A Pre-clinical Assessment of Lithium to Enhance Fracture Healing in Healthy and Impaired Bone Rat Model

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science- Biomedical Engineering
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Fracture healing is a complex biologic process that fails in 5-10% of cases. There is a critical need for an adjunct treatment that can enhance fracture healing, particularly in osteoporotic bone. Lithium, the standard treatment for bipolar disorder, has been reported to improve fracture healing via the canonical Wnt pathway. Recently, a pre-clinical study in our laboratory utilized a design of experiments approach to optimize the lithium administration parameters, resulting in improved strength (~46%) of healing femurs in otherwise healthy rats. In this thesis, the optimized lithium regimen was verified in healthy rats (~44% improvement). This regimen did not significantly impact healing of simple or comminuted fractures in osteoporotic rats. The lithium onset time may need to be extended to better target and enhance osteogenic activity in fractured osteoporotic bones. This thesis supports clinical evaluation of lithium for healthy long-bone fractures and will guide pre-clinical lithium optimization in impaired bones.
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In 2014, I began my Masters under Dr. Diane Nam and Dr. Cari Whyne. Two years later, I look back at my journey since then and realize how fulfilling this experience has been. The two women have been truly inspiring in their own ways. Their guidance and encouragement has been instrumental not only at times of success but, more importantly, when my experiments failed. Cari exemplifies a true leader, one that takes responsibility but not credit. Her active involvement and tremendous willingness to assist me throughout these years is clearly reflective of her dedication as a mentor. I was also very fortunate to have Diane as my co-supervisor. She was personally involved in all of the animal surgeries and her insight was crucial for the ultimate success of this project. Despite her expertise, Diane has always been highly receptive to my opinion. Her immense patience and humbleness have undoubtedly helped me to learn and grow in the process.

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1. Background

1.1 Bone

The human skeletal system is a sturdy framework of bones that is held in place by other connective tissues (primarily tendons, ligaments and cartilage) and muscles. Bone is a composite material made of hydroxyapatite crystals \([\text{Ca}_5(\text{PO}_4)_3(\text{OH})]\) embedded in a collagen-rich matrix (primarily type I) (Crockett et al., 2011). Its primary functions relate to structure, protection of internal organs and locomotion. Moreover, its marrow harbors mesenchymal and hematopoietic stem cells, which serve as the primary source for different bone cells and blood cells, respectively. A third important role of the bone is derived from its matrix composition- the bone tissue serves as the body’s main reserve for calcium and phosphorous (Crockett et al., 2011).

Osseous tissue is categorized as cortical or cancellous. Cortical bone consists of longitudinally organized concentric lamellae in a dense matrix (~10% porosity) and it forms the outer hard shell of the bone (Martin, Burr, & Sharkey, 2004). In contrast, cancellous bone is a porous network of thin rod and plate-like trabeculae (~90% porosity) lying within a cortical shell (Martin, Burr, & Sharkey, 2004).

Bones are optimally shaped for their physiological function and location. Accordingly, they are categorized as long, short, flat and irregular. Long bones have a characteristic “shaft” and are part of the appendicular skeleton. They provide structural support to the upper and lower halves of the body and enable limb motion. Short bones are small in size with roughly equal length and width, and they provide stability to other bones. Flat bones are thin with some marrow between the cortical layers. They protect vital internal organs such as the brain, heart and lungs, and also provide a large surface area for tissue attachment in the body. Irregular shaped bones include bones with unique shape and size, such as vertebrae and the mandible, which are designed according to their functionality and location.

The femur is the largest long bone in the body and is one of the most common sites for bone-related injuries. It is characterized by a large length-to-diameter ratio, and is comprised of three regions- epiphyses at the ends, inner metaphyses and a diaphysis that constitutes the length of its shaft (figure 1). Epiphyses and metaphyses have thin cortices surrounding a dense trabecular...
meshwork that is filled with hematopoietic red marrow. The diaphysis is exclusively thick cortical bone with a hollow center filled with fatty yellow marrow and some red marrow.

Figure 1: Anatomy of human femur (“Anatomy Human Body”, n.d.).

The bone’s biomechanical properties play an important role in conferring its load bearing behavior. Bone is anisotropic, meaning that is behaves differently based on the direction of the force. Accordingly, the bone is strongest in compression, followed by tension and weakest in torsion. Upon loading under these three conditions individually, the ultimate strength for a healthy femur has been found to be 190 MPa, 150 MPa and 80 MPa, respectively (Reilly et al., 1974). This anisotropy is physiologically justified as bones are generally subjected to axial forces rather than shear. The collagen fiber arrangement in the longitudinal direction (rather than perpendicular to its central axis) is crucial for resisting bending motion (Vashisht et al., 2007).
Bone is also viscoelastic, meaning that the rate of loading also dictates its response. When loaded, the stress versus strain curve includes elastic as well as plastic deformation region prior to complete failure. The curve begins with a small linear region, with its stiffness primarily defined by its hydroxyapatite mineral phase (Launey, Buehler & Ritchie, 2010). After the yield point, the curve transitions into the non-elastic region where the applied energy is absorbed by the bone leading to permanent changes in its microstructure or geometry. The toughness of the bone is governed by its collagen rich organic matrix (Launey, Buehler & Ritchie, 2010). As increasingly higher loads continue to be applied, cracks propagate and the bone ultimately breaks. Typically, in everyday motions, the bones of the human body are loaded slowly enough under physiologic loading such that it is able to adjust and sustain the load (0.1-1% strain/second) (Cristofolini et al., 2009; Keavey, Morgan & Yeh, 2004). However, when high forces/loads are applied, often rapidly as in an high impact scenario, the bone is unable to accommodate the loading resulting in damage or catastrophic failure.
1.2 Fracture Patterns

A healthy bone is injured when it experiences extrinsic forces that are greater than its yield capacity. Unable to be absorbed, the excess energy is dissipated through the bone causing a break in its continuity; the extent of damage depends on the bone involved, its underlying condition as well as the type and amount of force inflicted. The most common long bone fracture patterns include transverse, oblique, spiral and comminuted fractures (figure 2). All these fractures involve bicortical damage.

Transverse fractures typically occur in the diaphyseal region of long bones. They involve fracture lines propagating perpendicular to the long axis in a single horizontal plane. Bones subjected to high bending forces are likely to experience a transverse fracture pattern (McGee, Qureshi, & Porter, 2004).

Oblique fractures are characterized by a fracture line cutting through the cortices in a single, angled plane (30-45°). They result from high forces hitting the bone at an angle rather than perpendicularly (Pierce, Bertocci, Vogeley, & Moreland, 2004).

Spiral fractures are similar to oblique ones but with lines traversing multiple planes. They generally occur upon excessive torsional force rather than axial loading as in the case of transverse and oblique fractures. However, axial forces are involved to some extent and the fracture line propagates in a winding fashion around the bone (McGee, Qureshi, & Porter, 2004).

The above three patterns result in two primary bone fragments. In contrast, comminuted fractures result in multiple fragments of varying sizes and shapes consequent to infliction of a combination of forces in multiple directions (Pierce, Bertocci, Vogeley, & Moreland, 2004). They are generally associated with excessive trauma and soft-tissue injury; however, low-quality bones may experience such fractures even under low loads.
Figure 2: Femur fracture patterns (“Femoral Fractures”, n.d.).

The “AO Classification” for long-bone fractures is based on fracture location, pattern and severity (AO Foundation, 2014). Diaphyseal fractures may be either type A (simple), B (wedge) or C (comminuted). Each of these types is further subdivided based on the shape of the resulting fragments. Healthy patients mainly experience high-impact induced fractures of type B and C, while patients with reduced bone density (typically the elderly) may experience fragility fractures of type A and B. AO Classification is widely used for evaluation of surgical options and comparison of treatment outcomes.
1.3 Fracture Burden and Current Treatment Options

From fracture occurrence until recovery, the patient often requires one or more surgeries, post-surgical care and physiotherapy. Complications such as secondary fractures, implant failures and delayed or non union increase this period to recovery, and require additional resources to achieve complete union under a complicated biological scenario (Nikolaou et al., 2009). Clearly, fracture treatment is complex and burdens the healthcare system as well as the individual financially and socially. In United States, 5-6 million fractures occur annually and osteoporotic fractures alone costing nearly $20 billion in direct treatment (Reginster, & Burlet, 2006). High cost of fracture treatment is also typical in other countries around the world (Haussler et al., 2009, Johnell, & Kanis, 2004; Lee, Lim, & Lam, 2008; Reginster, & Burlet, 2006).

Treatment of long bone fractures generally involves a gradual increase in dynamicity and micro-motion at the fracture site (Dimitriou, Tsiridis, & Giannoudis, 2005). In contrast to humerus fractures that are generally treated non-surgically using arm casts, long bone fractures of lower limbs typically are fixed internally with customized implants such as screws, plates, intramedullary nails, and bone grafts (Lambiris et al., 2007). Non-conventional strategies to promote bone growth using electrical stimulation therapy, cell and growth factor rich scaffolds, and intermittent parathyroid hormone (PTH) therapeutic use have been aggressively pursued in the recent decade (Leung et al., 2009). Growth factors under evaluation include platelet derived growth factors and fibroblast growth factors, but the most extensively evaluated and effective candidates are bone morphogenic proteins (BMP) (Giannoudis, Psarakis, & Kontakis, 2007). Clinically, BMP-2 may be used for new fractures, while BMP-7 is applicable for nonunions (De Biase, & Capanna, 2005; Jones et al., 2006). However, both are primarily used in tibial fractures at present, and their application for long bone fractures still requires optimization of dosage and timing (von Ruden et al., 2016; White et al., 2007). Intermittent PTH therapy has proven to be effective in fracture models, but the dose used in many of these studies exceeded the safe administration range in humans (Li et al., 2012; Black et al., 2003). Thus, its translation is hindered by a lack of comprehensive knowledge of its therapeutic conditions for fracture treatment particularly since PTH administration can only be for a short duration. Overall, for various reasons including efficacy, cost and accessibility, none of these options are applicable in a widespread, quick manner with manageable concerns of their side effects.
Diaphyseal fractures are successfully treated in 90-95% of the cases and generally heal within 6 months. However, such long durations imply significant lost work hours and lengthy dependency on others for daily activities. Moreover, although delayed or non-union occurs in only a small fraction of cases, the number of these patients is significant based on the overall incidence of long bone fractures. Longer healing periods in these patients is associated with an increased risk of complications, co-morbidites and mortality.

