Study of chitosan microparticles with bone marrow mesenchymal stem cells for bone tissue regeneration

Yugandhar Kandimalla
Medical University of Ohio
THESIS PRESENTATION
For the Degree of
Master of Science in Biomedical Science
(Concentration in Orthopaedics)

CANDIDATE: Yugandhar Kandimalla

TITLE: Study of Chitosan Microparticles with Bone Marrow Mesenchymal Stem Cells for Bone Tissue Regeneration

TIME: 12:00 p.m.

DATE: May 18, 2009

LOCATION: Orthopaedic Center Conference Room

MAJOR ADVISOR: Champa A. Jayasuriya, Ph.D.

A Lecture Open to the Public
A Thesis Entitled

Study of Chitosan microparticles with bone marrow mesenchymal stem cells for bone tissue regeneration

Dr. Yugandhar Kandimalla, M.D

University of Toledo, Health Sciences Campus
2009
ACKNOWLEDGEMENTS

I would like to extend my gratitude to my advisor Dr. A Champa Jayasuriya and Program Director & chairman Dr. Nabil A Ebraheim and for their incredible support towards my academic and research work. I am very thankful to my mentors for being a vital driving force for my future success. I would also like to thank Dr. Vijay Goel, my committee member for offering learning and guidance throughout the process. Special Thanks to Archana Bhat who was always available when help was most needed. I thank my family members for their unwavering trust in me. I thank all my colleagues and staff at the Orthopaedic Surgery and Bioengineering Departments for creating a wonderful learning environment. This study was supported by NSF grant# 0652024 and UT start up funding.
ABSTRACT

Experiments were aimed at testing the hybrid microparticles (MPs) “Chitosan and Chitosan with Calcium Phosphate” as suitable constructs for “bone marrow mesenchymal cells” (BMSCs) attachment, proliferation in vitro and to evaluate the use of “ hybrid microparticles with bone marrow mesenchymal cells constructs”, aiding bone tissue regeneration/healing in a rat femur critical size defect model. The particle were prepared with emulsification process, BMSCs harvested from rats were femurs. Cells attachment was evaluated using manual cell counting with hemocytometer. Size and surface structure of particles was assessed with Scanning electron microscope images. In vivo studies included a partial thickness 5 mm length segmental defect model of femur in 8 rats. Control group had the defect treated with metal plate only and the experiment group received transplant containing both MP and BMSC’s. A semi qualitative analysis of bone regeneration performed using X-rays, showed significant bone regeneration in experiment group at end of 4 weeks as compared to 8 weeks in previous studies.
Table of Contents

ACKNOWLEDGEMENT........................................................................................................3
ABSTRACT.........................................................................................................................4

CHAPTER I- INTRODUCTION
SIGNIFICANCE OF BONE TISSUE REGENERATION..........................................................1
BONE MARROW MESENCHYMAL STEM CELLS FOR TISSUE ENGINEERING.....................4
CHITOSAN MICROPARTICLES ..........................................................................................6
MURINE MODEL FOR BONE REGENERATION STUDIES....................................................8
CRITICAL SIZE BONE DEFECT AND SURGERIES..............................................................12

CHAPTER II- MATERIALS AND METHODS
EXPERIMENTAL DESIGN..................................................................................................22
PREPARATION OF CHITOSAN MICROPARTICLES .............................................................23
BONE MARROW MESENCHYMAL CELLS ISOLATION..........................................................24
SCANNING ELECTRON MICROSCOPE IMAGING.................................................................25
CELL ATTACHMENT........................................................................................................26
ANIMALS..........................................................................................................................26
SURGERIES AND POST SURGICAL CARE..........................................................................27
REGENERATION ANALYSIS WITH X-RAY.........................................................................29
STATISTICAL ANALYSIS.................................................................................................30

CHAPTER III- RESULTS
CHITOSAN MICROPARTICLES CHARACTERIZATION......................................................31
SCANNING ELECTRON MICROSCOPE IMAGING.................................................................32
CELL ATTACHMENT STUDY.............................................................................................34
BONE REGENERATION ANALYSIS: X-RAYS.....................................................................35

CHAPTER IV-DISCUSSION.................................................................................................38
CONCLUSION....................................................................................................................42
FUTURE WORK...................................................................................................................44

CHAPTER V- CLINICAL RESEARCH PAPER: PUBLISHED
CLINICAL OUTCOME OF FRACTURES OF THE TALAR BODY.............................................46

REFERENCES......................................................................................................................59
CHAPTER I

INTRODUCTION

Significance of Bone tissue regeneration

The ability to generate new bone for reconstructive procedures is of paramount importance. A novel approach to above problem is use of autologous Bone marrow mesenchymal stem cells (BMSCs) as progenitor cells, for re-growth and incorporating these cells on to a biocompatible construct material such as Chitosan microparticles (MPs). Cells seeded with Chitosan microparticles when allowed to grow *in vitro* may provide with valuable cues for cells to grow, differentiate and secrete extra cellular matrix, enhancing likely hood of new bone formation. The formation of bone from osteoprogenitor cells is variable and is controlled by a number of factors including external support structure –Microparticles (MPs) constructs and internal cellular microenvironment. Bone loss causing structural abnormality leading for functional disability is a major challenge in orthopedic science. Reconstructive surgeons face an obstacle in terms of successful closure of bone defects to achieve comprehensive healing. While most often bone loss is secondary to trauma, it can also arise from congenital disorders, neoplasm, and infections (Mankani et al., 2001). Guidelines for treatment of severely traumatized lower legs are controversial. Traditionally two treatment pathways for management of bone tissue loss have been amputation or surgical reconstruction. It is suggested that functional outcomes are similar following either amputation or reconstruction of a severely injured lower extremity (Bosse et al., 2002). Practical problems arise in the diagnosis, the classification, and the derived treatment modalities for bone defect management. Nevertheless, since reconstruction efforts may be life-
threatening, early decision making is necessary. Arguments in favor of primary amputation include the limited risk of complications (especially fatal outcomes), the postulated potential for rapid rehabilitation, avoiding permanent social disintegration, and the postulated cost-effectiveness. Injury-dependent patterns such as devascularization, segmental or multilevel lesions, lesion of the posterior tibial nerve, degree of contamination, associated injuries, age, profession, all have their specific, but not clearly defined value in decision making. Although the complexity of the lesion determines whether reconstruction is feasible or not, but for potentially salvageable legs, reconstruction is justified because (a) the functional outcome for reconstruction was better than for amputation; (b) there was no permanent social disintegration due to the long treatment; and (c) total costs (including pensions) for reconstruction were far lower than for amputation (Hertel et al., 1996).

Cost of reconstruction surgery Vs Amputation, economic impact on health care

Cost of care from loss of bone integrity may include (1) the initial hospitalization, (2) all rehospitalizations for acute care related to the limb injury, (3) inpatient rehabilitation, (4) outpatient doctor visits, (5) outpatient physical and occupational therapy, and (6) purchase and maintenance of prosthetic devices. When costs associated with rehospitalizations and post-acute care was added to the cost of the initial hospitalization, the two-year costs for reconstruction and amputation were similar. When prosthesis-related costs were added, there was a substantial difference between the two groups ($81,316 for patients treated with reconstruction and $91,106 for patients treated with
amputation). The projected lifetime health-care cost for the patients who had undergone amputation was three times higher than that for those treated with reconstruction ($509,275 and $163,282, respectively). These estimates add support to efforts to improve the rate of successful reconstructions have merit. Not only is reconstruction a reasonable goal at an experienced level-I trauma center, it results in lower lifetime costs (MacKenzie et al., 2007). Also a separate study done on long term outcome of reconstruction Vs amputation by (Williams, 1994) suggests that Ilizarov limb reconstruction is cost-effective when compared with amputation when prosthetic costs are also considered. Thus it would be a cost effective initiative in long term perspective to expand research concepts in terms of using novel material, stem cells to regenerate lost tissue and to improve the process of reconstruction of bone.

**Bone regeneration**

The natural processes of bone repair are sufficient to effect timely restoration of skeletal integrity for most fractures, when an appropriate mechanical environment exists or is created with internal fixation or coaptation. However, some situations require manipulation or augmentation of natural healing mechanisms to regenerate larger quantities of new bone than would naturally occur to achieve surgical goals. Specific situations that may require additional interventions include substantial loss of host bone from trauma or tumor resection, arthrodesis, or spinal fusion, non- or delayed unions, metabolic disease, arthroplasty, or insufficient healing potential of the host because of local or systemic disease or old age. A wide variety of materials have been employed to repair bone defects, including autogenous cells, allogeneic tissues, and alloplastic
materials. Use of such a variety of approaches imply to the absence of an optimal method for restoring bone integrity, especially in the presence of a sizable defect. While surgeons have extensively used bone autograft to deliver osteoblasts and osteocytes to deficient sites, the transplantation of *ex vivo* expanded osteoprogenitor cells is a relatively recent and promising advance (Luria et al., 1987; Mankani et al., 2001; Owen and Friedenstein, 1988; Yoo and Johnstone, 1998). Materials and strategies that are employed must duplicate and amplify the events of secondary bony union to achieve the desired result (Bruder and Fox, 1999; Derubeis and Cancedda, 2004). Bone can be regenerated through the following strategies: osteogenesis—the transfer of cells, osteoinduction—the induction of cells to become bone, osteoconduction—providing a scaffold for bone forming cells, or osteopromotion—the promotion of bone healing and regeneration by encouraging the biologic or mechanical environment of the healing or regenerating tissues. The most efficacious strategies use as many of the fundamental components of bone regeneration as possible, and each facet of bone regeneration relies upon mesenchymal cells (MSCs) (Bruder and Fox, 1999; Muschler and Midura, 2002).

