of 50-60 MPa. Hardening can be accelerated with phosphate solution, sodium fluoride, and sodium hydrogen phosphate. Inclusion of porosity, with the aim to improve the osteoconductivity, can be introduced by the addition of soluble inclusions such as sucrose, sodium hydrogen carbonate, sodium hydrogen phosphate (Takagi. 2001). The low temperature of formation and inherent porosity also permits the addition of antibiotics, or growth factors that stimulate the differentiation of pre-osteoblastic cells.

Because of composition similar to natural bone apatite, the calcium phosphate apatite cements undergo more biological degradation compared to calcium sulfate. Experimental studies on animals have shown that after implantation the cement gets covered by numerous multinucleated osteoclast like cells which begin to break down the bone substitute as new bone formation begins. New bone formation by osteoblasts progresses centripetally along the scaffold provided by the apatite cements (Welch, 2003; Wiltfang, 2002; Sarkar, 2001). The average time of resorption of cement would depend on many factors like composition of cement, site of implantation, patients' metabolic rate and general health. Difficulty exists in comparing individual studies since every study has its own protocol of obtaining specimens for observing the degradation process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal Model

Male BB Wistar rats were obtained from the breeding colony established at the UMDNJ-New Jersey Medical School Research Animal Facility derived originally from Health Canada Animal Research Division (Ottawa, Canada). The animals were housed under controlled environmental conditions and fed ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at UMDNJ-New Jersey Medical School.

The animals were split into two experimental groups. One group received 0.1ml of calcium sulfate hemihydrate mixed with insulin and injected into the intrmedullary canal and the other received 0.1ml of tri-calcium phosphate mixed with insulin and injected into the intramedullary canal. Blood glucose levels (ACCU-CHEK Advantage, Roche Diagnostics, Indianapolis, IN.) were checked on post surgery days 1 and 3 from blood obtained from the tail vein of the animal.

2.2 Surgical Procedure

The animal is administered general anesthesia through intraperitoneal injection of Ketamine (60mg/kg) and Xylazine (8mg/kg). Once sedated, the animal is shaved and prepped with betadine and 70% alcohol.

A closed mid-diaphyseal fracture was created in the right femur of the animal using the following modification of the methods described by Bonnarens and Einhorn. A

medial parapatellar incision is made and the patella is dislocated laterally. Once exposed, an entry hole is created with an 18 gauge needle at the interchondylar notch of the distal femur. The femur is then reamed. The carrier is then place inside of the intremedullary canal. A 1mm Kirschner wire is then placed in the canal. The wire is secured into the proximal end of the femur to assure stability. The wound was then irrigated and closed with a 4-0 vicryl suture. The Einhorn fracture machine was then used to create a unilateral, closed midshaft fracture. X-rays were then taken to determine whether the fracture was of acceptable configuration. Animals that exhibit non-comminuted, transverse fractures confirmed by radiograph are used in this study. Post-fracture, the animals are permitted to ambulate freely.