Thus, fracture epidemiology and treatment-related limitations warrant the need for an adjunct treatment that can accelerate or enhance the process of healing. Such a treatment option should be safe, widely available, easily implementable and inexpensive to enable access worldwide.
1.4 Process of Fracture Healing

Fracture healing is a well-orchestrated, multi-stage repair process that aims to restore union and pre-fracture functionality. Long bone fractures heal through three main stages: inflammatory, reparative and remodeling (Figure 3) (Buckwalter, Einhorn, & Marsh, 2006; Frost, 1989). This process of fracture healing is applicable to rodent, larger animals and humans. Each stage is mediated by intricately linked signaling pathways with cross-talk among cytokines and the various cell types in the milieu, both affected by the mechanical forces experienced at the site of injury (Buckwalter, Einhorn, & Marsh, 2006; Einhorn, 1998).

Fracture injury is often accompanied by soft-tissue damage with disruption of blood supply at the fracture site. In response, the immune system infiltrates the region with pro-inflammatory cytokines and blood cells generating a hematoma (inflammatory stage). Fibroblasts then aggregate to form loose granular tissue as seen in wounds.

Next, the reparative phase begins. Mesenchymal progenitor cells differentiate into osteochondral progenitor cells that have the potential to form either chondrocytes or osteoblasts. Their fate is decided by the requirement, which is determined by mechanical stability at the site. Thus, there is either direct bone formation via intramembranous ossification or bone development following stabilization via endochondral ossification. In the former case, the bone fragments have to be separated by a very small gap (<2 mm) and should remain completely stable so that osteoblasts and blood vessels can directly travel across the gap. Thus, little or no soft callus develops and new bone directly forms in the gap. However, if the fragments experience considerable micro- or macro motion, a stiff material like bone cannot form. In this case, stability is first achieved by the endochondral pathway in which chondrogenesis occurs and forms a soft callus. Such a matrix, with an elongation ability of nearly 100%, is able to withstand motion while crudely affixing the fragments. As stability is achieved, this matrix begins to mineralize, ultimately forming hard osseous tissue. Thus, the mature chondrocytes begin to hypertrophy and, in combination with mineral deposition, the soft callus transforms to a stiffer bony callus. Osteoblast activity peaks at this stage and the gap is filled with unorganized “woven” bone tissue. Radiographically, the gap appears radiopaque and union is defined as restoration of continuity at three of the four cortices in antero-posterior and medio-lateral planes.
In the final remodeling stage, the woven bone is replaced by organized lamellar bone, which also restores the original shape of the bone. In humans, the first two stages of endochondral healing together take 3-6 months, depending on the bone involved and fracture severity (Frost, 1989). At the end of these stages, the patient regains functionality. However, remodeling continues for several months to even years. In contrast, intramembranous healing requires greater post-operative care to ensure stability and takes longer time period to achieve bony union (Epari et al., 2006).

![Fracture Healing in the Rat](image)

**Figure 3:** Timeline for long bone fracture healing via the endochondral pathway in rats (modified from Strohbach, Rundle, & Strong, 2011).
1.5 Biomechanical Progression in the Healing Process

Upon bicortical damage, biomechanical changes follow the biological progression during the course of healing. White III, Panjabi and Southwick (1977) were the first to classify these changes into four stages. While these findings were based on tibial osteotomies in rabbits, the stages are applicable to other species including rodents and humans.

Stage I is characterized by a low stiffness matrix that becomes the site of bone failure when loaded. This is associated with the initial granulation tissue or the soft callus prior to mineralization. Stage II involves a high stiffness matrix but is still mineralizing or is woven bone at best. Failure occurs completely through this site. Transition from stage I to II is often the time-point of interest for animal studies as its occurrence indicates a successful ongoing process to forming the bony tissue. Stage III has a high stiffness matrix that is in the process of restoring the pre-fracture strength and thus specimen failure occurs through both the old and new bone. Achievement of stage III suggests that union has been established and pre-fracture properties have been nearly attained. Stage IV is when the bone is completely healed and, undoubtedly, the matrix at this stage demonstrates high stiffness. If subjected to mechanical loading, it will fail through the intact portion only and demonstrate the strength and toughness of a normal bone. These stages always occur in the described order; however, the time required to achieve them varies between species.
1.6 Canonical Wnt Pathway in Fracture Healing

The Wnt signaling pathway is one of the key mechanisms for bone repair (Logan et al., 2004). β-catenin, a key downstream molecule in the pathway, is up regulated at time points as early as day 4 post fracture in mice (Chen et al., 2007). The pathway’s activity remains up regulated throughout healing compared to the baseline levels in a normal bone.

The Wnt pathway is complex and involves several cytokines (Logan et al., 2004). In its inactive state, the pathway’s membrane receptors, Frizzled and Lrp5/6, remain inactive. In the cytoplasm, Axin, casein kinase-1a (CK-1a) and adenomatous polyposis coli (APC) form a complex, which binds β-catenin for destruction. β -catenin is then dephosphorylated by glycogen synthase kinase-3β (GSK-3β) and removed by proteasome. Thus, the pathway’s targeted binding between B-catenin and transcription factors TCF/LEF does not occur, and transcription of osteogenic genes is inhibited. Additional cytokines such as dickkopf-1 (Dkk-1), sclerostin and secreted Frizzled-related protein 1 (Sfrp1) further regulate the pathway and prevent Wnt’s action.

![Figure 4: Wnt signaling pathway in the absence (A) and presence (B) of Wnt molecule (Deb, 2014). This figure is reproduced with permission from Oxford University Press (see Appendix).](image-url)

When a Wnt molecule is expressed, the pathway is activated. Wnt molecules bind to the cellular
receptors causing the disassembly of the destruction complex. Thus, β-catenin is free in the cytoplasm and its accumulation eventually leads to its translocation into the nucleus. Here, it binds to TCF/LEF and thus, the expression of Wnt-target genes is initiated. The key genes are related to osteoblast differentiation including receptor activator for nuclear factor k B ligand (RANKL) and runt-related transcription factor 2 (Runx2). An increase in osteoblasts is crucial for mineralization of the soft callus and its conversion from a cartilaginous form to stiffer bony matrix.

A main category of molecules pertains to inhibition of GSK-3β. Other cytokines of interest include antibodies for Dkk-1 and Sfrp1, which have been shown to be effective in promoting β-catenin in preclinical models. Anti-sclerostin is currently in early trial stages (discussed in next section), but its long-term risks are as yet unknown.
1.7 Potential Wnt Pathway Based Therapeutics for Fracture Healing

Several molecules have been considered for promoting bone regeneration at the fracture site including locally delivered growth factors and parathyroid hormone. Apart from these, the anabolic nature of the Wnt/β-catenin pathway for augmentation of bone has led to extensive research of its various molecules as potential therapeutic targets. However, no treatment based on this pathway has yet reached the fracture clinics.

Modulation of the canonical Wnt pathway can be achieved by selective up-regulation of Wnt agonists (norrin, r-spondin) or down-regulation of Wnt/β-catenin antagonists (secreted Frizzled-related proteins, Wnt inhibitory factors, dickkopf-1, sclerostin) (MacDonald, Tamai, & He, 2009). Antibodies targeting Dkk-1 and anti-sclerostin for bone formation under osteoporotic conditions have yielded promising results in in vivo studies (Cui, Cheng, & Song, 2014; Li et al, 2009). However, Amgen led phase II trials (NCT00907296 and NCT01081678) to evaluate anti-sclerostin antibody for fragility fractures have not progressed into Phase III and Amgen has announced to discontinue their further pursuit (Levin, 2013). A recent publication showed increased bone mass in fractured rats upon subcutaneous treatment with bispecific antibody for Dkk and sclerostin (Florio et al., 2016). However, their long-term systemic effects remain unknown and, if proven effective, their cost and long time period prior to clinical use are major limitations. A clinical trial for evaluating Dkk-Ab in myeloma patients with prior fractures began in 2009 (NCT00741377); the final results from this study are yet to be published. Even if successful, additional studies will be required for its use in other patient cohorts prior to its incorporation into clinical use.

Another widely considered option is inhibition of GSK-3β. AZD2858, an oral inhibitor molecule has been shown to up-regulate new bone formation in rats via intramembranous healing (Sisask et al., 2013). However, in general, systemic modulation of GSK-3β remains a primary concern as GSK-3β is involved in several important pathways related to cell proliferation, inflammation, apoptosis. Its expression has been associated with neurologic disorders, cancer and bipolar disorder (Ali et al., 2001). Thus, an ideal GSK-3β inhibitor molecule will be one that is highly selective in mediating its effect for fracture healing without impacting other pathways.
Osteoporosis and Its Impact on Fracture Healing

Fracture repair is affected when the underlying bone is of poor or abnormal quality. This happens in the case of bone metabolic disorders (osteoporosis and diabetes), metastatic cancer and bone related genetic disorders (osteomalacia and osteopetrosis). Of these, the most prevalent condition with the greatest burden on the healthcare system is osteoporosis.

Osteoporosis is characterized by a significant reduction in bone mineral density and increased susceptibility to low-energy fractures. The impaired bone condition is clinically evaluated by dual-energy X-ray absorptiometry; a bone mineral density of 2.5 SD lower than normal is clinically defined as osteoporotic. Osteoporosis is caused by the loss of physiological balance between bone absorption and bone resorption in the remodeling process, which gets tipped towards the latter, leading to bone mineral loss over time. Currently, nearly 200 million people are estimated to suffer from osteoporosis worldwide (Cooper et al., 1992). In Canada, an estimated $2.3 billion are spent annually in direct treatment of osteoporotic fractures with additional long-term rehabilitation pushing the number to $3.9 billion (Tarride et al., 2012).

Osteoporotic fractures mainly occur at loading sites with abundant trabecular bone due to rapid destruction of trabeculae prior to significant cortical damage. This is due to the larger exposed surface area of the trabeculae for the osteoclasts to act on in comparison to the compact cortical bone. Primary sites of osteoporosis include the vertebrae, the proximal and distal ends of long bones, and the wrist. Sites of trabecular bone are less radiopaque due to reduction in trabecular number as well as thinning of trabeculae. An osteoporotic long bone has a widened medullary canal and concomitant circumferential increase of the diaphysis due to endosteal resorption and periosteal apposition, respectively.