**Bone marrow mesenchymal stem cells for tissue engineering**

MSC are multipotential cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, and myoblasts (Jaiswal et al., 1997; Pittenger et al., 1999). Bone marrow and periosteum sources are richest in young animals with their numbers diminishing with age. MSC numbers and biologic activity are greatest in metaphyseal bone and areas of thick and vascular periosteum. The study of MSC has primarily utilized
those retrievable from bone marrow aspirates. Isolation techniques generally are based on the adherent properties of the MSCs. Density gradient centrifugation is employed initially to separate nucleated MSC. As the cells are cultured, MSCs adhere to the flask surface (Bruder et al., 1998b). The non-adherent cells are removed with the culture medium when it is changed, concentrating the MSC (Bruder et al., 1998b). The cells are repeatedly passaged, expanding the cell population, until a pure culture is produced. The isolated cells are confirmed to possess osteogenic potential by various techniques (Yoo and Johnstone, 1998), (Kadiyala et al., 1997), (Bruder et al., 1998b). *In vitro* culturing of purified MSC in the presence of dexamethasone, ascorbic acid, and glycerophosphate (Jaiswal et al., 1997) results in the cells progressing although a osteoblastic lineage (Haynesworth et al., 1992). The cells assume a cuboidal osteoblastic shape and there is a transient induction of alkaline phosphatase activity (Bruder et al., 1998a). The cells express bone matrix protein mRNAs and the deposition of a hydroxyapatite-mineralized extracellular matrix confirming that the cells isolated become bone forming cells (Jaiswal et al., 1997), (Kadiyala et al., 1997). *In vivo* studies that document osteoblastic potential of the MSCs include loading isolated and culture expanded cells into porous ceramic carriers and implanting them in subcutaneous tissues of a living animal (Bruder et al., 1997). Vascularized bone forms within the confines of the ceramic implants and not in acellular implants. In addition, cells can be labeled and have been shown to survive implantation and maintain their multilineage potential (Quintavalla et al., 2002). MSC can also maintain viability and multilineage potential after cryopreservation (Bruder et al., 1997). Therefore, MSC can be effectively isolated, culture expanded, preserved, and implanted (Kraus and Kirker-Head, 2006).
Chitosan Microparticles

In recent years, considerable attention has been given to Chitosan (CS)-based materials and their applications in the field of orthopedic tissue engineering. Interesting characteristics that render Chitosan suitable for this purpose are a minimal foreign body reaction, an intrinsic antibacterial nature, and the ability to be molded in various geometries and forms such as porous structures, suitable for cell in-growth and osteoconduction. Due to its favorable gelling properties Chitosan can deliver morphogenic factors and pharmaceutical agents in a controlled fashion. Its cationic nature allows it to complex DNA molecules making it an ideal candidate for gene delivery strategies. The ability to manipulate and reconstitute tissue structure and function using this material has tremendous clinical implications (Di Martino et al., 2005).

Chitosan (CS) is a deacetylated derivative of chitin, a high molecular weight, second most abundant natural biopolymer commonly found in shells of marine crustaceans and cell walls of fungi. Chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β (1–4) manner; the glucosamine/N-acetyl glucosamine ratio being referred as the degree of deacetylation. Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1000kD with a degree of deacetylation from 30% to 95% (Huang et al., 2003). In its crystalline form, CS is normally insoluble in aqueous solutions above pH 7; however, in dilute acids (pH 6.0); the protonated free amino groups on glucosamine facilitate solubility of the molecule. The cationic nature of CS is primarily responsible for electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged
molecules. This property is of great interest because a large number of cytokines/growth factors are linked to GAG (mostly with heparin and heparan sulphate), and a scaffold incorporating a CS–GAG complex may retain and concentrate growth factors secreted by colonizing cells. Moreover, the presence of the N-acetylglucosamine moiety on Chitosan also suggests related bioactivities. In fact, CS oligosaccharides have a stimulatory effect on macrophages, and both Chitosan and chitin are chemoattractants for neutrophils both in vitro and in vivo. Lysozyme is the primary enzyme responsible for in vivo degradation of CS through hydrolysis of acetylated residues. Another interesting property of CS is its intrinsic antibacterial activity. Studies have shown that CS can reduce the infection rate of experimentally induced osteomyelitis by Staphylococcus aureus in rabbits. Its cationic amino group associates with anions on the bacterial cell wall, suppressing biosynthesis; moreover, CS disrupts the mass transport across the cell wall accelerating the death of bacteria. When added to HA and plaster of Paris to obtain a composite for sustained vancomycin or fosfomycin release, the composite material was able to inhibit methicillin-resistant S. aureus in vitro for as long as 3 months, a period compatible with the treatment of most orthopedic infections (Zambito and Di Colo, 2003). Chitosan (CS) has been combined with a variety of delivery materials such as alginate, hydroxyapatite, hyaluronic acid, calcium phosphate, PMMA, poly-L-lactic acid (PLLA), and growth factors for potential application in orthopedics. Overall, CS offers broad possibilities for cell-based tissue engineering. Possible matrix preparations for cell cultures include gels (Shu and Zhu, 2001), sponges, fibers, or porous compositions of CS with ceramic or other polymeric materials such as collagen or gelatin to adjust cell seeding properties and
mechanical behavior of cell transplants for the intended clinical application (Kato et al., 2003).

**Murine model for Bone regeneration studies**

Many mammalian and avian species have been used as models to study fracture healing. The individual animals, based on their size and other characteristics, are used to show the effects of different interventions on healing bone. The models may have fractures created by closed or open means or osteotomies. Fracture healing can be evaluated through histologic, mechanical, chemical, or biological studies. Internal fixation, external skeletal fixation, or no fixation may be used in the experiments. Results of fracture healing studies among animal species may conflict even when the same model is used in each species. These differences may be explained by anatomic, biochemical, and gene expression differences. Evaluation of the animal model is an important consideration when applying the results of any particular study to humans. Consensus regarding fracture healing develops from agreement between results of animal models and human clinical studies (Nunamaker, 1998). Although *in-vitro* studies and clinical trials provide adequate techniques and instruments to improve our knowledge on bone repair, animal studies still represent an essential tool to analyze the biology of fracture healing (Holstein et al., 2009a). Accordingly, numerous mammalian species, ranging from the mouse to the horse, have been introduced as models to study fracture healing. Nonetheless, differences in the anatomy and metabolism of animals compared with humans must be considered in the experimental setup and in the interpretation of experimental results. In addition, the transfer of the experimental data to the clinical situation must be interpreted in light of
these limitations; because of a more primitive bone structure without a haversian system, small rodents, like mice and rats, are thought to be less appropriate for bone healing studies compared with larger animals. In contrast to larger rodents and other mammals, small rodents use resorption cavities for bone remodeling during fracture healing, and this process of remodeling has been shown similar to the haversian remodeling in larger animals (Nunamaker, 1998). There are several reasons why the murine model is considered as the ideal species for bone healing and regeneration studies. The expenses for purchase, breeding, and holding of mice are relatively low. Compared with larger animals, the space necessary for holding and the time necessary for bone healing are reduced. Thus, the implementation of larger groups in the experimental study design is more feasible. Finally and of utmost importance, a broad spectrum of antibodies and gene-targeted animals is available for mice, allowing mechanistic studies on molecular mechanisms of bone healing and regeneration.

**Anatomic and surgical considerations in murine fracture models**

The small size of mice is challenging for the development of a fracture model. Therefore, particularly large long bones, such as the femur and the tibia, are thought to be most appropriate for studies on fracture healing (Hiltunen et al., 1993). Nonetheless, previous studies have also used the rib, the radius, the ulna, the mandible, and the calvaria to investigate bone repair. However, these bones are unfavorable for biomechanical testing due to their small size and their anatomic configuration.
Tibia Vs Femur

The tibia fracture model, which was first described by (Hiltunen et al., 1993), is supposed to be the most established murine fracture model. However, due to the distally declining caliber of the tibia, slightly different fracture sites in closed models may result in markedly different callus sizes, which limit both standardization and comparability. The triangular configuration and the bowed longitudinal axis further afford a more sophisticated design for implants, guaranteeing stable fixation. In addition, the biomechanical test accuracy is limited due to the irregular shape of bone. Although the tibia is relatively easy to fracture because of its thin soft tissue cover, this anatomic condition is disadvantageous when analyzing the role of soft tissue in bone repair. The last issue that has to be considered when using tibia fracture models is the influence of the fibula. In closed tibial midshaft fractures, the fibula may also break, which results in either 2 different calluses or 1 combined callus. To avoid a fracture of the fibula, the tibia has to be broken very distally and closely to the metaphysis, which, however, is not feasible in a standardized fashion in closed models. In contrast to the tibia, the mouse femur is a tubular bone length about 15 mm with a relatively consistent inner and outer diameter (outer diameter about 1.5 mm). Accordingly, different fracture sites within the diaphyseal part of the bone show comparable callus responses. Because of the straight longitudinal axis, standardized fracture stabilization is easier in the femur than in the tibia. Unlike the tibia, the femur is capable of rotation testing, and also 3- and 4-point bending tests provide more consistent results in the femur than in the tibia. As mentioned above, the relatively bulky muscle cover of the femur is challenging when creating a closed fracture. But on the other hand, the muscle cover is an important element to
investigate the role of soft tissue during bone repair. Thus, in our opinion, the femur has to be considered more appropriate for fracture studies compared with the tibia.