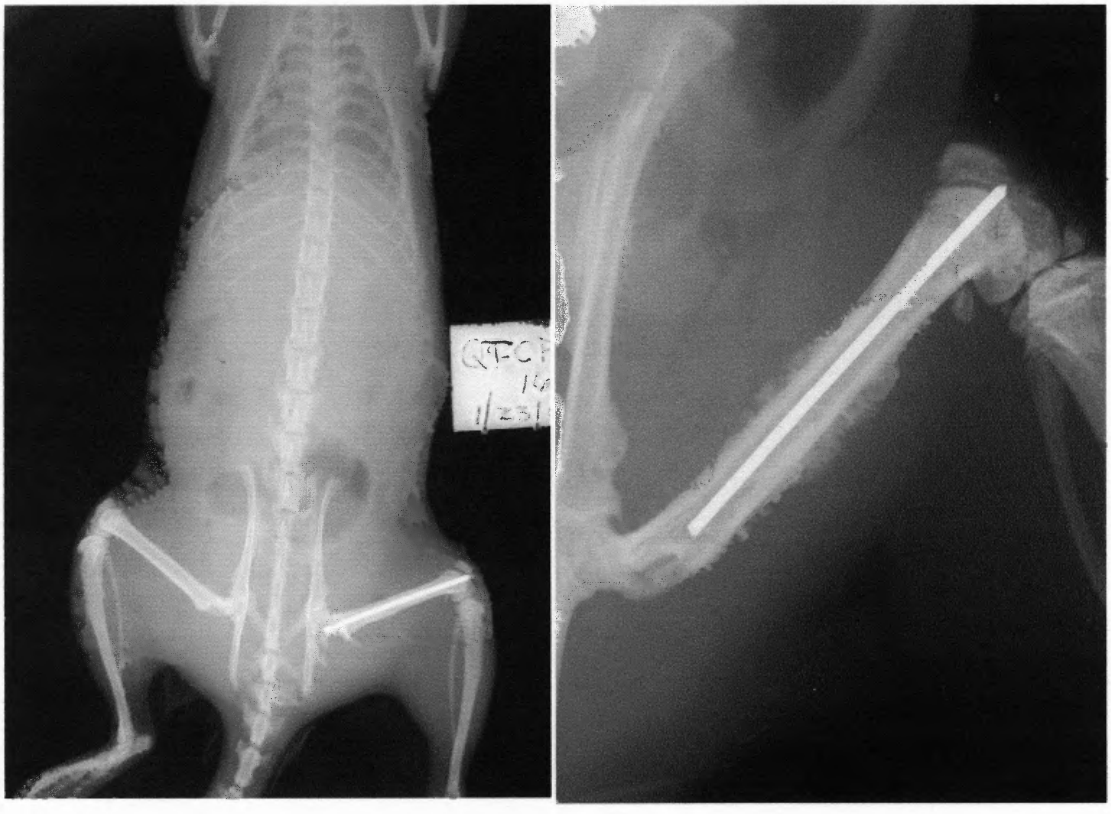

Figure 2.1 Radiograph of closed fracture.
A closed mid-diaphyseal fracture stabilized by a 1mm Kirschner wire.

2.3 Delivery System

Insulin was delivered to the fracture site using the carriers tri-calcium phosphate and calcium sulfate. The carrier materials were first sterilized in glass vials in a convection oven at 196°C for 6 hours. During the surgery, 0.8 grams of each carrier was mixed with 0.4ml of ultralente human insulin. Prior to the insertion of the Kirschner wire, 0.1ml of the mixture was injected into the intramedullary canal of the right femur of the animal.

2.4 Insulin Quantification

The study groups were assigned to days 2, 4 and 7. At the time of sacrifice, blood from each animal was collected by cardiac puncture and used to determine the systemic insulin level of the animal. The blood samples were centrifuged at 10,000 rpm for 10 minutes. The plasma was then collected and stored at -80°C until it was used for testing. The plasma was used to normalize the local insulin levels within the fracture to the total systemic protein concentration of the animal using bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

Local insulin levels were measured in both the fracture and contralateral femora in each study group. At the time of sacrifice, both femora of the animal were resected and the fracture callus and mid-diaphyseal region corresponding to location of the fracture callus on the contralateral femur were isolated. The callus and corresponding diaphysis were then flash frozen in liquid nitrogen. The frozen samples were then pulverized and total protein content was extracted using mammalian protein extraction reagent (MPER®) (Pierce, Rockford, IL). The extracted protein is then stored in -80°C until testing.

Insulin quantification of the samples was performed using a rat/mouse specific insulin ELIZA kit (EZRMI-13K, Linco Research, Inc., St. Charles, MO). The assay and analysis were performed in accordance with the manufacturer's instructions. This specific kit was chosen because of its ability to detect both rat insulin and human insulin, allowing for the contralateral leg of the animal to serve as the control. The kit was also able detect insulin in both plasma samples and serum samples made from the callus and corresponding mid-diaphyseal region of the control leg. This enabled the standardization of detection of both local and systemic insulin levels in the animal.