In vitro and in vivo studies have consistently demonstrated a changed molecular expression profile in osteoporosis (Kubo et al., 1999; Manolagas, & Jilka, 1995; Ozawa et al., 2002. Several key markers related to osteoblasts including osteoprotegerin, alkaline phosphatase (ALP) and osteocalcin have a lower expression level in comparison with a healthy bone (Marie et al., 2005; Tang et al, 2008). Simultaneously, receptor activator of nuclear factor- Kappa B (RANK), nuclear factor-Kappa B (NF-kB) and tartrate resistant acid phosphatase (TRAP), all associated with osteoclast activity, are significantly elevated. Analysis of bone marrow biopsies from
fracture patients with osteoporosis has also shown a significantly reduced proliferative ability of mesenchymal cells when cultured (MacNamara, 2010). These cells also had an increased tendency to undergo adipogenesis instead of osteoblastogenesis with restored balance upon in vitro stimulation with estrogen.

Clinically, it is unclear if osteoporosis is an independent risk predictor for impaired fracture healing or increased healing duration. This lack of definitive evidence may be in part because of the lack of standardized evaluation criteria among surgeons for defining long-bone (non)union (Bhandari et al., 2002). Regardless, osteoporotic fracture patients experience significantly more complications related to implant failures compared to healthy patients, thus requiring more surgeries and additional treatment to restore their bone integrity (Gruber et al., 2006). Longer union times increase the risk of secondary health issues and such complications further limit our understanding of osteoporotic fracture healing.

Current treatment options for osteoporotic fracture risk reduction are not always effective. They primarily rely on drugs that slow down bone resorption in order to reduce the bone loss. The most commonly prescribed drugs in this category include bisphosphonates (i.e. alendronate, residronate), raloxifene and denosumab (Reid, 2008). Bisphosphonates are inorganic pyrophosphates that are embedded in the bone matrix following resorption. Ralofixene (a selective estrogen receptor modulator) is an orally administered agonist molecule of estrogen that slows down the rate of bone loss. Denosumab is an antibody molecule that inhibits RANK ligand, thereby slowing the proliferation of osteoclasts. An exception to these drugs is teriparatide, which promotes new bone formation to maintain bone density. While the impact of these drug therapies on osteoporotic fracture risk reduction within a five-year term from start of treatment is positive, a long-term risk reduction is considered unlikely (Ott, 2005). In fact, it is suggest that prolonged use of anti-resorptive drugs increases fracture risk. This is due to increased bone brittleness as a consequence of abnormally increased mineral content and decreased micro-crack repair. For instance, bisphosphonates are incorporated in regions of high osteoclast activity, often leading to high, localized concentration (Fleisch, 1998; Ito et al., 1999). These drugs have to be discontinued during fracture healing as they hinder the early stages of the process when osteoclast-led resorption is crucial for removing necrotic fragment ends. This resorption also allows for subsequent deposition of new bone matrix by osteoblasts.
Hormone replacement therapy (HRT) with estrogen and/or progesterone has gained much attention in recent times due to its effect of significantly reducing the risk of osteoporotic fractures (Writing Group for the Women’s Health Initiative Investigators, 2002). However, they are only beneficial for risk reduction of hip and vertebral fractures. Moreover, several large studies have found a 5-10 fold increase in risk of breast and/or uterine cancer when HRT was continued for longer than 5 years. Clearly, the long-term risks outweigh the bone-related benefits and thus cannot become the standard for fracture risk reduction.

Thus, current fracture prevalence, limited treatment options and consequences of lengthy recovery times justify the need for an adjunct treatment that can reliably restore union despite of the status of the bone.
1.9 Animal Models of Osteoporosis

Commonly used species for the study of osteoporosis include mice, rats, rabbits, dogs and sheep (Jee, & Yao, 2001). Methods have been developed to induce bone loss, primarily through reduction of hormone levels, aging and bone disuse. However, the type and extent of bone loss achieved in each of these scenarios is different. For example, prolonged immobilization weakens the load-bearing bones but normal bone status is restored upon mobilization, whereas post menopausal estrogen deficiency leads to chronic weakening of bone micro-architecture that can be merely dampened by anti-resorptive drugs (Mazess, & Whedon, 1983). The choice of animal model is based on the bone-related changes desired with aspects such as feasibility and ease of evaluation being secondary.

Osteoporosis is significantly correlated to estrogen deficiency, thus the most common approach for achieving osteoporotic bone loss in animals is through ovariectomy or orchidectomy (Lelovas et al., 2008). However, this approach is not suitable for all species. Dogs show minimal deterioration in bone health post ovariectomy (Shen et al., 1992). Sheep studies have found an inconsistent reduction in BMD post surgery (Newman, Turner, & Wark, 1995). Rabbits experience a different rate of bone turnover than humans, thus failing to simulate the human bone healing conditions. Moreover, large animals are expensive to maintain and evaluate as well as time-consuming if additional surgical work is to be performed. Rodents, specifically rats, are advantageous as osteoporotic conditions can be satisfactorily achieved and they are relatively cost-effective to house and maintain, which allows for sufficiently large sample sizes. Several studies document their systemic and bone related changes post ovariectomy (Bagi et al., 1997; Lelovas et al., 2008; Miller, & Wronski, 1993).

Rat models fall under two broad categories- mature and aging osteoporotic bone models (Kalu, 1991). The former mimics the bone status in early years of menstrual cessation and has significantly reduced trabecular bone density. The latter mimics elderly women with age associated bone weakening and prolonged estrogen deficiency, yielding significantly increased porosity in trabecular and cortical bone. The mature model is preferred in order to avoid age-related complications and involves ovariectomy of two to five month old rats and a disease development period of another two to six months prior to experimental work (Levolas et al., 2008). The earliest trabecular bone loss has been reported after 30 days in the femoral neck
followed by a decrease in the lumbar vertebrae after 60 days (Jee, & Yao, 2001). Significant changes in cortical bone (related to its width and marrow cavity) occur between 90 and 120 days in the femoral shaft.

A large proportion of osteoporotic fractures occur at trabecular rich sites. However, the choice of the femoral diaphysis for studying osteoporotic fractures is justified at the pre-clinical level. More consistent fracture patterns can be obtained at the diaphyseal site as opposed to the epiphyses or metaphyses, which have irregular and fragile bone structure that can further fragment post fixation (Histing et al., 2011). Diaphyseal fractures are easier to stabilize and the fixation implants have more predictable post-operative behavior. From a clinical perspective, while fractures in proximal regions of long bones are often deemed to be suggestive of osteoporosis, recent studies suggest that fractures of femoral diaphysis in elderly patients should also be considered as an indication of underlying osteoporosis.
1.10 Lithium

Lithium is a small, alkali metal and is the third element in the Periodic Table. With a total of three electrons in its ground state, it is highly reactive and readily forms ionic bonds to acquire the stable cationic form with a charge of +1. Lithium is most commonly found as $^7\text{Li}$ (92.5%), while the remaining exists as $^6\text{Li}$ isotope. The abundance of lithium is about 0.005% in the Earth’s crust and is primarily obtained from ores or saltwater.

Lithium was first found in the petalite mineral ore; hence its name stems from the word *lithos*, meaning stone in Greek language. It was discovered by Johan August Arfvedson in 1817 but he could not isolate it then. A couple years later, both William Thomas Brande and Sir Humphry Davy individually extracted the metal in its elemental form.

Lithium is widely employed in wide-ranging applications. The most significant of these are in glass and ceramics industry to strengthen the products, and in commercial production of aluminum from its oxide form. Lithium stearate, a fatty acid of lithium, is highly stable grease and is extensively used in automotive, military and cosmetics industries. Lithium alloys with magnesium and aluminum have found tremendous use in aerospace industry due to the combined advantage of being strong and lightweight material. Lastly, lithium-based batteries are very efficient and are used in products ranging from toys to spaceships.

The use of lithium in the medical field began in the 19th century. Alfred Garrod began administering lithium to patients with gout in 1849. The first use of lithium in psychiatry occurred later in 1871 when William Hammond began to prescribe lithium for mania. However, it was after 1949 that lithium salts began to gain attention for psychotherapy. In that year, John Cade reported improvements in the mental status of manic patients whom he treated with lithium. Later, larger trials were conducted and research on its dose and toxicity began.

For over 60 years now, lithium has been safely and effectively administered in patients with bipolar disorder. Apart from mood disorders, lithium is actively being evaluated for reduction of cancer risk, for treatment of other psychological and cognitive disorders. Moreover, its effect on bone biology has recently emerged as lithium treated patients have been noted to have higher bone density and lower bone turnover.
1.11 Lithium and Bone

Lithium is known to inhibit GSK-3β, thereby increasing the activity of the canonical Wnt pathway. Consequently, expression of Wnt’s target genes is up regulated, leading to osteoblast proliferation and bone formation. Lithium has been shown to compete with magnesium ions and cause direct inhibition of GSK-3β. Recent studies also speculate its indirect inhibitory effect via inducing its phosphorylation. Thus, lithium is a downstream modulator of the canonical pathway that leads to accumulation of β-catenin in the cytoplasm and ultimately the transcription of osteoblastogenic genes even in the absence of Wnt molecules.

In 1984, Broulik and colleagues found that nearly 65% of the manic patients on lithium had higher alkaline phosphatase levels and concluded that lithium intake increased osteoblast activity. Several studies around that time showed lithium impact on the bone matrix composition. Birch and Jenner (1973) reported reduction calcium content in rat bone, while Brudevold et al (1975) and Curzon & Losee (1977) found lithium in dental enamel. Since lithium was known to strengthen the material and strengthen properties of a crystal lattice, lithium was speculated to augment bone quality.

In the past decade, with the knowledge of lithium’s mechanism of action, it has been extensively studied in the context of bone biology. A number of in vitro and in vivo studies collectively demonstrate lithium’s association with increased bone formation and decreased bone resorption (Galli et al., 2013; Tang et al., 2015; Wang et al., 2015). In vitro experiments involving cell treatment with lithium have shown an increase in osteogenic response with reduced mRNA expression of osteoclastic markers such as RANKL and TRAP as well as an increase in osteoblast activity markers, primarily ALP and collagen type I. Simultaneously, expression level of nuclear β-catenin is elevated following treatment with lithium chloride, thereby confirming the antagonistic action of lithium on GSK-3β (Chen et al., 2007).

One of the early animal studies exploring lithium for bone was carried out by Zaidi and colleagues (1989) who found an augmented bone quality in healthy treated rats. Clément-Lacroix et al. (2005) treated Lrp5-knockout (low bone mass) and SAMP (accelerated osteoporosis) mouse models with lithium chloride. They found an increased bone mass and improved bone microarchitecture especially at the trabecular site, thereby rescuing the phenotypes in both the
models. Similarly, in 2010, Warden et al. showed that healthy mice treated with lithium had significantly higher BMD in comparison with untreated controls. Meng and others (2010) used a rodent hind limb unloading model to induce regional bone loss similar to that associated with prolonged immobilization or anti-gravity atmosphere. Upon lithium treatment, there was significant improvement in molecular, histomorphometric and biomechanical strength measures.