**Closed Vs Open fracture models**

To analyze bone repair, both closed and open fracture models have been used. Closed femur fracture models in mice are based on the model described by (Manigrasso and O'Connor, 2004). A standardized transverse fracture pattern associated with minor soft tissue trauma is accomplished in these models by a 3-point bending device. Using a parapatellar approach, the patella is dislocated to insert the intramedullary implant retrogradely at the intercondylar notch. The advantage of closed models is that the parapatellar approach and the closed fracture production cause only a minor soft tissue trauma. However, the intramedullary implant might affect the endosteum and the bone marrow (Holstein et al., 2009b). In open models, the femur is fractured or osteotomized visibly using a lateral approach (Cheung et al., 2003; Garcia et al., 2008a). Thereby, the entire lateral muscle layer has to be split to expose the full length of the femur from the greater trochanter to the lateral femoral condyle. In fact, the open surgery procedure produces a major soft tissue trauma, which may have a critical impact on the vascularization of the femur. In contrast to closed models, which use intramedullary implants, open models use extramedullary implants, which preserve the endosteum and the bone marrow. However, care has to be taken during the surgical procedure to avoid damage to the periosteum.
Age of animals

Previous studies have shown that the age of the animals has a critical impact on fracture healing (Lu et al., 2005). Therefore, animals with an age of completed bone growth should be used to mimic fracture healing in adults. In humans, the ratio of age at growth plate closure and life expectancy is about 20%. This ratio is quite comparable to that of mice, whereas it markedly differs from that of other species of established models such as rats (30%), rabbits, dogs, and sheep (5%-10%) (Kilborn et al., 2002).

Critical size bone defect and surgeries

A number of animal models have been used to study effectiveness of regenerative agents in alveolar bone loss. Primates are considered suitable because the morphological features of their teeth and periodontal tissues closely resemble those of humans (Fritz et al., 2000). However, the low cost, short study intervals, small variation among strains and known genetics make murine models extremely attractive in the study of bone loss (Wilensky et al., 2005). Rats have the advantage over mice because of the size and the accessibility of their bone tissues, and therefore have potential as the ideal animal model of alveolar bone loss. It has been suggested that the repair of bone defects depends on the size of the defect; that is, a bone defect larger than a certain size (a critical size) cannot be healed with bone. The remaining defect is filled with fibrous connective tissues (Honma et al, 2008). Calvarial critical-size bone defects have been used in various studies to investigate the effectiveness in bone defect repair of bone regenerative agents such as growth factors, biomaterials, cell or tissue implantation, or any combination of these (Kamakura et al, 1999, 2004, 2006; Lutolf et al, 2003; Cowan et al, 2004, 2005).
The relevance of biomechanically standardized osteosynthesis in murine femur fracture models

Because of the lack of stable fixation techniques, most of the former femur fracture studies in mice were conducted with an unstable intramedullary pin fixation (Manigrasso and O'Connor, 2004), or even without fracture stabilization. However, the course of fracture healing has been demonstrated to depend on the stability of the osteosynthesis technique. In large animal models, it has been shown that interfragmentary movements lead to an increased formation of fibrocartilage, which is associated with a significantly decreased bone formation (Claes et al., 2002; Lienau et al., 2005). Thereby, bone mineral density and bending rigidity are strongly reduced in fractures that experience shear compared with those that are exposed to axial compression. It has been further demonstrated that interfragmentary motion disturbs angiogenesis (Claes et al., 2002; Lienau et al., 2005). In fact, the expression of angiogenic and osteogenic cytokines is affected by the biomechanical environment in the fracture gap (Lienau et al., 2005).

Murine studies have illustrated that mechanical factors during fracture healing alter the chronology of chondrogenic and osteogenic cytokine induction, the differentiation of mesenchymal cells, and the size and tissue composition of the callus (Garcia et al., 2008a). Standardized osteosynthesis techniques that guarantee defined biomechanical and biologic conditions for fracture research are a matter of course in large animal models. In contrast, it is commonly argued that murine fracture models do not require biomechanical standardization because the predominance of research using mouse models has focused on the molecular aspects of fracture healing. However, as outlined above, the cellular and
molecular mechanisms of fracture healing are critically affected by the mechanical
environment in the fracture gap. Several femoral fracture models have been developed in
mice (Cheung et al., 2003; Garcia et al., 2008a; Manigrasso and O'Connor, 2004).
Additional models have been introduced for the analysis of bone repair in segmental bone
defects (Garcia et al., 2008b).

**Intramedullary Pin**

The intramedullary pin provides simple but unstable closed fracture (Fig 1 A)
stabilization (Manigrasso and O'Connor, 2004). Using a medial parapatellar incision, the
patella is dislocated to ream the intramedullary canal at the intercondylar notch. A
stainless steel wire (diameter 0.25 mm) is applied for intramedullary fracture
stabilization. Migration of the pin is prevented by an additional wedge (length 2 mm and
diameter 0.3 mm) at the distal end of the intramedullary canal. The diaphyseal fracture is
produced by a 3-point bending device after the insertion of the implant.

**Intramedullary Locking Nail**

To overcome the lack of rotational stability observed with pin stabilization, a locking nail
has been developed (Fig 1B) (Holstein et al., 2007a). The nail system consists of a
modified injection needle (diameter 0.55 mm) and a tungsten guide wire (diameter 0.1
mm). Rotation stability is accomplished by flattening the proximal and distal ends of the
needle. Using the same surgical approach as described for the pin stabilization, the guide
wire is inserted into the intramedullary canal. After the production of a closed diaphyseal
fracture, the locking nail is pushed over the guide wire. The guide wire is then removed.
Due to the application of the guide wire, it is possible to produce the fracture without
prenailing the bone. This avoids alterations to the implant and also more closely mimics the clinical setting.

**Fig 1:** X-rays of fractured mouse femurs after stabilization with (A) an intramedullary pin (Manigrasso and O'Connor, 2004), (B) an intramedullary locking nail (Holstein et al., 2007a), (C) an intramedullary compression screw (Holstein et al., 2009b), (D) an external fixator (Cheung et al., 2003), (E) a pin-clip (Garcia et al., 2008a), and (F) a locking plate (Gröngröft et al; Matthys and Perren). X-rays in figures B, C, and F were taken at the day of fracture and those in figure A, D, and E were taken at day 7, day 21, or 35 after fracture. Figures A-C represent closed femur fracture models and figures D-F show open models (Holstein et al., 2009a).
**Intramedullary Compression Screw**

Recently, an intramedullary compression screw (length 18 mm and diameter 0.5 mm) has been engineered (Fig1C) (Holstein et al., 2009b). It provides not only rotational but also axial stability after osteosynthesis of closed femoral fractures in mice. The technique uses also a guide wire to allow the production of the fracture before insertion of the implant. Rotational and axial stability after fracture fixation is achieved by means of interfragmentary compression through the screw, which consists of a proximal cone-shaped head and a distal thread. According to a pilot study in senescence accelerated mice, strain P6 (SAMP 6), osteoporotic bones can be stabilized by the use of the intramedullary compression screw (Holstein JH, Histing T, Garcia P et al, unpublished data).

**External Fixator**

The application of an external fixator also allows stable fracture fixation (Fig1D)(Cheung et al., 2003). In addition, segmental bone defects might be stabilized by an external fixator. As an external stabilization device, the fixator preserves the endosteum and the bone marrow. However, a traumatizing surgery with a significant injury of the soft tissue is necessary to insert the fixator pins (diameter 0.3 mm) and to fracture the bone. Furthermore, the bulky design of the external fixator might restrict the physiologic activity and gait of the animals.
**Pin-clip Device**

The pin-clip device represents an open model using an intramedullary pin in combination with an extramedullary clip for fracture fixation (Fig 1E) (Garcia et al., 2008a). Of interest, the pin-clip technique permits also the stabilization of segmental bone defects. In accordance, it is possible to create different gap sizes and thereby to affect the healing process resulting in critical size defects or even nonunions. Compared with the bulky external fixator, this internal stabilization device causes no obvious alteration of the animals' gait. In contrast to the external fixator, however, the intramedullary pin may affect the endosteal bone repair. Also, the insertion of the pin-clip device requires a traumatizing surgical approach.