2.5 Histomorphometry

To compare the effect of calcium sulfate alone and calcium sulfate combined with insulin on chondrogenesis and osteogenesis in the fracture callus, histomorphometric analysis was performed. The animals were separated into two groups. Both groups underwent the fracture model described in Section 2.2. The control group received 0.1ml of calcium sulfate mixed with 0.9% saline solution. The experimental group received 0.1ml of calcium sulfate mixed with ultralente human insulin. The mixtures were injected into the intramedullary canal of the right femur prior to fracture.

The fractured femora were resected at days 4, 7 and 10. The samples were fixed with formalin, decalcified, embedded and sectioned using standard histological techniques. To identify cartilage formation, the sections were stained with Weigerts iron hematoxylin, biebrich scarlet and aniline blue using Masson's Trichrome Staining technique. Mineralized tissue appears red-blue and cartilage appears dark blue.

The areas of cartilage formation as a percentage of total fracture callus were determined through histomorphometric analysis using Scion Image software (Scion Corp, Frederick, MD). The fracture callus is defined as the region located on either side of the cortices, external to the intramedullary marrow cavity. Digital photomicrographs of the sections were taken using an Olympus BHZ-RFCA microscope (Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo, Japan).

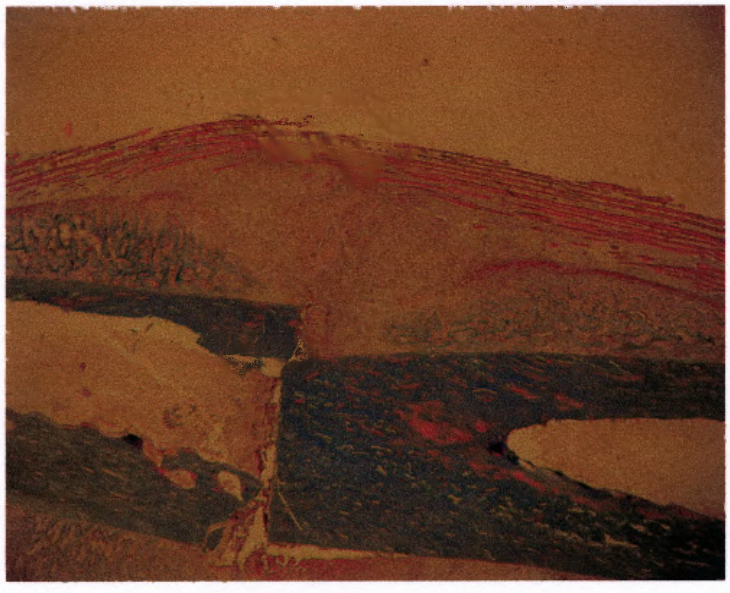

Figure 2.2 Early fracture callus histology.
Slides were stained with Weigerts iron hematoxylin, biebrich scarlet and aniline blue according to Masson's Trichrome staining protocol. Muscle tissue appears red, bone tissue appears dark blue, cartilage appears light blue and nuclei appear black.