Despite of the overwhelming evidence in support of a positive impact of lithium on bone, there has been little work done to explore its use in fracture healing. One of the few in vivo studies towards this goal was conducted by Chen et al. (2007) who evaluated the efficacy of lithium chloride in a mouse fracture model. They compared bone healing in mice that were given lithium three weeks prior to fracture with those treated 4 days post fracture. Interestingly, they found that pre-fracture treatment with lithium worsened healing compared to healing in untreated fractures, indicated by presence of higher number of undifferentiated mesenchymal cells and reduced amount of calcified bone. In contrast, post-injury lithium administration led to accelerated healing marked by increased bone volume and density in the healing callus.

Currently, there is sufficient clinical evidence that links lithium treatment with bone augmentation in psychiatric patients. This comes from clinical studies by Nordenstrom and colleagues in 1994, Vestergaard et al in 2005, Witling et al in 2007 and Zamani et al in 2009. In the works published in 2007 and 2009, fracture risk was significantly lower in patients on regular lithium medication than for the untreated control subjects. They also reported an increase in bone mineral density at trabecular-rich sites.

Lithium may be beneficial for enhancing bone healing, however, its potential for clinical use in fracture management is hindered by the current gap in knowledge of its precise administration parameters (dose, treatment onset and duration). Lithium has a narrow therapeutic range, above which its side effects may become evident. Therefore, determination of these administration parameters is imperative for further consideration of lithium use for fracture treatment.
1.12 Previous Work

A pre-clinical study was undertaken to optimize lithium administration parameters - drug dose, treatment onset and treatment duration to best enhance fracture healing in rodent femoral shaft fracture model. This study used a design of experiments (DOE) approach, which enables the evaluation of multiple input factors simultaneously on the output responses. Although used commonly in engineering design, this method is only very recently gaining use in biomedical research. The DOE is comprised of three stages - screening, optimization and verification. Prior to this thesis, the first two stages were completed. The DOE utilized a three factorial (dose, duration and onset) two level (low and high) approach to find the optimal combination of parameter values. This method enabled the three administration parameters to be evaluated individually to determine their relative importance in improving fracture healing as well as identify any interactions between them. DOE based experimentation is beneficial in that it can minimize the number of treatment groups required in comparison with a more common “one factor at a time” approach.

For both screening and optimization, three-month old female Sprague Dawley rats were subjected to unilateral, closed, mid-shaft transverse femoral fractures using a load drop technique to stimulate a trauma-type injury (Bernick, 2013; Pagotto, 2014). Over the course of the healing period, the rats received lithium or saline according to their group’s administration conditions. They were then sacrificed at day 28 post fracture and their femurs excised. Healing was evaluated based on torsion load testing (primary outcome) and μCT based stereology (secondary outcome). These will be discussed in detail in section 3.1.2 since the outcomes were analyzed identically in the entire study. Briefly, both the femurs, fractured and contralateral, of each rat were scanned at 14.8µm voxel size. The scans were then processed in AmiraDEV 5.3 (Visage Imaging, CA, USA) and analyzed in CTAnalyser software (SkyScan, Belgium) to obtain micro-structural properties of the bone. Next, the femurs were subjected to torsion load to evaluate their load bearing strength. They were twisted until failure or for a maximum of 50°, whichever occurred first. The generated load displacement curve was utilized to obtain torsional strength at yield point.
1.12.1 Screening: Experimental Set Up

Eight different combinations of the three administration parameters at minimum/maximum values were evaluated. The low/high values were: 20 or 100 mg/kg for dose, day three or seven for onset time point, and one or two weeks for treatment duration (Figure 4). Each of these values was chosen based on previous studies related to the use of lithium and/or fracture healing timeline, as described in more detail below.

Dose: Hamamura and others (2000) aimed to understand the impact of lithium chloride in dose (a comparison of 20mg/kg to 100mg/kg) on rat brain cells in the context of mood disorder treatment. The low dose was effective in eliciting a response, meaning that a dose as low as 20mg/kg was able to make a significant physiologic difference. Similarly, Ghasemi and colleagues (2011) found the rat equivalent dose of 15mg/kg lithium in mice was efficacious as an anti-depressant but lower doses did not have any significant impact. Thus, the lower limit was chosen at 20mg/kg. The upper limit of 100mg/kg for the rats was derived from the 200mg/kg dose found to be the maximal dose that can be administered without causing negative effects in mice (Reagan-Shaw et al., 2007).

Treatment onset: The onset time point of three days was based on the results published by Chen et al in the 2007 paper. In their study, the day four onset time post fracture for lithium treatment was effective in improving tibial shaft fracture healing in mice. Accordingly, day three was opted as the lower limit since this time point marks the initiation of mesenchymal stem cell activity in the fracture milieu. Day 7 represents the point when osteoblastic lineage cells begin to differentiate and mineralize the matrix.

Treatment duration: The one and two week treatment periods were chosen based on the knowledge that rat bones require ~ 4-6 weeks to achieve union. Thus, for the treatment to be of significance, the healing should improve before the normal union time. Considering the mechanism of lithium’s action, the osteoblast-induced bone formation can be captured within this time frame.

The screening stage consisted of 11 experimental groups in total: one group for each combination occupying the corners of the cubic DOE space as depicted in Figure 5, one group treated at middle values for the three administration parameters (central point) and two control
groups. The first control group received only vehicle (saline) and the second control group did not receive any treatment. Each group consisted of 6 rats.

**Figure 5**: Experimental design for screening stage (number of rats per group shown in the square) (Bernick, 2013).

### 1.12.2 Screening: Results

The results from stereology and biomechanical testing were comprehensively evaluated using DOE modeling with ANOVA, comparison between mean values and Pearson correlation analysis (Bernick, 2013). The data was normalized prior to statistical analysis using BOX-COX transformations. DOE modeling did not find any of the models to be predictive based on their overall coefficient of determination. However, one model was found to be significant ($p=0.017$). This model, for maximum yield torque (primary outcome measure), displayed an inverse relation with onset time ($p=0.013$) and dose ($p=0.111$), and direct relation with duration ($p=0.141$). Scatterplot analysis for each of the parameters versus yield torque showed a clear trend for the onset time. More points for higher onset time (day 7 versus day 3 or 5) were clustered at greater torque values. No such trends were seen for dose or duration. Onset parameter had the greatest
contribution to the model space (55%) in comparison to other parameters (dose: 22%, duration: 18%). All together, these results implied that late onset (day 7 versus day 3), low dose (20mg/kg versus 100mg/kg) and high duration (two weeks versus one week) were optimal for maximal improvement in fracture healing (maximum yield torque).

Equation 1: Predicted relation of yield torque and the administration parameters from screening phase

\[ \text{Sqrt(Maximum Yield Torque)} = +18.20 -0.85*\text{Dose} +1.36*\text{Onset} +0.79*\text{Duration} \]

The results for stereologic measures and torsion load testing for three treatment groups (optimal combination, combination directly opposite to the optimal values and saline only) are shown in Table 1. No significant differences were observed for the stereologic parameters. For torsional load testing, the highest yield torque was demonstrated by the group treated with 20mg/kg lithium chloride from day 7-21. In comparison to saline control, the group’s healing femurs could bear a 46% higher load prior to the yield point (p<0.05).

Table 1: Results of stereological analysis (top) and biomechanical testing (bottom) from the screening phase. This table shows average values for the primary outcome measure for three of the eleven treatment groups. Conditions for group 5 are opposite to the optimal values, group 6 received saline only and group 10 had the highest yield torque, hence proposed as the most optimal treatment combination (Bernick, 2013).
1.12.3 Optimization: Experimental Set Up

This stage aimed to further optimize the parameter values based on the findings from the previous screening stage. Since only the onset time was significant in the DOE model (higher value being better), optimization evaluated onset timing extended to day 10 and day 14. Callus transformation from soft to hard type begins between day 7 and 10, so by day 14 mature osteoblasts would be present in the callus. Hence, beginning the treatment later than day 14 was not considered to be beneficial. These onset times were evaluated at high dose/low duration and low dose/high duration (Figure 6). The dose/duration combinations were chosen based on the opposite association observed for the two parameters. Dose values were 20 and 100 mg/kg, and duration times were one and two weeks. These values were kept the same as in screening since dose and duration did not have a significant impact on the outcome measures.

![Figure 6: Experimental design for optimization stage (Pagotto, 2014).](image)

1.12.4 Optimization: Results

The torsional strength for the healing femurs of the four groups were similar. Yield torque ranged from 243 N-mm to 278 N-mm, as shown in table 2. This was lower than the torque values for the saline group (302.2 N-mm) or the optimal treatment group (481.1 N-mm). There were no differences observed for any of the stereologic parameters.
Table 2: Biomechanical testing results for the treatment groups in the optimization stage (Pagotto, 2014).

<table>
<thead>
<tr>
<th>Onset (days)</th>
<th>Dose/Duration (mg/kg-wt/day)/weeks</th>
<th>Max Torque (N-mm)</th>
<th>Angle (°)</th>
<th>Stiffness (N-mm°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20/2</td>
<td>242.8 (62.0)</td>
<td>35.9 (15.5)</td>
<td>10.1 (5.8)</td>
</tr>
<tr>
<td>14</td>
<td>20/2</td>
<td>250.2 (67.4)</td>
<td>22.9 (11.3)</td>
<td>14.4 (9.0)</td>
</tr>
<tr>
<td>10</td>
<td>100/1</td>
<td>252.1 (101.0)</td>
<td>24.3 (10.0)</td>
<td>10.4 (4.2)</td>
</tr>
<tr>
<td>14</td>
<td>100/1</td>
<td>278.4 (91.6)</td>
<td>27.8 (17.8)</td>
<td>13.1 (5.4)</td>
</tr>
</tbody>
</table>

Statistical analysis was performed using DOE modeling, ANOVA and correlation analysis. The results found none of the four combinations to be superior in comparison with the optimal combination from screening phase. This was inferred from the generated DOE models, none of which showed significant differences between the day 10 and 14 onset times or for the combinations of dose and duration. AVOVA analysis was performed for yield torque and it showed a significant effect of onset time for the treatment condition of low dose/high duration \( (F_{\text{critical}} = 4.2, F= 11.2, p=0.002) \). When evaluated with Student-Newman-Keuls post-hoc test, day 7 onset time was found to be the best (Figure 7).
Hence, results from screening and optimization phase together implied that the best lithium-based treatment for fracture healing in rats was a daily dose of 20 mg/kg-wt started seven days after fracture for a duration of two weeks.
2. Motivation, Aims and Hypotheses

Lithium induced bone growth may have a direct clinical application in treatment fracture patients. The proposed optimal lithium treatment is advantageous since the dose is below the clinically administered dose for bipolar disorder, and the treatment onset time is well after the injury has occurred, giving the patient sufficient time to visit a doctor and begin the lithium treatment. Moreover, it is inexpensive and its side effects are well known, so the patient can be well informed and monitored during the treatment process. To complete the DOE study, the final stage of verification is required to validate the findings. As such, the efficacy of the optimized treatment must be evaluated in an additional set of healthy animals with fractures. A critical impediment in osteoporotic fracture management relates to poor healing in estrogen-depleted state. Hence, it is important to determine if lithium administration can also positively impact fracture healing in osteoporotic bones. Together, this research will form a robust foundation for future translational work aimed at assessing the clinical potential of lithium drug for fracture treatment.