**Locking Plate**

The locking plate is a third implant that enables a stable fracture fixation in the mouse femur but does require open surgery (Fig 1F) (Gröngröft et al; Matthys and Perren). The plate is fixed to the bone by 4 interlocking screws. The plate is designed to minimize the implant to bone contact area, avoiding major damage to the periosteum. Nonetheless, the plate fixation may affect the external callus formation at the implant site. Instead of a rigid plate, a flexible plate can be used in which the medial part is replaced by 2 elastic splinting wires. These 2 plate models allow either stable fracture fixation or standardized flexible stabilization of the mouse femur. Comparable to the pin-clip device, the plate also permits the stabilization of different gap sizes. As a common drawback of open fracture models, the insertion of the locking plate is also associated with a traumatizing surgical approach, which affects the soft tissue cover of the bone. As the intramedullary
compression screw, the locking plate can also be used in osteoporotic bones (Histing T, Holstein JH, Garcia P et al).

**Biomechanical ex vivo testing of osteosynthesis devices**

To evaluate the biomechanical properties of different osteosynthesis devices, biomechanical ex vivo testings have been performed. For these analyses, murine cadaver femora are osteotomized and stabilized by the implant of interest. Using highly sensitive testing devices, the rotational or axial stiffness of the osteosynthesis devices can be analyzed. These analyses revealed highly significant differences in rotation stiffness of the different devices. The locking plate showed a rotational stiffness, which was comparable to that of unfractured control femora. In contrast, the conventional pin failed to provide any rotationally stable fracture fixation.

**Methods for the analysis of bone repair in murine femur fracture**

**Imaging Techniques**

To study femur and tibia fracture healing in mice, the animals have in most cases to be killed at different time points after fracture. This has to be done to resect and prepare the bones for further radiologic, histologic, biomechanical, cytologic, and molecular analyses. However, beside these postmortem analyses, noninvasive imaging techniques have been introduced to evaluate bone repair, including micro-positron emission tomography and micro-magnetic resonance imaging. In addition, a variety of molecular imaging techniques, such as bioluminescence, near-infrared fluorescence, and nuclear and magnetic resonance imaging, have been applied for noninvasive real-time studies on
gene expression, protein degradation, cell migration, and cell death in living animals. Nevertheless, the most established imaging techniques to study murine fracture healing are high-resolution radiography and 2-dimensional and 3-dimensional microcomputer tomography (CT). Using conventional x-ray techniques, the size and radiologic density of the fracture callus can be analyzed in living animals and in resected bone (Garcia et al., 2008a; Holstein et al., 2007b). Although micro-CT scans can be applied also \textit{in vivo}, \textit{ex vivo} micro-CT scans reveal a significantly higher resolution than in vivo scans (Cano et al., 2008; Cano et al., 2007). Common parameters that are evaluated by micro-CT are tissue mineral density, total callus volume, and bone volume fraction of the callus. By the postmortem injection of a chromate-based contrast agent, the vasculature of the callus can also be visualized and quantitatively assessed by 3-dimensional micro-CT techniques (Duvall et al., 2007).

\textbf{Histologic Analysis}

The quantitative histologic analysis of the fracture callus (histomorphometry) has been proven to be an important parameter to assess bone repair (Gerstenfeld et al., 2005). Thereby, the American Society of Bone and Mineral Research has provided recommendations regarding a standardized nomenclature, appropriate indices for assessment, and methodologic approaches for histomorphometric analyses. In addition to histomorphometric studies, immunohistochemical analyses allow the in situ detection of different proteins like cytokines and cell markers within the fracture callus. Because the callus is a heterogeneous, 3-dimensional structure comprising several different types of tissue, it is a great challenge to accurately analyze the different tissue areas using 2-
dimensional histologic sections. Therefore, it is crucial to define representative, standardized longitudinal or transverse bone sections that allow a reproducible calculation of the size and tissue composition of the callus. From the technical aspect, decalcified and undecalcified bone sections are used for a variety of different staining methods (Gerstenfeld et al., 2005). In general, the implant has to be removed; however, when applying the sawing and grinding technique, the implant can also be left in place (Donath and Breuner, 1982).

**Biomechanical Analysis**

Three-point and 4-point bending tests and torsion tests have proven to be applicable for biomechanical studies on bone repair in murine femur fracture (Holstein et al., 2007b; Murnaghan et al., 2005). In addition, axial testing, which has been reported for the *ex vivo* analysis of osteosynthesis devices, might be additionally appropriate for the evaluation of bone repair. As mentioned above, the small size of the murine femur is a great challenge for biomechanical tests because an inaccurate, nonstandardized fixation of the bones in the testing machine critically affects the testing results. As a matter of course, highly sensitive testing devices are required for biomechanical tests in mouse bones. Using nondestructive 3-point and 4-point testing devices, usually the bending stiffness of the healed bone is assessed from load displacement curves. When applying destructive devices, the ultimate load at failure of the bone is determined (Murnaghan et al., 2005). In accordance, torsion stiffness and ultimate torque and angle at failure can be quantitatively analyzed in torsion testing procedures (Holstein et al., 2007b). In general, the results of the biomechanical analysis of the healing bone are expressed as percentage
of that of the contralateral intact bone to account for individual differences of the animals (Holstein et al., 2007b; Murnaghan et al., 2005).

**Cytologic and Molecular Analysis**

Beside the new exciting field of molecular imaging, conventional techniques are available to investigate cellular and molecular aspects of bone repair. Numerous proteins like cytokines, cell metabolism markers, and cell surface proteins are detectable in situ by immunohistochemical methods. Results of immunohistochemical assessments can be supported by semiquantitative protein analyses using biochemical methods such as Western blotting and enzyme-linked immunosorbent assay techniques. In situ hybridization studies provide further information on the corresponding messenger RNA expression in the different cells. Also, the assessment of the in situ messenger RNA expression can be additionally supported by semiquantitative techniques such as Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR).

Furthermore, cell counting methods like FACS analysis have been reported to be effective to evaluate the effect of different bone marrow cells on bone repair. Cell apoptosis can be assessed by in situ labeling of nuclear DNA fragments using the TdT-mediated duTP-biotin nick end labeling (TUNEL) technique. Finally, cells of the fracture callus can be harvested for further cell culture studies (Toyosawa et al., 2004; Uusitalo et al., 2000).
CHAPTER II

MATERIALS AND METHODS

Experimental design

Experiments were aimed at testing the hybrid microparticles (MPs) Chitosan and Chitosan with Calcium Phosphate as suitable constructs for bone marrow mesenchymal cell attachment, proliferation in vitro. To further evaluate the use of hybrid microparticles with bone marrow mesenchymal cells constructs aiding bone tissue regeneration/healing in a rat femur critical size defect model in vivo. Two particle groups prepared with emulsification process, included 1.5% Chitosan and 1.5% Chitosan with Calcium Phosphate to evaluate cell attachment and proliferation and in vivo bone regeneration. Bone marrow mesenchymal cells harvested from male rats DA Harlan were processed and passaged using tissue culture techniques to allow particle-cell attachment in vitro and in vivo studies. Size and surface structure of particles was assessed with Scanning electron microscope images. Cells attachment was evaluated using manual cell counting using Hemocytometer. Statistical analysis was performed with 2-way-Anova for the attachment studies using Minitab software (v) 15.

In vivo studies included a partial thickness 5 mm length segmental defect model, which was surgically created and stabilized under strict aseptic conditions in mid diaphysis of femur in 8 rats. The animals were divided in to two groups. One group of rats had the defect treated with metal plate only and the other group received transplant containing both MP and BMSC’s and metal plate. Animals were euthanized at 4 weeks and femurs harvested. A semi qualitative analysis of bone regeneration was performed using X-rays.
Preparation of Chitosan microparticles (MPs)

Chitosan research grade powder (Sigma) component was used for preparing 1.5% Chitosan batch particles using the emulsification method. 750 mg of Chitosan (1.5%) and 75 mg of calcium phosphate powder were stir mixed with 50 ml 1% acetic acid to form acetic acid-Chitosan mix. 600 ml of cotton seed oil (Sigma) with 4 ml of Span 85 as emulsifier (Sigma) in a 1000 ml beaker with stir bar was stirred continuously at speed 870 rpm on magnetic stir plate. 25 ml of acetic acid-Chitosan mixture was mixed with 25 ml of acetone to form Acetone-Chitosan mixture and 36 ml of this mixture was injected with 21 gauze needle and 20 ml syringe in a drop wise manner to cotton seed oil and span 85 solution. This preparation was set to stir for 14 hours at 37°C, after which 172 mg (64%) cross linking agent Sodium Tripolyphosphate (TPP) (Sigma) in 4 ml distilled water is added and continued to stir for another 4 hours. Particles were allowed to settle down for 30 minutes and the oil solution was eliminated by treating with equal volume of hexane and vacuum filtration method. Particles collected on a filter paper were air dried. A second particle group with 1.5% Chitosan only, included similar process but with-out calcium phosphate. Particles were washed with 0.01 N sodium hydroxide solution and air dried. Before and after wash with sodium hydroxide solution pH were 6.85 and 7.08 respectively. For all the batches run, expected yield and actual yield were calculated at 0.469 g and 0.350 g respectively with yield ratio of 0.748 g. Yield ratio of replicates was noted to be 0.436 g. Yield ratio was calculated as a ratio of actual yield obtained after final process to expected yield from weight of ingredients used in the process. Several such batches were run as replicates to obtain particles for cell attachment and in-vivo
studies. Particles were labeled, weighed and stored in mini glass test tubes. Resultant particles were fine spherical particles with neutral pH.