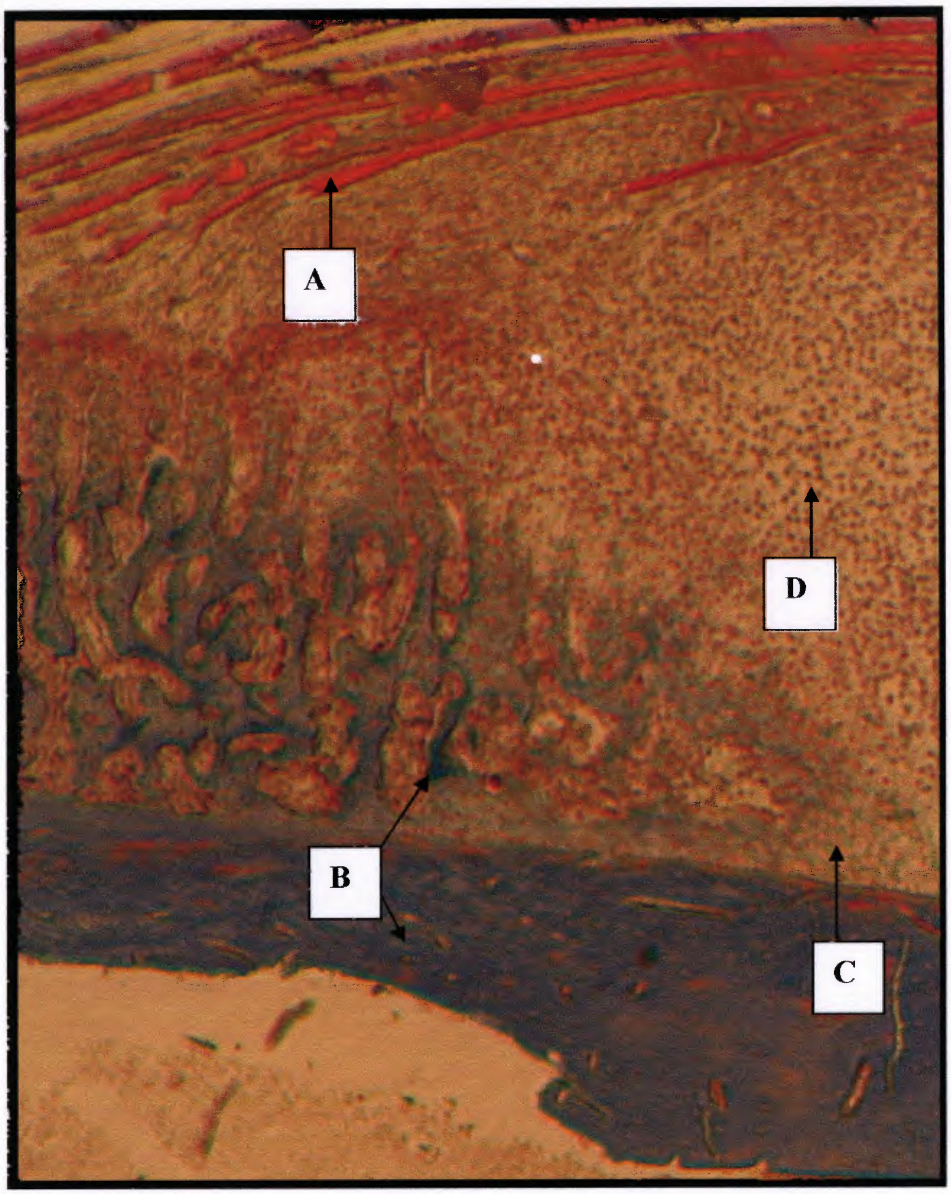

Figure 2.3 Magnification of early fracture callus histology.
Using Masson's Trichrome staining and standard histological techniques the samples are then viewed as digital photomicrographs. Above: A. Muscle tissue appears red, B. bone tissue appears dark blue, C. cartilage appears light blue and D. nuclei appear black.

2.6 Experimental Design

Two separate experiments were conducted in this study, insulin quantification and histomorphometric analysis, as described in Sections 2.4 and 2.5.

Insulin quantification was the first experiment conducted. The ability of calcium sulfate and tricalcium phosphate to continuously release insulin to the fracture site from day 0 to 7 was examined. Twelve animals were used in each experimental group. The experimental groups were then split into 3 time points, day 2, day 4 and day 7. Four animals were assigned to each time point. The contralateral femur of the animal served as the control. The total number of animals for this experiment was 24.