**Lithium Treatment in Healthy Rats**

**Aim:** To validate the effect of the proposed optimal lithium administration conditions for femoral diaphyseal fracture healing in healthy rats (20 mg/kg for two weeks beginning at seven days post fracture).

**Hypothesis:** Systemic administration of lithium in healthy rats will enhance the structural and mechanical properties of the healing femur, as observed in the screening and optimization stages of the DOE.

**Lithium Treatment in Osteoporotic Rats**

**Aim:** To evaluate the efficacy of the optimal lithium treatment (20 mg/kg for two weeks beginning at seven days post injury) in improving the quality of fracture healing in OVX rats.

**Hypothesis:** The optimized lithium treatment in osteoporotic rats will improve the extent of femoral diaphyseal bone healing in comparison to the saline treated osteoporotic rats despite the impaired nature of the bone tissue.
3. Healthy Bone Model

This chapter is focused on the verification stage of the DOE study to evaluate the proposed optimal lithium treatment in healthy rats. The healing outcomes of the femoral diaphyseal fractures following the proposed regimen are compared to the outcomes of saline treated controls. In section 3.1, the in vivo experiment and the evaluation processes are described. The results and discussion follow in sections 3.2 and 3.3, respectively.

3.1 Methods

The rat model for verification was identical to that in screening and optimization. 10-12 weeks old female Sprague Dawley rats were obtained from Taconic Laboratories (Albany, NY, US) and were acclimatized at the animal facility for one week. They were housed for an additional duration (to a maximum of two additional weeks) to allow for weight gain. At 13-15 weeks of age, all rats were entered into the fracture study with an average weight of 265g.

3.1.1 Experimental Outline

Sample size calculation

Sample sizes were determined from the differences obtained between the optimal treatment group and the saline control group in the screening stage. The effect size was calculated from the groups’ averages and standard deviations for maximum yield torque (primary outcome measure). In order to achieve 80% power at the 5% significance level, a total of 20 samples were required. Thus, the lithium and saline groups comprised of 10 rats each.

Fracture Creation

A closed diaphyseal fracture of the right femur was created following the technique outlined by Manigrassi and O’Conor (2004). First, each rat was anesthetized using isofluorane (induction at 4%, maintenance at 3%), its right hind limb shaved and disinfected with Betadine and alcohol. A shallow incision, approximately 5mm long, was made on the parapatellar surface. An entry point was created with a 25G needle, through which a 1mm diameter intramedullary pin was drilled.
into the medullary canal to pre-stabilize the bone. The pin position within the canal was evaluated radiographically and then engaged at the proximal end. The incision site was closed with a 3-0 absorbable suture. Next, each rat was positioned on the fracture jig apparatus, which is a guillotine platform designed earlier in the pilot stage of this study. The right femur was positioned such that the blade, when dropped, would hit the diaphysis perpendicularly. The load dropped was approximately 280g; it was increased or decreased based on the weight of the rat in order to avoid severe comminution. The fracture occurrence and its severity were determined in medio-lateral and antero-posterior planes using fluoroscopy. The incision site was ligated again (if required) and stapled. Postoperatively, the rats were housed individually, allowed to weight bear, and had access to food and water ad libitum. Temgesic was subcutaneously injected at the fracture site for the first two days post fracture.

**Lithium Treatment and Sacrifice**

The fracture was allowed to heal over the next 28 days. The rats were randomized into either lithium or saline treatment groups. Accordingly, they received either 20mg/kg lithium chloride or a volumetric equivalent of saline via oral gavage under light anesthetic from day 7-21 post fracture. After every dose administration, 2 mL of 0.9% NaCl was subcutaneously injected to prevent dehydration due to lithium. All animals were sacrificed 28 days post fracture, and their fractured and contralateral femurs were harvested. The intramedullary pin was removed, and each femur was wrapped in saline-soaked gauze and stored at -20°C until evaluation.

A summary of the experimental outline is shown in figure 8.
3.1.2 Evaluation of Healing

μCT-based 3D bone stereology and torsion load testing were used to assess the impact of treatment on the overall quality of bone healing in comparison to saline control. Any healing femur that involved comminution in the epiphyseal or metaphyseal region, a simple transverse fracture or extensive fragmentation was excluded from analysis.

The contralateral femurs were also analyzed using these techniques to determine potential off-target skeletal effects of lithium treatment.

Figure 8: Experimental outline for verification stage.

Figure 9: Summary of outcome measures for the evaluation of fracture healing.
Stereological Analysis

Stereology is a standard technique for quantitative evaluation of bone microstructure using high-resolution μCT scans. Accordingly, each harvested femur was imaged at an isotropic voxel size of 14.8µm using Scanco 100 μCT scanner (Scanco Medical, Switzerland). The settings were 55kV, 200µA with a beam hardening correction factor of 1200mgHA/ccm. The intensity-bone density relationship was generated using the scanner’s pre-calibration with four hydroxyapatite phantoms of 100, 200, 400 and 800 mgHA/ccm.

Reconstructed scans were exported as DICOM images into AmiraDEV 5.3 (Visage Imaging, CA, USA) to crop and align the region of interest (ROI). The ROI was denoted as a fixed distance from the mid-point of the lesser trochanter proximally to the start of the patellar notch distally (Figure 10). These boundaries were chosen to ensure that the entire fracture callus was consistently included in all the femurs, which was crucial since the fracture location and extent of fragmentation varied from rat to rat. The femur was aligned vertically using the transformation tool and cropped to obtain the desired region. It was then exported to CTAnalyser software (SkyScan, Belgium) for quantitative analysis.

To segment out the unmineralized region, a global threshold of 25% of the maximum native gray scale value (365 mgHA/ccm) was applied. This value was chosen in the screening stage based on the results published by Morgan and colleagues (2009). An automated custom processing module, which included processing of the ROI and calculation of the bone properties, was applied.

The stereological parameters calculated were bone volume (BV, mm³), total volume (TV, mm³), bone volume fraction (BV/TV, %), mean bone mineral density (BMD, mgHA/ccm), mean tissue mineral density (TMD, mgHA/ccm) and mean bone mineral content (BMC, mgHA). They were defined as follows:

Bone volume: total voxels that are segmented as bone.
Total volume: all voxels that constitute the volume of interest, including bone and non-bone voxels.
Bone volume fraction: fraction of the total volume that is occupied by mineralized bone, calculated as bone volume divided by total volume.
BMD: average attenuation value of bone and non-bone voxels that is converted to mineral density using the calibration set by hydroxyapatite phantoms.
TMD: similar to BMD except that only the bone voxels are used.
BMC: mineral content within the ROI calculated as the product of bone volume and tissue mineral density.

![Definition of the region of interest (ROI) utilized for stereological analysis (Bernick et al., 2014). This figure is reproduced with permission from Wolters Kluwer Health Inc.]

**Theoretical Torsional Rigidity**

The μCT scans were also used to calculate the torsional rigidity of the healing fracture callus. This is a computational approach for estimating an otherwise experimental parameter determined by biomechanical testing. The product of the slope of the generated angular displacement curve and the specimen gauge length gives the sample’s torsional rigidity. Theoretically, torsional rigidity is defined as the product of the polar moment of inertia and shear modulus, and thus incorporates both geometry and quality of the callus. CT-based rigidity analysis (CTRA) was first published by Hong and colleagues in 2004 for predicting pathologic fracture risk. Later,
their group utilized this method to evaluate fracture healing, and demonstrated a high correlation between the theoretical and experimental minimum torsional rigidity in a rat model. Based on the procedure outlined in their publication from 2010, an AmiraDEV (Visage Imaging, CA, USA) code was written and implemented in the screening phase. The steps included in this code are briefly described below.

Shear modulus: The scan was converted from intensity-based (Hounsfield units) to its density equivalent using the scanner’s calibration curve. This density was then scaled to ash density using a correction factor. The resulting ash density was the input for obtaining the shear modulus-based scan (Nazarian et al., 2009). The below equations were used in the process:

**Equation 2: Conversion of intensity to ash density**

\[
\text{Ash Density} = (0.1485 \times \text{Hounsfield Unit}) - 14.074
\]

**Equation 3: Calculating shear modulus from corrected ash density**

\[
G = 3.16(r_{\text{app}})^{1.24}
\]

Polar moment of inertia: The centroid for each slice of the scan was calculated using the equation below. ‘da’ is the area of pixels (14.8*14.8 um²).

**Equation 4: X and Y co-ordinates of the centroid point for a slice.**

\[
\bar{x} = \frac{\sum_{i=1}^{n} x_i E_i da}{\sum_{i=1}^{n} da} \quad \bar{y} = \frac{\sum_{i=1}^{n} y_i E_i da}{\sum_{i=1}^{n} da}
\]

All the centroids were joined to obtain the neutral axis, along which the forces were zero. Finally, the shear modulus and centroids were combined in the following formula to calculate the minimum (GJ_{min}) and average (GJ_{avg}) CT-based torsional rigidity (CTRA, kN*mm²). The minimum value corresponded to the weakest point in the bone while the average value was the mean of rigidities for all the slices.
Equation 5: Formula for calculating the torsional rigidity using the shear modulus and centroid.

\[
\text{Torsional Rigidity: } GJ = \sum [G_i(p) \cdot (x_i^2 + y_i^2)] \, da
\]

Biomechanical Testing

The femur’s biomechanical strength was evaluated by subjecting the specimen to torsion. This method was chosen over other approaches such as axial loading or three-point bending in order to determine the maximum load that the femur could bear prior to its first point of failure. This point is associated with torsional failure, rather than tension or compression, since a bone is weakest in shear. The failure load can be determined in an unbiased manner when the bone is loaded uniformly. One of the primary concerns with bending tests is that the load is applied at a particular point, hence prescribing the point of failure, instead of distributing the load equally over the whole sample and allowing for failure to occur at the weakest point.