**Rat bone marrow harvesting and in-vitro expansion**

Bone marrow mesenchymal cells processing for in-vitro expansion and attachment: BMSCs were harvested after euthanasia using CO₂ inhalation performed in compliance with AVMA panel on euthanasia and UTHSC animal studies guidelines. Animals are carefully wrapped in sealed bags, transported to dissection area which was kept sterile with ethanol 70% spray. All surgical instruments were sterilized in autoclave prior to procedure. Scalpel blade No-7 was used to make incision starting at hip extending to knee on lateral aspect of lower extremity, underlying tissues were separated to expose femur. Micro dissection instruments were used to detach femur from hip joint and knee joint. Similar procedure was performed on opposite lower extremity to extract femur. Femurs in sterile PBS were subsequently processed under tissue culture hood, both ends are snipped with scissors and a 21 gauge needle with syringe was used to flush out marrow, which was collected in sterile PBS. Marrow cells were subjected to centrifugation at 1000 rpm for 10 minutes, supernatant was discarded leaving the cell pellets which were resuspended in 10 ml of α-MEM (minimum essential medium) containing Dulbecco’s modified eagles medium (GIBCO-BRL, Life technologies), with 10% Fetal bovine serum, and antibiotics penicillin 100 units/ml, streptomycin 100g/ml. The suspension is re-centrifuged for 10 minutes; cell pellet thus formed is suspended in 10 ml of fresh α-MEM and pipetted thoroughly to avoid cell aggregations for a uniform suspension. The single cell suspension is further transferred to T-flasks, labeled and
observed under microscope followed by incubating flasks in incubator with 5% CO$_2$ at 37$^\circ$ C.

**Seeding of BMSCs on Microparticles (MPs)**

Rat BMSCs from tissue culture flasks after 2-5 passages, were trypsinsed for 5-10 minutes with trypsin to aid detachment from flask. Cells were then washed and centrifuged for 10 min with PBS to get rid of residual trypsin. All procedures are performed under tissue culture hood with strict aseptic precautions. 12 mg particles from each formulation were weighed into 96 well plates. Aliquots of 50 µL cell suspension at a cell density 400,000 cells/ml were added into each well. Cells seeded in wells without MPs served as control. Manual counting of cells with Hemocytometer was undertaken to prepare cell suspension after adequate dilution to contain 20,000 cells. Cells suspended in 100 µL of α-MEM, are carefully added coating/immersing particles in to 96 well plate containing replicates of 12 mg of Chitosan group, 12 mg of Chitosan+10% Calcium Phosphate microparticles group, and particle free control group. After observing under microscope 96 well plates are labeled and incubated in incubator with 95% room air and 5% CO$_2$ at 37$^\circ$ C till four time points under study for 5, 10, 20, 30 hrs respectively.

**Scanning Electron Microscope (SEM) Imaging**

Size and surface morphology of Chitosan microparticles prepared by emulsification were observed by scanning electron microscope after gold coating and double side tape application. Although some disintegrated particles were also noted most of the Chitosan particles were found to be smooth and spherical. The size of particle ranged from 40-60µm in diameter.
Cell attachment study

Cell attachment was done in a 96 well plate, it was filled with 12 mg of Chitosan and Chitosan with Calcium Phosphate and was sterilized with UV radiation for 10 min. Cell culture flasks after 2-3 passages was treated with trypsin to detach cells and incubated for 10 min, after which the cells were transferred to a 15 ml test tube, washed with PBS and later with α-MEM. The cells were subjected to centrifuge at 1000 rpm for 10 min. Concentrated cell suspension is derived in 1 ml of α-MEM after suction of supernatant. All eight chambers were used in Hemocytometer cell counting method. Volume in the 15 ml test tube was adjusted to get cell suspension containing 20,000 cells. The 96 well plates with two groups of particle, their replicates were added with cells suspension containing 20,000 cells and incubated in incubator with 5% CO₂ at 37°C till four time points under study for 5, 10, 20, 30 hours respectively. Media was changed at end of two days. At end of every set time point each 96 well plate in treated with trypsin for cell detachment and counted manually with hemocytometer. The data was gathered and compiled using Minitab software.

Animals

DA male rats from Harlan, Indianapolis, IN were used as transplant recipient subjects. Each rat age ranging 12-14 weeks, weighing 350-450 grams were selected with intention of having a fully matured bone structure in rats understudy. Pre surgery examination for general physical well being was assessed on each rat. Prophylactic antibiotic penicillin 40,000 U/Kg was administered. Pre-defined surgical procedure was practiced in two
euthanized rats and six live rats under anesthesia with inhalant isoflurane, subsequently rats were euthanized before weaning of anesthesia using CO₂ inhalation. Construct material MPs with BMSCs transplant were transplanted in critical size defect in four rats with supporting metal plate and K-wire fixation formed experiment group. Four rats which only received metal plate with K-wire fixation functioned as control group.

**Pre surgery, surgeries and post surgical care**

**Pre surgery**

The eight DA Harlan rats used in the study weighed an average of 400 g (350-450 g). The *in vivo* study protocol was approved IRB protocol # 3105818 IACUC. All animal handling, surgeries and post surgery monitoring was done at pre inspected and approved UTHSC DLAM facility. The animals received a pre surgical evaluation for general well being and a prophylactic antibiotic dose of Intramuscular penicillin 40,000 U/Kg 12-24 hrs before actual surgery. Rats’ paws were covered with adhesive tape to avoid contamination of surgical area. Anesthesia: All rats were anesthetized with inhalant isoflurane 100% O₂ at flow rate of 200-500 ml oxygen mix. The lower extremity site was denuded of hair with animal hair clippers and was cleaned with povidine iodine. Anesthesia was assessed periodically at 10-15 min by tail twitch. Animal was draped with sterile cloth and prepared with alcohol and povidine iodine. All surgical instruments were sterilized by autoclaving prior to surgery. MPs were sterilized with gamma radiation for 10 minutes appropriate training and approvals were obtained for dept of health and safety at UTHSC.
Surgery

Entire length of antero-lateral aspect of femoral shaft was exposed through a 25-30 mm longitudinal incision, with careful hemostatis. Vastus lateralis and biceps femoralis were held back in position with micro muscle retractors (Life science Technology). Periosteum was longitudinally incised then retracted with periosteum elevator, to expose shaft of femur. A 5 mm critical size longitudinal area was marked with tissue marker sketch using measuring scale; this was followed creation of critical size defect with mini power driver (Life science Technology) using a 1.8 mm bone trephine (Life science Technology). Adjacent tissue was protected with muscle retractors (Life Science Technology). Defect was irrigated with saline intermittently to remove debris with suction. The defect was filled from lateral side with MPs with BMSCs in experimental group and none in control group. Only one implant/defect per animal was used. A custom made Stainless steel plate (18 mm x 3.5 mm x 1.5 mm) (UT tool shop) with two pre drilled 1 mm diameter holes on either side of center of plate, was placed on proximal end of defect. A 1 mm Kirschner wire (K-wire) (Zimmer) was driven carefully holding plate in place with a hemostat and subsequently distal end of defect was also driven with K-wire using a gas operated K-wire driver (Zimmer- Hall Surgical). This process was repeated again to have 2 K-wires driven on either end of defect to hold the supporting stain less steel plate. K-wires were carefully flush cut to avoid sharp edges. This provided our rat femur defect model with very good stability. Periosteum and muscles were reapposed in step wise manner with Ethicon 4-0 suture, the subcutaneous fascia, and skin was closed with Ethicon 4-0 suture. Skin was cleaned with povidine iodine, each animal received injection of Buprenorphine
(0.01-0.05 ml) subcutaneously for analgesia and anesthesia was discontinued before transferring animal to Incubator for next 12 hours.

**Post surgery and recovery**

All rats received careful post operative activity and feeding observation and twice daily evaluation for complications in terms of wound dehiscence, hematoma, and infection for 3 days or until complications are resolved. Surgical wounds site were kept clean and dry. All animals received appropriate analgesic Buprenorphine (0.01-0.05 ml) subcutaneous injections for 3 days to subdue pain. Sutures were removed at end of 7-10 days.

**Regeneration analysis with X-ray**

Animals were monitored for specific time points in study in DLAM facility for activity, pain and infections. At the end of time point of 4 weeks animals were euthanized with CO\textsubscript{2} inhalation. Femurs were extracted via dissection procedure, separating the stainless steel plate to avoid interference in X-ray images and stored in PBS. After appropriate approval and training at UTHSC radiology dept, samples containing rat femurs and control samples were subjected to X-rays with and with-out stainless steel plate both AP and Lateral views. Out-put X-rays images were saved on a CD and reviewed with Dr. Carlos MD orthopedics, for bone tissue regeneration in comparison with control groups at four weeks.
**Statistical analysis**

Minitab version 15 was used with 2-way-ANOVA for cell attachment study. At end of each time point data was gathered from Hemocytometer manual cell counting and recorded in laboratory record book. After completion of all time points the data was compiled using Minitab software with 2-way-analysis of variance. Statistical analysis showed significant difference in attachment between each time point, for all the groups (p<0.05).
CHAPTER III

RESULTS

Chitosan Microparticles (MPS) characterization

CS is a deacetylated derivative of chitin, a high molecular weight and is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β (1–4) manner. In its crystalline form, CS was normally insoluble in aqueous solutions above pH 7; however, in dilute acids like 10% acetic acid (pH 6.0) facilitated solubility of the molecule. MPs obtained from the emulsification process and air drying appeared porous, small spherical structures without any clumps.