Table 2.1 Insulin Quantification Experimental Design

Group	Day 2	Day 4	Day 7
Experimental Calcium Sulfate + Insulin n=12	n=4	n=4	n=4
Experimental Tricalcium Phosphate + Insulin n=12	n=4	n=4	n=4
Control Calcium Sulfate n=12*	n=4*	n=4*	n=4*
Control Tricalcium Phosphate n=12*	n=4*	n=4*	n=4*
Total Number of Animals n=24*	n=8*	n=8*	n=8*

*The contralateral leg of the animals in the experimental groups served as the control.

The second experiment used histological techniques to examine the direct effect of insulin upon the parameters of the early fracture callus. Total callus area, cartilage content and new bone content were compared between the experimental and control

groups. Again, twelve animals were used in each experimental group and twelve animals were used in the control group. Both the experimental and control groups were split into 3 time points, day 4, day 7 and day 10. Four animals were assigned to each time point. The total number of animals for this experiment was 24.

Table 2.2 Histomorphometric Analysis Experimental Design

Group	Day 4	Day 7	Day 10
Experimental Calcium Sulfate + Insulin n=12	n=4	n=4	n=4
Control Calcium Sulfate n=12	n=4	n=4	n=4
Total Number of Animals n=24	n=8	n=8	n=8

2.7 Statistical Analysis

An analysis was performed on the data in each study using Statistical Analysis Software 9.1 (SAS®9 Carry, NC). The experimental and control groups that were assigned the same time point were analyzed. The groups were found to be independent with normally distributed sampling distributions and sample means. Once equality of variances was determined, a t-test was conducted upon the data. A p-value < 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Animal Model – General Health

The fasting blood glucose levels of the animals were measured prior to surgery and again prior to sacrifice. The levels remained within the range that was expected for normal BB Wistar rats throughout the duration of the study. Systemic rat insulin levels in both groups were not affected by the intramedullary insulin delivery systems.

Table 3.1 General Animal Health

Group n=12	**Blood Glucose (mg/dl)** Prior to Surgery	Time of Sacrifice	**Weight** g
Insulin Quantification			
Calcium Sulfate w/ Insulin	83.4 ± 17	87.2 ± 12.3	435 ± 26.4
Tricalcium Phosphate w/ Insulin	80.7 ± 23	79.8 ± 18	417 ± 19
Histology			
Calcium Sulfate with Insulin	74.6 ± 28	89 ± 24.7	398 ± 32.8
Calcium Sulfate with Saline	86.2 ± 16	80.3 ± 19	424 ± 29.2

The data represent average values ± standard deviation.

3.2 Quantification of Insulin

The amount of insulin collected from the fracture sites was normalized to total protein levels of the individual animal. The total protein levels were determined through a BCA assay of plasma. Plasma was collected prior to sacrifice at days 2, 4 and 7.

Table 3.2 Plasma Insulin Levels

Group	Day 2	Day 4	Day 7
Calcium Sulfate + Insulin n=4	938 ± 670	400 ± 25	806 ± 454
Tricalcium Phosphate + Insulin n=4	442 ± 191	306 ± 131	305 ± 176
Control * n=6	398 ± 14	381 ± 15	352 ± 11

The data represent average values ± standard deviation in pg/ml.
* Gandhi et al. 2005

The tricalcium phosphate group showed immediate release of insulin upon injection into the intramedullary canal. All of the time points have similar amounts of insulin at the fracture site. These levels were not significantly different from the control group (contralateral leg). The calcium sulfate group shows a burst of insulin release in the 2 day time point and an increase in the amount of insulin released at day 4 followed by a smaller quantity left at the fracture site by day 7. Days 2, 4 in the calcium sulfate group had significantly higher amounts of insulin at the fracture site as compared to the control groups ($p < 0.05$).