Each femur was loaded under the same conditions as performed previously in screening and optimization. The femur was aligned longitudinally with respect to the loading axis of the MTS Bionix 858 materials testing system (MTS Systems, MN, USA). Its proximal and distal ends were potted in bone cement (polymethylmethacrylate) at a constant gauge length of 15mm (Figure 11). Since the fractures were diaphyseal, this length was sufficient to subject the entire shaft, and thus the callus, to torsion. A 1.4N-m reaction torque transducer (Futek, CA, USA) measured the applied torque load. An angular displacement of 1.5°/second was applied until failure occurred or until a maximum angular displacement of 50° was achieved. Maximum yield torque, twist angle at failure and experimental torsional stiffness were determined based on the generated torque – angular displacement curves. Maximum yield torque was used as the primary study outcome measure to assess the quality of fracture repair.
3.1.3 Data Analysis

Healing femurs in the lithium-treated group were compared with the respective saline control group. Statistical differences were calculated using a Student t-test and p-value lower than 0.05 was considered significant. Additional comparisons were made between contralateral femurs in the lithium and saline groups in order to assess for lithium’s potential impact on the off-target limbs. Finally, healing and contralateral femurs within a treatment group were compared to understand the extent of restoration to a pre-fracture state. Correlations between parameters of biomechanical strength and microstructure were explored using Pearson correlation analysis.

Figure 11: Set-up showing the potted femur for torsion load testing
3.2 Results

All rats were radiographed after pin insertion and immediately after load drop to evaluate fracture location and severity. Fractured femurs were also radiographed one day post fracture and then weekly until sacrifice. In the first week, any rat with extensive comminution or a severely displaced pin was sacrificed. Fractures were well tolerated in most cases and the rats began to weight bear within one week after injury. Post fracture, one rat had to be sacrificed on the next day due to significant displacement of the bone fragments. Two additional rats were excluded from analysis; both belonged to the lithium group. The first rat had bone fragmentation extending to metaphyseal region, and the second rat had simple transverse fracture pattern, which differed from the comminuted nature in other rats. After exclusion, the group size was 8 and 9 for Lithium and saline groups, respectively.

3.2.1 Qualitative Healing

Radiographs taken throughout the healing period demonstrated gradual formation of soft callus and eventual disappearance of distinct radiolucent fracture lines in the fourth week. Due to their poor resolution, cortical bridging was difficult to visualize.

Reconstructed μCT scans of the harvested femurs were compared to evaluate for any consistent differences in the healing callus of the lithium group versus the saline group. Overall, femurs of the lithium group had restored periosteal continuity with periosteal callus generally present and inter-cortical callus visible at some gaps (figure 12). These hallmarks of healing were inconsistently present in the saline group and calluses in some rats appeared larger. No difference in radiopacity (mineralized callus) was evident.

Figure 12: Transverse sections of healing femurs at 28 days post fracture from lithium treated rat (left) and saline treated rat (right).
3.2.2 Stereology

**Fractured Femurs:** The two groups did not differ significantly with respect to any stereologic measures (table 3). However, the lithium group trended towards a lower average bone mineral density ($609.56 \pm 122.66$ vs $692.60 \pm 82.23$ mgHA/ccm, $p=0.13$).

**Contralateral Femurs:** The two groups differed only for bone volume fraction, which was slightly lower for the lithium group ($66.22 \pm 2.44$ vs $69.19 \pm 2.21$ %, $p=0.04$). Average values for all the stereologic measures are summarized in table 3.

**Table 3: Stereology data (average with standard deviation) for fractured and contralateral femurs.**

<table>
<thead>
<tr>
<th></th>
<th>Bone Volume (mm$^3$)</th>
<th>Total Volume (mm$^3$)</th>
<th>BV/TV (%)</th>
<th>BMD (mgHA/ccm)</th>
<th>TMD (mgHA/ccm)</th>
<th>BMC (mgHA)</th>
<th>Theoretical GJ min (N-mm$^2$)</th>
<th>Theoretical GJ avg (N-mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fractured Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>286.63 (49.52)</td>
<td>498.24 (105.77)</td>
<td>58.23 (6.76)</td>
<td>609.56 (122.66)</td>
<td>1313.97 (225.50)</td>
<td>371.41 (67.96)</td>
<td>216285 (58871)</td>
<td>340314 (106257)</td>
</tr>
<tr>
<td>Saline</td>
<td>268.97 (39.37)</td>
<td>435.34 (65.27)</td>
<td>61.99 (4.12)</td>
<td>692.60 (82.23)</td>
<td>1287.98 (227.47)</td>
<td>350.56 (95.92)</td>
<td>183386 (62284)</td>
<td>294762 (73940)</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.43</td>
<td>0.17</td>
<td>0.20</td>
<td>0.13</td>
<td>0.82</td>
<td>0.61</td>
<td>0.28</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Contralateral Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>185.34 (43.91)</td>
<td>279.40 (63.27)</td>
<td>66.22 (2.44)</td>
<td>881.36 (135.65)</td>
<td>1508.67 (356.91)</td>
<td>268.72 (36.89)</td>
<td>95393 (15635)</td>
<td>157033 (37860)</td>
</tr>
<tr>
<td>Saline</td>
<td>180.28 (11.55)</td>
<td>261.02 (21.87)</td>
<td>69.19 (2.21)</td>
<td>943.41 (176.30)</td>
<td>1499.80 (333.89)</td>
<td>267.88 (50.98)</td>
<td>95393 (6046)</td>
<td>157033 (108592)</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.79</td>
<td>0.52</td>
<td><strong>0.04</strong></td>
<td>0.46</td>
<td>0.96</td>
<td>0.97</td>
<td>0.24</td>
<td>0.63</td>
</tr>
</tbody>
</table>

3.2.3 Biomechanical Testing

**Fractured Femurs:** The two groups differed significantly for average maximum yield torque (primary outcome measure), the lithium group being stronger (44%; $261.73 \pm 63.69$ N-mm vs
181.58 ± 71.40 N-mm, p=0.03), as shown in figure 13. No differences were found for average twist angle at failure or average torsional stiffness (table 4).

**Contralateral Femurs:** There were no intergroup differences for any measures of torsional strength (figure 13 and table 4).

**Table 4: Torsional testing data (average with standard deviation) for fractured and contralateral femurs.**

<table>
<thead>
<tr>
<th></th>
<th>Max Torque (N-mm)</th>
<th>Angle at Failure (°)</th>
<th>Torsional Stiffness (N-mm/°)</th>
<th>Experimental GJ (N–mm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fractured Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>261.73 (63.69)</td>
<td>14.46 (4.96)</td>
<td>22.56 (6.36)</td>
<td>19387 (5466)</td>
</tr>
<tr>
<td>Saline</td>
<td>181.58 (71.40)</td>
<td>11.77 (3.65)</td>
<td>18.13 (6.88)</td>
<td>15578 (5911)</td>
</tr>
<tr>
<td><strong>T-test</strong></td>
<td><strong>0.03</strong></td>
<td>0.23</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Contralateral Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>376.05 (76.60)</td>
<td>12.75 (1.88)</td>
<td>30.10 (6.34)</td>
<td>25701 (5326)</td>
</tr>
<tr>
<td>Saline</td>
<td>378.17 (79.34)</td>
<td>12.08 (2.84)</td>
<td>32.19 (3.29)</td>
<td>27676 (2754)</td>
</tr>
<tr>
<td><strong>T-test</strong></td>
<td>0.96</td>
<td>0.58</td>
<td>0.45</td>
<td>0.40</td>
</tr>
</tbody>
</table>

An intragroup comparison within the lithium group noted the average yield torque for fractured femur was different but approaching the average for the contralateral femurs (261.73 vs. 376.05 N-mm, p=0.10). However, within the saline group, the fractured femur had significantly lower average torque than its contralateral (181.58 vs 378.17 N-mm, p=0.0005), as shown in figure 13.
3.2.4 Correlation Analysis

Several significant correlations were found between measures of torsional strength and stereologic measures. Maximum yield torque positively correlated with bone volume (r=0.554, p=0.014), total volume (r=0.454, p=0.051) and theoretical minimum torsional rigidity (r=0.556, p=0.014). Twist angle at failure negatively correlated with TMD (r=-0.548, p=0.015) and positively with theoretical minimum torsional rigidity (r=0.497, p=0.030). Torsional stiffness positively correlated with TMD (r=0.506, p=0.027) and BMC (r=0.474, p=0.040).

Figure 13: Average maximum yield torque for fractured and contralateral femurs of Lithium and saline group. F: fractured femur, and C: contralateral femur.
3.3 Discussion

Healthy adults typically experience femur fractures upon a traumatic incident. Femoral fracture healing is reliably achieved in 90 to 95% of cases. The standard approach to diaphyseal fractures involves surgical reduction of the fragments and stabilization by intramedullary nailing or plating to restore gross anatomical structure. Surgical treatment is followed by early controlled mobility to provide a dynamic microenvironment essential for formation of new bone tissue. However, this method is not always successful and patients often require additional surgeries or longer recovery periods due to slow or delayed healing.

Modulation of the Wnt pathway as a therapeutic approach to improve fracture repair is under active exploration due to the pathway’s critical role in promoting osteogenesis. Many of the potential therapeutic molecules associated with this pathway either remain in preclinical stages or have advanced to clinical trials but their results have yet to demonstrate definitive efficacy. If successful, their long-term side effects will remain initially unknown, and their incorporation into clinical management may take years. Interestingly, lithium has been a long-standing GSK-3β inhibitor that has been safely administered for treatment of mood disorders. As long as serum lithium levels are maintained below 1.2mEq/L, its use has been deemed safe with side effects being non-severe and manageable (Schou et al., 1970).

The overarching goal of this preclinical study was to delineate the optimized values for lithium administration parameters in a rodent femoral fracture model using a three stage DOE approach. The screening and optimization stages evaluated different combinations of doses, onset times and durations simultaneously (in contrast to the “one factor at a time” approach). The proposed optimized lithium treatment from the results of screening and optimization stage formed the basis of this thesis. The final verification stage was performed in this thesis to confirm the efficacy of this proposed treatment for femoral fracture healing in otherwise healthy rats. The proposed values for the administration parameters were: 20 mg/kg daily lithium dose, day 7 onset time and 14 days duration. Using this regimen, a 44% increase in yield torque was found for the lithium treated group compared to saline control, hence validating the earlier findings in this study.