Fig 2: Chitosan microparticles physical appearance.
**Scanning electron microscope (SEM)**

Size and surface morphology of Chitosan microparticles prepared by emulsification were observed by scanning electron microscope and most of the Chitosan particles were found to be smooth and spherical although some disintegrated particles were also noted. The size of particle ranged from 40-60 micro meters in diameter. The images obtained are labeled as Fig 3a showing the surface structure, contours and size of Chitosan micro particles and Fig 3b shows similar data for Chitosan with Calcium Phosphate group.
Fig 3 (a) Scanning electron microscope image of CS only MPs

Fig 3 (b) Scanning electron microscope image of CS-10% CaHPO₄ MPs
**In Vitro Studies: Cell attachment study**

The 96 well plates with two groups of particle, their replicates were added with cells suspension containing 20,000 cells and incubated in incubator with 5% CO₂ at 37 °C till four time points under study for 5, 10, 20, 30 hrs respectively. Media was changed at end of two days. At end of every set time point each 96 well plate in treated with trypsin for cell detachment and counted manually with Hemocytometer. The data was gathered and compiled using Minitab software. The compiled data is represented in **Fig 4**.

**Fig 4.Cell attachment study analysis**

![Cell attachment study analysis graph](image-url)
Attachment of MSCs on MPs

Statistical analysis showed significant difference in attachment between each time point, for all the groups (p<0.05). There was also significant difference in cell attachment between each group, at each time point (p<0.05). It was observed that more than 50% of the cells that were initially seeded attached to the MPs after 30 hrs of incubation. Attachment in MPs was significantly lesser than the control wells at the earlier time points. However, at the 30 hr time point this difference was insignificant.

In vivo studies: Bone regeneration analysis with X-ray study

Radiographical examination of the amount of new bone tissue formation in the rat femoral defect sites was evaluated clinically under supervision of Orthopedic surgeon. Via serial radiographic examination, a clear bone repair/bone remodeling process was demonstrated in the femoral bone defect site in the group of rat which received Chitosan MP and BMSCs. X-ray examination demonstrated that abundant density bone-like tissue was formed in the femoral defect sites as early as 2 weeks post-implantation. At 2 weeks post-implantation, newly formed low-density tissue filled the entire defect site and also exceeded the size of the bone defect. At 4 weeks post-implantation, newly formed tissue in the defect site was of higher density, the trabecular structures could be observed, and the two ends of the femoral defect were united by dense osseous bone tissue. Four of four femoral defects in the Chitosan MP seeded with BMSCs group were forming bone by week 4. On X-ray examination; abundant low-density bone-like tissue was observed at 2 weeks post-implantation. This newly formed tissue became denser and smaller, to fit the size of the defect and united the ends of the femoral defect at week 4.
implantation. A mature bone structure that healed the defect site was observed at week 4. Four of four rats in this group demonstrated complete healing of the defect sites, although the medullary cavity had not fully developed. Very little new bone formation was observed in the control group which received only metal plate and defect, 4 weeks after the surgery. X-ray images of rat femurs showing bone regeneration at the defect site with only metal plate Fig 5 (a) and after implantation of MSCs seeded MPs Fig 5 (b).
Fig 5 (a): X-ray images of rat femurs showing bone regeneration at the defect site with metal plate only, at end of four weeks.

Fig 5 (b): X-ray images of rat femurs showing bone regeneration at the defect site after implantation of MSCs seeded MPs at end of four weeks.
CHAPTER IV

DISCUSSION

Stem cell and tissue engineering

Tissue engineering applies biological and engineering principles to the study of cells and tissues to develop functional cell and tissue substitutes. It has recently emerged as a new interdisciplinary science that can be used to repair injured body parts. Skin, bone, and articular cartilage are the first success stories: the FDA has already approved a living skin product, autologous chondrocyte implantation for cartilage repair, and others are flowing through the regulatory pipeline. Techniques that use scaffolds, matrices, hybrid microparticles, transforming growth factors, and pluripotent mesenchymal cells have substantial treatment potential. Stem cell technology utilizes cells that have multiple differentiation potential and may be converted (in vitro or in vivo) to the tissue types needed for functional tissue substitution. Embryonic stem cells are derived from the blastocyst. They may be maintained as undifferentiated cells. They are responsive to differentiation signals in vitro and in vivo and can be used for generating transgenic animals and modeling gene mutations. Adult stem cells are derived from marrow stroma and connective tissues. They may be culture expanded as undifferentiated cells. Stem cells can be used for the following applications: stably transfecting selected cell lines, introduction into blastocoel cavity, production of chimeric and transgenic animals, and potential uses in tissue engineering. Multipotential stromal mesenchymal stem cells can be used to develop articular cartilage, bone, and so forth. Important issues with
mesenchymal or differentiated cells used as tissue or organ substitutes include availability, exendability, inducibility, proliferation, programmed maturation, response to environment, immunocompatibility, and their ability for recombinant manipulation.

Resorbable or nonresorbable matrices or scaffolds, used for tissue repair or replacement can be natural or synthetic (ceramic or metallic). The important features of scaffolds include mechanical stability, cell retention, endo- and exo-biocompatibility, bioactivity, and degradability. The future of tissue engineering will explore the application of paradigms of developmental biology to cell and tissue engineering, cells sources, their isolation, maintenance, and expansion, the development of scaffolding biomaterials, growth factors, and differentiation genes. Stem cells can be isolated from adult bone marrow, but they are rare cells, capable of extensive expansion and multilineage differentiation. The number of mesenchymal stem cells found in bone marrow appears to decline with age, as they are normal diploid human cells that are not immortal.

Mesenchymal stem cells are progenitors of structural and connective tissues, such as bone, cartilage, tendon, ligament, muscle, marrow stroma, and fat. MSCs uniformly differentiate in vitro to multiple lineages, including bone, cartilage and fat. Colonies of MSCs derived from single cells display multilineage differentiation comparable to the parental cells. Factors that influence MSC differentiation are polypeptide growth factors and cytokines, basal nutrients, cell density, spatial organization, mechanical forces. MSCs can differentiate to osteoblastic cells in vitro and in vivo and show the molecular markers of osteogenic cells. In studies of segmental gaps in the rat femur, ceramic implants containing syngeneic MSCs form substantial bone in 8 weeks. In studies of segmental gaps in the canine femur, implantation of autologous MSCs results in callus
formation at 8 weeks and a complete bridge at 16 weeks. Allogeneic MSCs used for bone regeneration do not express large amounts of major histocompatibility complex (MHC) II or co-activators. In a mixed lymphocyte reaction, MSCs do not stimulate T-cell activation or proliferation. Ectopic implants of allogeneic MSCs do not show lymphocytic infiltration and are not rejected. In ceramic cubes, ectopic implants of allogeneic MSCs form bone, and therefore, the goal is a universal MSC product for bone repair. Also, tendon differentiation from allogeneic MSCs appears feasible. Cartilage implants with allogenic MSCs show no rejection. Tissue engineering is likely to revolutionize orthopaedic surgery, engineered tissue substitutes, like bone and articular cartilage, are already available. This technology will take the field of orthopaedics beyond the reactive treatment to the replacement and regeneration of bone, osteochondral defects and ligaments.

**Biodegradable synthetic materials**

One of the goals of tissue engineering technology is bone replacement. However, the complexity of bone makes it difficult to replace it with traditional biomaterials. Allogeneic or autogeneic tissue can be used, but serious concerns about safety and donor morbidity remain. The functions of scaffolds include substrate for anchorage-dependent cells, stimulant for specific cellular response, carrier for growth factors, and retention of cells in defect. Synthetic biodegradable materials are optimal for the purpose. The important properties of materials are its porosity, pore size, and pore structure. Highly porous scaffolds with interconnected pores of diameter 200 to 400 µm are ideal. Mechanical properties are also important as the materials must minimize stress shielding
while providing support. Degradative properties, such as degradation time coupled to rate of tissue regeneration, and degradation mechanism (bulk vs. surface erosion) are important. Degradation products should be non cytotoxic and the materials should be sterilizable. The final product should be reproducibly manufactured, have a long shelf life, be easily handled in surgery, and should fit irregularly shaped defects. Applications for materials include tissue induction, migration and proliferation of host bone cells, cell transplantation, and delivery of bioactive molecules. Bone morphogenic proteins, angiogenic factors, and other bioactive molecules are important for the activation of materials. Polyglycolic acid (PGA) and poly L-lactic acid (PLLA) are the most common, most widely investigated preformed materials. Scaffolds made of these materials can be used with in vitro culture systems to produce large pieces of tissue before implantation. Another exciting option is injectable constructs. They can fill defects of all sizes and shapes; can be used in minimally invasive procedures and cross-link in situ to create highly porous substances. Injectable composite materials are particularly suitable for cavities as they are highly unsaturated materials that can be polymerized in vivo.
Conclusion:

This study shows that CS based MPs support the attachment and proliferation of the MSCs in vitro. These implanted male MSC are likely to have differentiated into osteoblasts because the cells were integrated into the newly formed bone tissue and exhibit function similar to the other cells by forming extracellular matrix in the tissue culture flasks. These results indicate that implanted male MSCs not only act as cell deliverer, but also participated directly in the bone formation process. SEM images show that the MPs from both the formulation were spherical in shape. The spherical and smooth surface structure provides particles with increased surface area for attachment. The particles had a diameter range between 30 to 50 µm which is ideal for cell attachment and proliferation making Chitosan microparticles an ideal delivery vehicle. Attachment studies of MSCs on hybrid MPs showed a significant difference in attachment between each time point, for all the groups (p<0.05). There was also significant difference in cell attachment between each group, at each time point (p<0.05). It was observed that more than 50% of the cells that were initially seeded attached to the MPs after 30 hours of incubation. Attachment in MPs was significantly lesser than the control wells at the earlier time points. However, at the 30 hours time point this difference was insignificant. This result may guide our future studies for replication of results or further enhancement as cells need at least 30 hours for 50% cell attachment. These studies conclude that microparticles (MP) Chitosan and Chitosan with Calcium Phosphate as suitable constructs for bone marrow mesenchymal cell attachment, proliferation in vitro.
X-ray imaging shows very little new bone formation in the control defects 4 weeks after the surgery. When in the defect site was implanted with the MSCs seeded MPs mature bone formation was observed at the defect site, suggesting the Chitosan MPs + BMSCs also aid in bone regeneration in vivo at 4 weeks after bone defect. Suggesting an earlier bone tissue formation with Chitosan microparticle and bone marrow mesenchymal cells construct as compared to previous studies.
**Future direction**

Considering improvement in regeneration at 4 weeks time period it would be worthwhile to investigate progress over subsequent time points using Chitosan with calcium phosphate group *in-vivo*. We are currently looking at *in vivo* bone regeneration at 8 weeks of implantation at with 1.5% Chitosan group. Chitosan being a versatile bio polymer provides the opportunity to use and correlate studies involving growth factors and Neovascularisation. At present, CS is one of the most promising biopolymers for tissue engineering and possible orthopedic applications. In particular, the possibility to generate structures with predictable pore sizes and degradation rates make CS a suitable material as a bone graft alternative in orthopedic procedures. However, efforts to improve the mechanical properties of CS-based composite biomaterials are essential for this type of application. Of great importance is the ability of CS to bind anionic molecules such as GFs, GAGs and DNA. In fact, the combination of good biocompatibility intrinsic antibacterial activity, ability to bind to growth factors and to be processed in a variety of different shapes makes CS a promising candidate scaffold material for cartilage, intervertebral disc and bone tissue engineering in clinical practice. Moreover, the ability to link CS to DNA molecules renders this material a good potential as a substrate for gene activated matrices in gene therapy applications in orthopedics. Imaging analysis can be extended in future to incorporate Semi quantitative methods and micro CT techniques for better resolution and quantification. Histopathological analysis can provide clues for type and evidence of on new bone formation. Biomechanical testing for
strength, bio-compatibility and degradation of the regenerated bone may advance our knowledge to enhance its application in future.
Clinical outcome of fractures of the talar body
Nabil A. Ebraheim & Vishwas Patil & Christopher Owens & Yugandhar Kandimalla

Abstract

Fractures of the talar body present a great challenge to surgeons due to their rarity and high incidence of sequelae. This study reports the medium-term results of displaced fractures of the talar body treated by internal fixation. Nineteen patients (13 M, 6 F, mean age 31) with talar body fractures were studied retrospectively to assess outcome after operative treatment. The fractures were classified as coronal (11), sagittal (6) and crush fractures (2). Six patients sustained open fractures and two had associated talar neck fractures. Average follow-up was 26 months (range: 18–43). Clinical outcome based on American Orthopaedic Foot & Ankle Society (AOFAS) ankle-hind foot scoring was excellent function in four patients, good in six, fair in four and poor in five. Early complications included two superficial wound infections, one partial wound dehiscence, one instance of skin necrosis and one deep infection. Other complications included delayed union in one, avascular necrosis in seven and malunion in one patient. Talar injuries are serious because they can compromise motion of the foot and ankle and result in severe disability. Crush fractures of the talar body and those associated with open injuries and talar neck fractures are associated with a less favorable outcome.
Introduction

Fractures of the talar body are highly uncommon. Fractures of the talar body, although constituting one of the rare injuries of the body, present a very grave prognosis. They present a greater risk for avascular necrosis than talar neck fractures. They constitute less than 1% of all fractures and 13–23% of talus fractures [2, 9]. They usually follow high energy injuries and are therefore associated with considerable soft tissue damage. Furthermore, fractures involving the talar body can be difficult to visualize adequately because of the shape of the tibiotalar articulation and the overhang of the anterior and posterior tibial plafond. Generally, it is well-protected by the tibial plafond superiorly and malleoli on either side. Sneppen et al. found that only certain non-physical forces, e.g. pronounced caudal compression, force during pronation and especially supination trauma, will injure the body of the talus. They found that with regard to talar body fractures, a medial site fracture was typical of supination trauma (compression or shear type), whereas a lateral site fracture was typical of pronation or pronation-external rotation trauma (compression fracture) [18]. The other common mechanism of injury resulting in fracture of the body of the talus is a fall from a height, producing an axial compression of the talus between the tibial plafond and the calcaneus. These fractures are often associated with other ankle, foot and skeletal injuries which complicate the treatment. Since fastening of seat belts has become mandatory, more patients involved in high-velocity motor vehicle accidents survive, which increases the number of injuries to the distal extremities. Only few studies in the past have reported on the talar body
fractures detailing the results of their management as compared to the more common fractures of the talar neck [6, 7]. Hence the aim of this study was to report the medium-term results of displaced fractures of the talar body treated by internal fixation.

**Materials and methods**

A retrospective study was performed to analyze the outcomes of patients with talar body fractures between February 1998 and January 2004. Those included in the study were treated by operative methods with a minimum follow-up of 18 months after the injury. The exclusion criteria were patients with an isolated talar neck fracture without talar body fracture, patients with an isolated posterior process fracture and those with solitary transchondral lesions. Nineteen patients who met the inclusion criteria were considered for this study. Six patients were female and 13 were male. The mean age was 31 years with a range of 21–68. Twelve fractures were to the right talus compared to seven on the left side. Seven patients were injured due to a fall, nine as a result of motor vehicle accident (MVA), two after motorcycle accidents and one patient was injured as a pedestrian in a MVA. The talar body fractures were classified according to Boyd and Knight’s classification [3]. The surgical approach depended upon the fracture pattern and hence a single or combined approach was used. Medial malleolus osteotomy was also performed if a better exposure was needed. Cancellous screws (4 mm) and small Herbert screws were used according to the fracture pattern. Bone grafting was not performed in any cases. Patients were kept non-weight-bearing by wearing an off the-shelf fracture boot with early foot and ankle motion for 10–12 weeks, until radiographs and clinical examination revealed evidence of union. Anteroposterior, lateral and mortise radiographs
were routinely made at 2 weeks, 6 weeks, 10–12 weeks and 6 months post-operatively. Additional radiography, computed tomography scans and magnetic resonance imaging was performed as often as needed.

Clinical results were evaluated using the clinical rating scale of the American Orthopaedic Foot & Ankle Society (AOFAS) (100 points total) [12]. The questionnaire assessed the level of pain, function and alignment. Patients were asked to rate pain on a scale of 0–40 points. Function was calculated as the total score of activity limitations (0–10), maximum walking distance (0–5), walking surface (0–5), gait abnormality (0–8), sagittal motion (0–8), hindfoot motion (0–6) and ankle hindfoot stability (0–8). Alignment was rated from 0 to 10 points (good, fair, poor), giving a total score of 100 points. Excellent was defined as a score of 85–100, good as 75–84, fair as 70–74 and poor as less than 70 [12]. Post-traumatic arthritis was defined as a decreased joint space, juxta-articular osteophytes, subchondral sclerosis and/or subchondral cysts seen either on plain radiographs or on computed tomography scans. Osteonecrosis was documented as present or absent as seen on standard radiographs.

**Results**

The 19 patients who were evaluated in clinic following operative treatment constitute the cohort of this study. The type of talar body fractures according to Boyd and Knight [3] was as follows: 17 type I fractures, 11 coronal and 6 sagittal and 2 crush fractures. Six patients sustained open fractures: one type I, three were type II, one was type IIIA and one was type IIIB. Furthermore, two patients had associated talar neck fractures: one Hawkins type I and another Hawkins type II. The average time from injury to surgery
was 2 days with seven patients undergoing surgery less than 24 h after sustaining the fracture; the range was 9 h to 13 days. During surgery, the medial approach was used in six procedures, the lateral approach was used in three and a combined approach was performed in ten. A medial osteotomy was performed in six patients for better exposure of the fracture. No fibular osteotomies were performed. The average duration of follow-up was 26 months (range: 18–43).