Table 3.3 Local Insulin Levels

Post Fracture (pg/ml)		Day 2 n=4	Day 4 n=4	Day 7 n=4
Calcium Sulfate n=12	Fractured femur	149.5 ± 61.2[a]	373.6 ± 123[a]	45.8 ± 32.7
	Contralateral femur	23.8 ± 14.7	19.7 ± 3.3	31.6 ± 10.9
Tricalcium Phosphate n=12	Fractured femur	28.6 ± 9.1	36.8 ± 18.4	29.9 ± 12.1
	Contralateral femur	18.7 ± 3.8	19.7 ± 2.3	23.4 ± 6.0

The data represent average values ± standard deviation.
[a] Represents values significantly greater than groups receiving insulin via TCP and control groups.

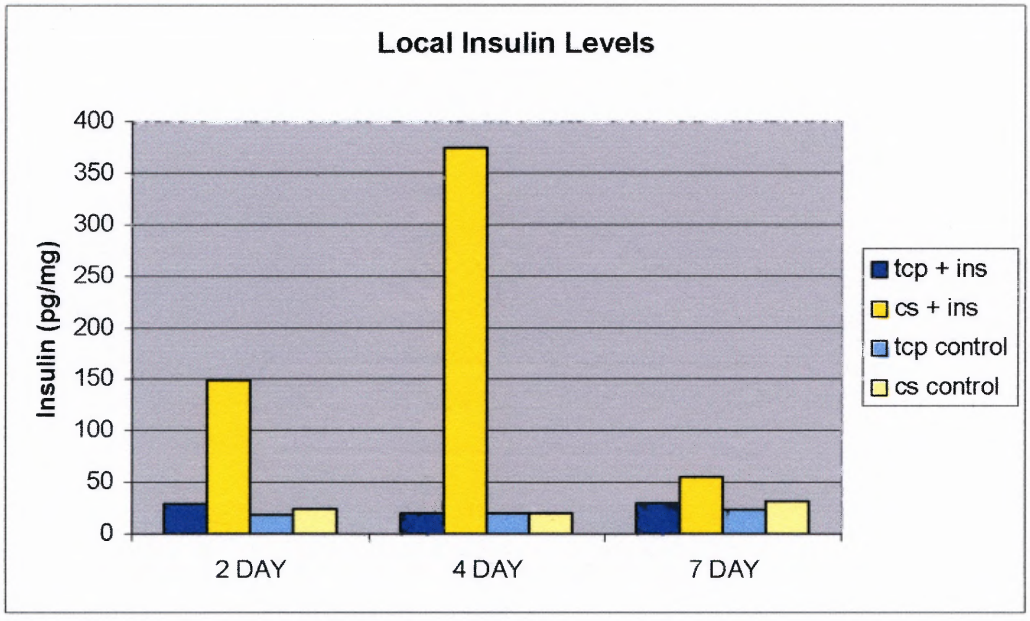

Figure 3.1 Average local insulin levels.
Average amount of insulin found locally at the fracture site at days 2, 4 and 7 in the calcium sulfate, calcium sulfate control, tricalcium phosphate and tricalcium phosphate control groups. The amount of insulin released by the calcium sulfate carrier was statistically greater than the amount of insulin released by tricalcium phosphate at days 2 and 4.

3.3 Histomorphometry

In the first experiment, the calcium sulfate group succeeded in a sustained release of insulin locally at the fracture site throughout the seven day study. The tricalcium phosphate did not continuously release insulin throughout the seven day time period and was not used in the second experiment of the study.

In the second experiment, a histomorphometric analysis was performed on a group of animals that received calcium sulfate plus insulin at the fracture site and compared to a group that received calcium sulfate plus saline to serve as the control group. The femurs were resected at days 4, 7 and 10. The total callus area, percent cartilage and percent bone that the calluses were comprised of were compared.

The total callus area of the two groups was equivalent at the three time points. The amount of cartilage found in the calluses was also equivalent. After four days, very little cartilage was found in the calluses of both groups. At days 7 and 10, both the $CaSO_4$ with Insulin group and the $CaSO_4$ with saline group show similar amounts of cartilage formation.