The fractures created using the closed drop weight approach were fragmentary indicating infliction of considerable trauma. Thus, at the end of four weeks, the healing bones had
mineralizing calluses but cortical union was not fully restored at any gaps. There is contradicting evidence in literature for the duration required to achieve union in broken rat femora. Several studies note the initiation of bone-remodeling around 25 days based on protein expression profile of the callus. On the contrary, Ekeland et al. (1981) observed union only at 13 weeks. Such broad range of healing period can be attributed to the differences in the age of animals, bone under evaluation, fracture severity and healing conditions. Thus, the healing achieved after four weeks in our rats is considered normal and the calluses can be expected to continue healing in a normal manner, whereby any chondrocytic matrix would be resorbed and replaced by a mineral-rich, tough bone matrix.

3.3.1 Biomechanical Testing and Stereology

The optimized low-dose lithium treatment was efficacious in comparison to the control treatment with saline. The lithium group demonstrated ~1.5-fold (44%) higher yield torque (primary outcome measure), which seemed to progress towards restoring its pre-fracture strength (i.e., the yield torque of the contralateral femora). This 44% difference was nearly equivalent to the 46% difference found in the screening stage when this lithium regimen was first evaluated in smaller group of rats (6/group, yield torque: 481.1 ± 104.4 N-mm for lithium group vs. 302.2 ± 159.7 N-mm for the saline group). The averages from the screening stage are higher than those obtained in verification, but this was unrelated to the treatment itself. Two likely reasons for it were: differences in calibration of the load transducer cell and potential genetic variation between the rats used for the two stages. Instead of Harlan Laboratories, verification experiments utilized rats from Taconic Laboratories, thereby introducing biological variations as well as the chow that they were fed. Furthermore, in all stages of the DOE the average torque values for each sub-group had high variation. This is not surprising as animal experiments are associated with inherent variability and a study can overcome these variations if it is sufficiently powered. The variation may have also resulted from the variable degree of trauma and fragmentation.

The calluses of the lithium and saline groups broke at similar twist angles, which were also comparable to the results from screening. The average torsional stiffness, which is the slope of the torque vs. twist angle curve, was higher for the lithium group but did not reach significance (p=0.19). This was similar to the findings in screening (lithium: 30.5 ± 5.8 N-mm/°, saline: 19.6
Overall, the results of biomechanical testing in the verification phase were in agreement with the preliminary results obtained in the earlier stages of the DOE.

Contralateral limbs were also analyzed. They served as internal controls within the treatment group to evaluate any off-target impact of lithium on other bones. The contralateral femurs had similar torsional strength regardless of their treatment, hence validating the attribution of differences in healing to lithium administration. The results obtained in verification paralleled those in the screening stage. The yield torque in the screening stage was 404.6 ± 87.9 N-mm for lithium and 363.5 ± 123.5 N-mm for saline group (p=0.62). Torsional stiffness results in the two stages were: Screening: lithium: 31.2 ± 7.4 N-mm/°, saline: 30.2 ± 14.1 N-mm/°, and Verification: lithium: 30.1 ± 6.3 N-mm/°, saline: 32.2 ± 3.3 N-mm/°.

Despite the significant differences found in maximum yield torque, the stereological parameters of the healing femurs was similar between the treatment groups. For the contralateral femurs, bone volume fraction of the lithium group in verification was slightly lower (4.5%) compared to the saline group, however this was not observed in the screening stage. None of the other parameters, including bone mineral content or density, differed between the two groups and this difference in bone volume fraction also did not compromise the bone’s torsional strength. This observation was unexpected since lithium is known to induce bone formation. For instance, in a study by Sisask et al. (2013), 2-month old rats (unfractured) were treated with AZD2858 (a GSK-3β inhibitor) for 14 days. No differences in bone volume fraction were found, and significant increases in bone mass and BMD were observed in the vertebrae. Their results concur with other studies that show an anabolic effect of lithium on bone health via reduced bone turnover and greater bone formation than resorption. It is possible that when lithium is administered for a short duration (like 14 days), lithium’s impact is evident on the more exposed trabeculae but not on the compact cortical bone. In consideration of the potentially different outcome of lithium on fractured versus intact bone, future work related to lithium should assess for off-target skeletal changes to understand the full spectrum of the molecule’s impact.

3.3.2 Correlation Analysis

Load-bearing performance of the healing femurs correlated with certain parameters of their microstructure. Yield torque positively correlated with the bone’s geometry (higher bone volume
and total volume). Biologically, larger calluses during remodeling are suggestive of poor healing as they generally result from poor soft callus resorption. Ideally, most of the soft callus is resorbed and replaced by mineralized matrix. Thus the callus is expected to decrease in size. However, similar bone mineral content in lithium and saline treated rats implied there was no impairment in the overall mineralization upon lithium treatment. Mathematically, polar moment of inertia is an integral function of second power of radius, and thus torsional rigidity is directly proportional to the sample’s geometry. Therefore, the larger callus of lithium treated femurs likely conferred greater torsional strength upon loading.

Two significant correlations were found with respect to TMD. Twist angle at failure was inversely related to TMD (r=-0.55), and this was consistent with the correlation noted in screening (r=-0.28). Thus, the TMD was poorer for the femurs that withstood greater twisting under torsion. While a highly mineralized specimen may have greater strength and stiffness, it is the toughness provided by the soft tissue / collagen matrix that would enable a greater twist angle before failure. This explains for the inverse relation between TMD and twist angle at failure. The other correlation was found between TMD and experimental torsional stiffness (r=0.51). This relation is intuitive based on the primary role of mineral crystals in a composite matrix. The mineralized matrices of transforming calluses demonstrated greater stiffness compared to soft calluses with relatively less progression through the mineralizing phase or lesser mineral deposition. Overall, a less mineralized matrix will have greater cartilaginous content, yielding a lower stiffness and a larger twist angle before failure.

\(\mu\)CT-based torsional rigidity analysis is an approach for estimating the strength of healing bone without destructive mechanical testing. Nazarian et al (2010) applied this method to healing long-bone fractures and demonstrated its ability to reflect trends in the experimental data. They showed a significant correlation between biomechanical testing results and minimum theoretical torsional rigidity (r=0.88, p=0.03). \(\mu\)CT-based torsional rigidity analysis was similarly applied in our study. Results from the verification phase found a positive correlation between maximum yield torque and minimum rigidity (r=0.56), but not with the average rigidity. This is justified since the experimentally determined yield torque in this study is defined as the first (weakest) point of failure, corresponding to the lowest bearable load prior to plastic deformation. Thus, this value is related to the minimum rigidity rather than the sample’s average rigidity at all points within it. The correlation value was weaker than the r=0.88 reported in the 2010 paper but much
stronger than that found in the screening phase of this study (r=0.24). Differences related to the animal model as well as experimental set-up probably led to such discrepancy in the final results.

It is important to note that biomechanical performance is resultant of various factors related to structure and geometry; thus the significance of the correlations between theoretical calculations and experimental findings is in their predictability of the overall healing rather than an individual outcome measure.

### 3.3.3 Potential for Clinical Translation

The timing of the optimal treatment regimen parallels the biological progression during healing. Mature chondrocytes of the soft callus begin to hypertrophy around day 7 to 9 post fracture and a concurrent increase in mineral deposition by osteoblasts occurs. While endochondral ossification continues until day 25-28, its initial period involves increased differentiation and proliferation of osteoblast lineage committed stem cells in the fracture milieu. Since lithium increases osteoblastic activity, starting lithium administration on day 7 would further amplify the already upregulated osteoblast activity. Continuing this treatment for 14 days would capture the entire duration in which enhanced bone formation has maximal impact on restoring union.

The optimal lithium regimen is highly relevant for clinical application. First, the onset time point of one week post fracture means that the patient will have sufficient time to receive a prescription for lithium. This is particularly important in military scenarios and underdeveloped parts of the world where patients may need to travel long distances to receive medical aid. Second, lithium can be orally ingested, and has been used safely via this mode of delivery for decades. Oral administration is simple and cost effective in comparison with other routes of administration. Third, lithium only needs to be taken for a short duration (two weeks) and would overall be an inexpensive therapy with minimal risk of side effects. Fourth, this regimen will not interfere or delay the surgical treatment that a patient with fragmentary fracture may require. Standard treatment can proceed as deemed crucial for fracture stabilization followed by physiotherapy, with no expected negative impact from daily oral intake of lithium.

Despite of lithium’s benefits, its narrow therapeutic range is considered an important concern for its use. Serum level of lithium in bipolar disorder patients is maintained between 0.8mEq/mL and
1.2mEq/L, above which the patient may experience side effects. Common adverse effects include diarrhea, weakness, dry mouth, weight gain and acne, while the more severe effects seen at higher serum levels (above 1.2mEq/L) are seizure, hyperparathyroidism, hypercalcemia and depression (Schou, 1970). Thus, the initial therapeutic dose of 1200-1700 mg/day for bipolar patients is reduced to 600-1500mg/day for maintenance therapy as soon as the serum level stabilizes. As such, close monitoring is required in the initial phase when starting lithium treatment. In contrast, the lithium dose of 20mg/kg in rats translates to approximately 250-300mg/day in healthy adults. This dose is half to a third of that administered clinically and is equivalent to the safe low starting dose that bipolar patients are recommended to avoid overwhelming the neurological and renal systems. Moreover, the treatment duration for fracture healing is very short compared to the typical duration for current lithium treatment for bipolar disorder that ranges anywhere from 3 months to years. Thus, a two week treatment with a very low dose is not expected to elicit any side effects or changes that would require medical attention in short or long term.

3.4 Conclusion

The efficacy of the proposed lithium treatment from earlier work in screening and optimization stages was successfully verified in an additional group of rats. The results were in agreement with those from the screening stage when the regimen was first evaluated; in both the stages nearly 1.5-fold improvement in the primary outcome measure of mechanical strength was found. Successful completion of this preclinical DOE study provides the foundation for subsequent evaluation of lithium drug in healthy fracture patients.
4. OVX Bone Model

Since the optimized lithium treatment was highly beneficial in healthy rats, the regimen was also evaluated in an impaired bone scenario, specifically osteoporosis. This bone condition is associated with an altered bone turnover rate leading to significantly diminished quality of bone tissue. Thus, it is important to understand if a therapeutic for fracture augmentation could aid in overcoming the poor bone healing response and lead to timely repair of osteoporotic bone fractures.

This chapter is focused on the second aim of the thesis, i.e. to evaluate the optimal lithium treatment in the ovariectomized rat fracture model.