According to the AOFAS rating system, four patients had an excellent function, six had good function, four had fair function and five had poor function. The average total score was 68.6 (range: 44–94). (Table 1)

**Table 1: Average American Orthopaedic Foot & Ankle Society (AOFAS) ankle-hindfoot score at the last follow-up Score.**

<table>
<thead>
<tr>
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<th>Score</th>
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<tbody>
<tr>
<td>Pain</td>
<td>27.4</td>
</tr>
<tr>
<td>Activity limitations</td>
<td>6.9</td>
</tr>
<tr>
<td>Walking distance</td>
<td>3.2</td>
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<tr>
<td>Walking surface</td>
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<td>Gait abnormality</td>
<td>4.4</td>
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<td>Sagittal motion</td>
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<tr>
<td>Hindfoot motion</td>
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</tr>
<tr>
<td>Ankle-hindfoot stability</td>
<td>8</td>
</tr>
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<td>Alignment</td>
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Total score: 68.6 (range: 44–94)

Early complications occurred in five patients and included two superficial wound infections, one partial wound dehiscence, one instance of skin necrosis and one deep
infection. Late complications were also assessed. One patient had delayed union and another had malunion. Both these patients had a fair outcome. Seven patients suffered from avascular necrosis (AVN); two of these were in patients who had suffered neck and body fractures while the other five were in fractures that only involved the body. In five of the cases of AVN, collapse occurred. Additionally, 11 patients suffered from subsequent ankle arthritis while 6 patients had subtalar arthritis. Fractures with open injuries had a high incidence of complications. Among the six patients (three coronal, one sagittal, two crush fractures) with open fractures, avascular necrosis developed in five, superficial wound necrosis in two, deep infection in one, arthritis of the ankle joint in six and subtalar joint in five. The clinical outcome was poor in four patients and fair in two. Two patients (both coronal fractures) with associated talar neck fractures had a poor outcome. One patient (Hawkins type I) had an open injury (grade II) and developed AVN along with superficial wound infection. The other patient (Hawkins type II) had skin necrosis and AVN. Radiographs demonstrated osteoarthritic changes in both ankle and subtalar joints. Among the various talar body fractures, two patients (both had open injuries, grade IIA and IIIB) with crush fractures had a poor outcome. In the six patients with sagittal fractures (two open injuries, grade I and II), three patients had a good outcome, one excellent, one fair and one poor outcome. Of the 11 patients with coronal fractures (2 open injuries of grade II and 2 talar neck fractures), 3 patients had excellent outcome, 3 good, 3 fair and 2 poor outcome. Radiographs of a patient with a coronal fracture are depicted in Figs. 6, 7 and 8.
Fig. 6: Lateral (a) and anteroposterior (AP) (b) radiographs of a patient with fractures of the talar head and lateral process of the posterior tubercle. Anterior talofibular ligament was also disrupted.

Fig. 7: Immediate post-operative lateral (a) and AP (b) films show internal fixation with Herbert screws and repair of anterior talofibular ligament.
Fig. 8: Lateral (a) and AP (b) radiographs after 23 months of follow-up. The patient had an excellent clinical outcome.

Discussion

Talus is derived from the French word talo which means ankle; its origin is from Greek and Roman gambling games. The Romans used dice that were the heel bones of horses called taxillus. Greeks, however, used the second vertebrae of sheep, which they called the astragalus. Both of these derivations eventually became associated with the foot bone known today as the talus [8]. In 1952, Coltart described the largest series ever reported of injuries of the talus that he collected from the Royal Air Force during World War II, which included 15 cases of fracture of the talar body [4]. A literature search reveals that in many of the previous studies, the majority of patients were treated nonoperatively [5, 11], and talar body fractures were not distinguished from other fractures of the talus [6, 7]. Injuries to the talus are serious because this bone is the critical link between the subtalar, transverse tarsal and ankle joint complex. The variety of coupled actions
occurring from the motion of this bone or surrounding bones permits the effortless flexibility of the hindfoot and midfoot. If the talus is damaged, the universal combined motion of the foot and ankle becomes compromised, and severe disability can result [17]. Inokuchi et al. differentiated talar neck and body fractures by critically analyzing the inferior surface fracture line. They defined a talar body fracture as one in which the fracture line on the inferior surface extends into the subtalar joint [10].

The body of the talus is supplied by vessels that enter through five surfaces: the superior surface of the talar neck, the anterolateral surface of the talar body, the inferior surface of the talar neck, the medial surface of the talar body via the deltoid ligament and the posterior tubercle [16]. The artery of the tarsal canal is the most significant supply of blood to the body of the talus. It supplies the middle one-half to two-thirds directly and through anastomosis potentially supplies the entire body (Fig.9).
Fig. 9: Blood supply to the various regions of the talus. The artery of the tarsal canal provides the predominant blood supply to the talar body.

Its largest branches enter through the inferior surface of the neck. The medial one-third of the body is directly supplied by the deltoïd branch which enters from the medial surface of the talus and supplies even more due to anastomosis. The artery of the tarsal sinus branches and then enters the anterolateral body and supplies the lateral one-eighth to one-half directly. The superior neck vessels also make a minor contribution. The system of vessels over the posterior tubercles mostly supplies the tubercle itself [14]. The vascular supply varies among the regional sections of the talus with the anterolateral surface of the talar body and the posterior tubercles of the talus being relatively avascular [15]. Boyd and Knight have classified talar body fractures according to the plane of the fracture line. A type I fracture is a coronal or sagittal shear fracture, compared to a type II fracture which occurs in the horizontal plane [3]. The most common mechanism of injury
resulting in a shearing-type talar body fracture is a fall from a height resulting in axial loading. Nondisplaced or minimally displaced shearing-type fractures of the talar body can be sometimes difficult to recognize on the plain radiographs. A delay in diagnosis and treatment may be associated with an increased risk of osteonecrosis, malunion, secondary osteoarthritis and other complications. Abrahams et al. reported on two cases of nondisplaced shear fractures of the talar body that were not recognized initially and recommended that these fractures should be suspected in patients with a history of axial loading injury and diffuse ankle swelling when no other fracture, particularly calcaneal, is identified on radiographs [1]. Displaced talar body fractures often result in significant morbidity. Vallier et al., reporting on radiographic findings of 26 talar body fractures with a minimum follow-up of 1 year, noted a 38% incidence of AVN, 65% incidence of post-traumatic tibiotalar arthritis and 34% incidence of posttraumatic subtalar arthritis. Worse outcomes were noted in association with comminuted fractures, associated talar neck fractures and open fractures [20]. Lindvall et al., in 2004, reported on 26 isolated talar neck and body fractures with a minimum follow-up of 48 months and found a 50% incidence of AVN and 100% incidence of post-traumatic arthritis. Timing of fixation did not appear to affect the outcome, union or prevalence of AVN in the later study because the fractures that were stabilized within 6 h did not have a lower incidence of AVN than those stabilized after 6 h [13]. Both of these studies concluded that patients with these injuries should be counseled on the long-term complications and that arthritis is an expected outcome in displaced talar body fractures despite accurate reduction with stable fixation. Sneppen et al. (1997) reviewed 51 patients with talar body fractures. In cases in which significant talar compression occurred they found that 50% of patients had ankle
osteoarthritis; if the talus exhibited a shearing pattern of injury, the incidence of post-traumatic arthritis in both the ankle and subtalar joints was 41%, with a further 24% of patients having osteoarthritis in either ankle or subtalar joints. They concluded that results in talar body fractures are directly related to the severity of the initial injury and emphasized that if subluxation and articular damage to the subtalar and talotibial joints occurred at the initial injury, long-term prognosis is poor [18].

Talar body fractures have been associated with a high incidence of complications including osteonecrosis, malunion, nonunion, secondary osteoarthritis, subtalar bony ankylosis, skin infection and skin necrosis. The incidence and severity of these complications appear to relate to several factors including the intrinsic talar vascular supply, the initial extent of displacement, the presence of associated dislocation and the adequacy of reduction [1]. This associated with the fact that more weight per area is borne by the talar dome than any other joint in the body means that post-traumatic arthritis and long-standing disability are frequent complications to fractures of the talus [14]. Avascular necrosis of the posterior fragment of the talar body or the superior fragment in the case of a horizontal fracture is particularly common [19].

Usually the fracture pattern and location will determine the choice of surgical approach [20]. However, the shapes of the tibiotalar articulation and the overhang of the posterior tibial plafond sometimes limit access to the posterior parts of the talus. Distraction of the tibiotalar joint can be used for reconstructive procedures but is risky in the acutely injured situation. When necessary, a medial or lateral osteotomy should be performed to provide exposure to all aspects of the talar body. There are a few limitations in this study. First, as
the study was retrospective in nature, the results were derived from the charts and radiographs. Second, no meaningful statistical derivations were obtained because of the small study sample. Further multi-centre prospective studies are needed to provide better and more definite conclusions on the relationship between type of fracture, open injuries and other factors with the clinical outcome.

In conclusion, talar body fractures are unique and very challenging injuries for the surgeon. Crush fractures of the talar body and those associated with open injuries and talar neck fractures are associated with a less favorable outcome.
References: For clinical published paper


**References: For MSBS thesis**


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