The amount of new bone content of the fracture calluses was significantly different in the two groups. The percent new bone content of the calluses treated with calcium sulfate and insulin were greater than those treated with calcium sulfate alone at all three time points ($p < 0.05$). This finding supports earlier studies that show insulin promotes osteogenic differentiation in the early fracture callus (Lu et al. 2003).

Table 3.4 Histomorphometry

	Total Callus Area mm2			
	Day 2	Day 4	Day 7	Day 10
$CaSO_4$ + Insulin	----	8.75 ± 0.8	14.5 ± 5.13	18.5 ± 2.3
$CaSO_4$ + Saline	----	7.68 ± 2.9	15.83 ± 4.6	13.8 ± 5.5
Systemic Insulin Beam et al. 2002	0.84 ± 0.2	3.76 ± 1.0	11.5 ± 2.35	----
Local Insulin Gandhi et al. 2005	0.96 ± 0.2	4.05 ± 1.13	12.51 ± 3.0	----

	Percent Cartilage Content (cartilage / total callus area)			
	Day 2	Day 4	Day 7	Day 10
$CaSO_4$ + Insulin	----	0	0.04 ± 0.004	0.19 ± 0.17
$CaSO_4$ + Saline	----	0	0.05 ± 0.014	0.13 ± 0.07

	Percent Bone Content (new bone / total callus area)			
	Day 2	Day 4	Day 7	Day 10
$CaSO_4$ + Insulin	----	0.13 ± 0.12[a]	0.24 ± 0.15[a]	0.21 ± 0.06[a]
$CaSO_4$ + Saline	----	0.07 ± 0.05	0.12 ± 0.06	0.14 ± 0.01

The data represent average value ± standard deviation.
[a] Represents values that are statistically higher than control values.

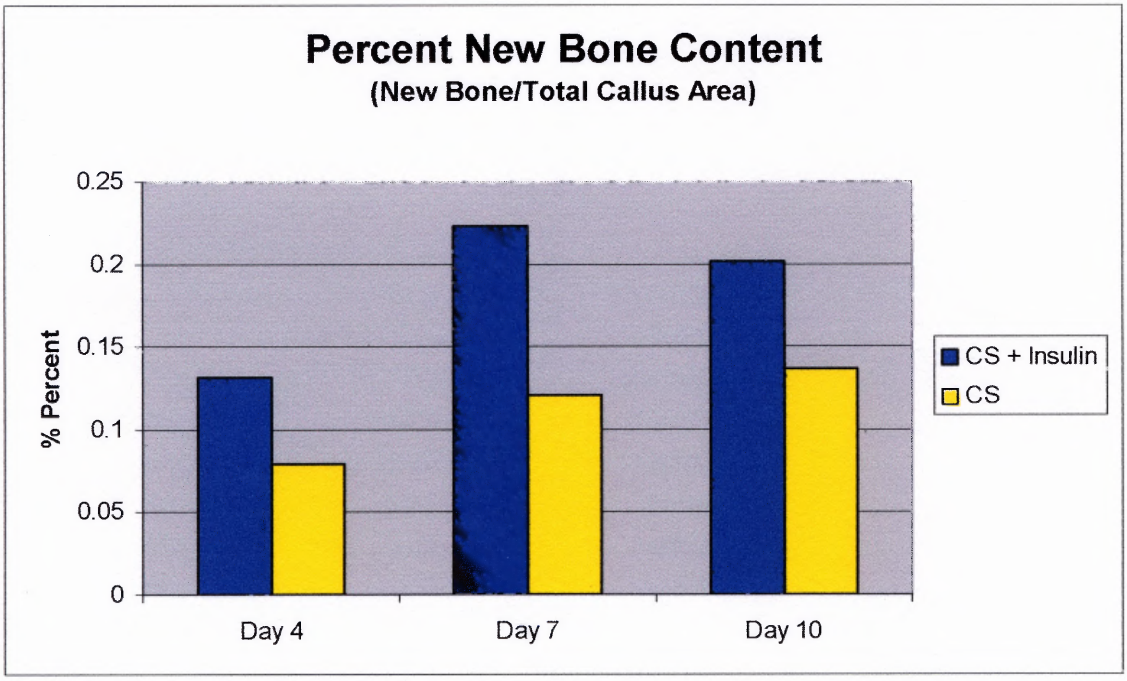

Figure 3.2 Percent new bone content in the fracture callus.
The amount of new bone found in the calluses of the experimental group, calcium sulfate plus insulin, was statistically greater than the amount of new bone found in the calluses of the control groups at the 4, 7 and 10 day time point.

CHAPTER 4

CONCLUSION

In this study, the direct effect that insulin plays on the fracture callus and fracture healing process in normal animals was studied. This initial study is the first to be conducted in a healthy normoglycemic animal model. Insulin's role in fracture healing has been examined both in vitro and in vivo, but it's direct and indirect effect upon bone healing have not been fully determined. It is clear that insulin treatment reverses impaired bone healing in diabetic animal models. In a diabetic fracture model, systemic blood glucose control maintained through subcutaneous insulin treatments achieved normalized early cellular proliferation, chondrogenesis and biomechanical properties of the diabetic fracture callus (Beam et al. 2002). However, these results do not distinguish between the indirect actions that insulin has on blood glucose levels and its direct effect upon fracture healing.

Gandhi et al. (2005) examined the effects of local insulin treatment upon diabetic fracture healing by administering insulin to the fracture site through the intramedullary canal. Immediate insulin treatment to the fracture site normalized mineralization, callus bone content and biomechanical properties in the late diabetic callus indicating that insulin is critical to the fracture healing process. Gandhi et al. (2005) also showed that the local delivery of insulin did not affect the animals' systemic blood glucose levels. This would indicate that intramedullary insulin treatment would not affect the blood glucose levels of a non-diabetic animal.

The current study was developed to test the hypothesis that intramedullary insulin delivery to the fracture site in a normal healthy animal model would improve normal fracture healing parameters without affecting the systemic insulin levels of the animal. Research concludes that insulin is necessary in the early proliferation phase of fracture healing which takes place during the first seven days after fracture (Gandhi et al. 2005). Although genetic engineering has produced numerous insulin analogs which can sustain the duration of insulin's effectiveness, the analog (ultralente) with the longest duration of effectiveness only lasts 24-28 hours. A carrier is necessary to sustain continuous insulin release for the duration of the proliferative phase and the length of our study. Ultralente human insulin was combined with carriers calcium sulfate and tricalcium phosphate. The results of the insulin quantification conducted in this study show that combination of ultralente insulin with calcium sulfate sustained the efficacy of the insulin for seven days. This result is consistent with studies that have shown a prolonged release rate of antibiotics combined with calcium sulfate administered in vivo.

Histomorphometric analysis indicates that the presence of insulin in combination with the osteoconductive ceramic calcium sulfate promotes osteogenesis in the early fracture callus. There was significantly more new bone found in the fracture callus when calcium sulfate combined with insulin is administered locally to the fracture site as compared with the administration of calcium sulfate alone. Further studies should be conducted to determine the effects that this novel biocompatible carrier has on the late parameters of fracture healing, including mineralized tissue content, cartilage content and mechanical strength.

Pending its effects upon the late parameters of fracture healing in the normal animal model, administration of insulin at the fracture site has invaluable indications. Intramedullary insulin therapy may lead to a reduction in recovery time in patients with bone deficiencies, such as the elderly and osteoporotic, as well as in healthy individuals. The relief of pain and restoration of mobility would enhance the quality of life for patients. Injury time for collegiate and professional athletes can be shortened, allowing for a quick return to the field. Accelerated fracture healing would reduce medical costs and prevent long-term disability caused by fracture nonunion.

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