4.1 Methods

*Animal Model:* Bilaterally ovariectomized Sprague Dawley rats were obtained from Taconic Laboratories (Albany, NY, USA). The rats were three-months old at the time of surgery. The procedure for ovariectomization is standard and non-risky (Steele, & Bennett, 2011). Briefly, under general anesthetic, the lower portion of the dorsal surface is sterile prepared and an incision of approximately 3cm is made along the midline closer to the base of the tail. The adipose tissue is then pulled apart to gain access to the periovarian fat; this is done for both the ovaries. Next, each ovary is exposed and a small incision is made between the fallopian tube and uterine horn. Once both ovaries are dissected, the uterine horn is placed back inside and the soft tissue is ligated with an absorbable suture. The incision site is closed and stapled.

The rats were housed in the animal facility at Sunnybrook Research Institute for three months post ovariectomy to allow for osteoporotic bone loss to occur. Throughout the housing period, they were fed with normal chow and water *ad libitum.*

*Fracture Experiment:* The fractures were created using the procedure as described in 3.1 for healthy rats with slight modifications. The femur was pre-stabilized with an intramedullary pin, but instead of drilling, the pin was simply inserted manually through the marrow cavity. The guillotine blade was dropped on the right limb diaphysis; however, the load utilized was varied based on the weight of the rat, ranging from 300-370g.
**Lithium Administration and Sacrifice:** The rats were closely monitored during the first week after fracture and radiographed weekly throughout the healing period. Lithium chloride (20mg/kg) or saline was orally gavaged daily from day 7-21 as performed in the healthy rats. The rats were sacrificed on day 28 post fracture and their femurs harvested bilaterally.

**Evaluation of Healing:** The healing was evaluated based on the same outcome measures as in the verification stage, with maximum torsional yield load as the primary outcome measure. Acquisition and processing of µCT scans and stereological analysis was performed at the same settings as described in 3.1.
4.2 Results: Impaired Bone Condition in OVX Rats

An established model of osteoporosis involving bilateral ovariectomy was utilized in this study. All the ovariectomized rats rapidly gained weight in the initial phase of the three-month housing period, which plateaued after 2.5 months (initial weight: 200-250g, weight prior to fracture: 350-400g). Such a dramatic weight change is expected due to the metabolic changes that follow chronic estrogen deficiency.

During the fracture experiment, the femurs of the OVX rats were notably weaker compared to those of healthy rats utilized in verification. First, the pin was easily inserted through the medullary canal without the need to drill and was then engaged at the proximal end simply by tapping, while in healthy rats it had to be drilled for insertion and engagement. Second, the drop load required to fracture the femurs of the OVX rats was similar or only slightly higher than that used for the healthy rats despite the significant weight differences and additional muscle and fat tissue surrounding the OVX bone.

The bone loss was evident in the reconstructed μCT scans: the OVX rat femurs had wider medullary canals (due to endosteal resorption) and significantly reduced trabecular bone at the epiphyses and metaphyses in comparison to the femurs of healthy rats. Transverse sections also revealed a thinner periosteum surrounding the callus/cortical bone (Figure 14). These bone-related changes, not generally seen in healthy 6-month old rats, occurred due to the ovariectomy in a manner that mimics post-menopausal changes in women.

Figure 14: Transverse slices through the healing femur of a healthy (left) and an OVX (right) rat.
4.3 Results: Sample Size and Fracture Classification

A power analysis based on the average and standard deviation values of the lithium and saline groups from the verification phase suggested the total number of samples required to be 26 (13 per treatment group) to identify at 20% difference for the primary outcome at a significance level of 5% and power of 80%. With the progression of our experimental work, a total of 53 rats were utilized. The initial study design, however, did not anticipate the further subdivision based on the fracture patterns.

Two different fracture patterns were obtained in the osteoporotic rats - simple and fragmentary fractures. Since these two types of fracture heal under different biological and mechanical conditions, the impact of lithium was evaluated separately. All the fractures of the pilot study were fragmentary. Rats were randomly chosen to receive lithium or saline at the time of fracture creation, but were segregated to either fracture type based on the fracture pattern seen in the µCT scan of each harvested femur. A fracture was classified as simple if it had a clean transverse or short oblique fracture line in the diaphyseal region; typically, it was associated with a clear two-part femur. If the diaphysis had more than three fragments, it was classified as fragmentary. The simple fracture type had 10 and 9 rats in the lithium and saline group, respectively. The fragmentary fracture type had 11 and 13 rats in the lithium and saline group, respectively. The remaining 10 rats were not included due to complications (details provided in 4.4 and 4.6).
4.4 Results: Simple Fractures

The exclusion criteria, similar to that for the healthy bone model, were applied. Upon evaluation of the fracture patterns, one rat was found to have some fragmentation around the fracture line and was included in the fragmented fracture category. Another rat was excluded from analysis since its healing femur demonstrated an unusual load-displacement curve with no yield point. Thus, the final group sizes were 10 and 9 for the lithium and saline groups, respectively.

4.4.1 Qualitative Healing

*In vivo* radiographic images prior to sacrifice (fours weeks after fracture) revealed visible fracture lines in all the rats irrespective of their treatment group. The healing calluses of both the groups were structurally identical with a periosteum that remained discontinuous at the fracture gap, and a wedge-shaped periosteal callus present on either side of this gap (Figure 15). These calluses had not sealed even in perfectly reduced fracture cases. The width of the callus varied, but qualitatively it appeared to be wider for femurs of the lithium group in comparison with the saline group. The callus structure resembled that which is typically seen in the early stages of the healing process where cartilaginous matrix fills the gap prior to periosteal bridging of fragments.

*Figure 15: Coronal sections of OVX rat femurs with simple fracture 28 days after fracture from saline-treated rat (left) and from lithium-treated rat (right).*
4.4.2 Stereology

**Fractured Femurs**: The two groups differed significantly for the following measures: bone volume ($p=0.029$), total volume ($p=0.039$) and bone mineral content ($p=0.019$), all three being higher for the lithium group (Figure 17). Bone volume fraction, BMD and TMD were similar. Additional analysis was performed using a small region around the fracture line to evaluate the properties of the callus tissue only (excluding the original bone) as shown in Figure 16. Three hundred sixty slices were taken above and below the fracture line and the total of 720 slices formed the new region of interest (ROI$_{\text{new}}$). This volume was sufficient to include all of the newly developed callus tissue. Using this modified ROI, no significant differences were found for any of the stereologic parameters. Only average total volume demonstrated a trend towards a higher value for the lithium group (24.6%, $p=0.08$).

**Figure 16**: Cross-sections through the healing femur with a simple fracture; The left image shows the old and newly mineralized bone, which was segmented to obtain only the new mineralized callus. This segmentation was used in 720 slice to form ROI$_{\text{new}}$ for analysis.

**Contralateral Femurs**: Similar to the trends for the original ROIs of the fractured femora, the contralateral femurs of the lithium group had significantly higher bone volume ($p=0.012$), total volume ($p=0.004$) and bone mineral content ($p=0.013$) (Figure 17).
Figure 17: Bar graphs showing average with standard deviation for the three stereologic parameters: bone volume, total volume and bone mineral content, which varied significantly between the treatment groups based on the original ROI.

4.4.3 Biomechanical Testing

**Fractured Femurs**: Average values for yield torque, twist angle at failure and torsional stiffness were similar between the lithium and saline group (109.2 vs 136.2 N-mm; 16.5 vs 15.3 °; 8.8 vs. 11.3 N-mm/°) (Table 5).
**Contralateral Femurs**: Average yield torque was higher for the lithium treated intact femurs (440.31 vs. 368.38 N-mm, p=0.145). Twist angle to failure and torsional stiffness were similar for the two groups.

Table 5: Results from torsion load testing of fractured and contralateral femurs of OVX rats with simple fractures.

<table>
<thead>
<tr>
<th></th>
<th>Yield Torque (N-mm)</th>
<th>Angle at Failure (°)</th>
<th>Torsional Stiffness (N-mm/°)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fractured Femur</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>109.19 (40.44)</td>
<td>16.51 (7.06)</td>
<td>8.80 (3.01)</td>
</tr>
<tr>
<td>Saline</td>
<td>136.23 (58.96)</td>
<td>15.34 (6.68)</td>
<td>11.28 (7.89)</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.268</td>
<td>0.717</td>
<td>0.394</td>
</tr>
<tr>
<td><strong>Contralateral Femur</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>440.31 (105.49)</td>
<td>11.29 (2.97)</td>
<td>39.45 (5.13)</td>
</tr>
<tr>
<td>Saline</td>
<td>368.38 (87.47)</td>
<td>10.52 (2.79)</td>
<td>35.77 (5.24)</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.145</td>
<td>0.591</td>
<td>0.165</td>
</tr>
</tbody>
</table>
4.5 Discussion: Simple Fractures

Simple patterns generally occur in elderly patients who are susceptible to low-energy fractures such as when they fall from standing height. Consequently, there is a low level of soft tissue damage and little impact to the cortical bone other than at the fracture line. Surgical fixation is a primary challenge in osteoporotic patients due to the weakened bone architecture leading to poor bone purchase and implant loosening. Overall, challenges related to bone status, co-morbidities, surgical success, and post-operative weight bearing often complicate the process of healing. While there is little conclusive evidence to suggest longer healing times in older patients, several retrospective studies have shown a greater need for secondary surgeries (~20%) in this cohort. This is accompanied with longer hospital stays, more rehab visits, and longer duration off work, all bearing financial consequences on the patient as well as the healthcare provider.

Stereological and Biomechanical Testing Analyses

In the OVX model, simple osteoporotic fractures were created such that the fractured femurs had cortical discontinuity without impact-associated comminution. After four weeks of healing, femurs of both the lithium treatment group and saline control had failed to unite at the fracture gap and a distinct unbridged mineralizing periosteal callus was evident. The lithium group had significantly higher bone volume (14.1%), total volume (21.4%) and bone mineral content (13.1%), but these stereological differences did not lead to any improvement in torsional strength. This was likely due to the unhealed fracture plane that was devoid of the periosteal layer and mineralizing callus that would have otherwise resisted some of the applied load. Thus, any improvement in the microstructural properties of the callus was not beneficial for the bone’s primary ability to bear load. When the fracture callus was reanalyzed using a redefined ROI without the old bone, the stereological differences disappeared.

Interestingly, among the contralateral femurs, these three stereological measures were significantly higher for the lithium group vs. the saline control group (BV: 12.9%, TV: 20.5%, BMC: 12.6%). Together, these results imply that the anabolic effect of lithium may be on the underlying bone rather than on the newly forming callus. In addition to improved micro-architecture, the contralateral femurs of lithium-treated OVX rats yielded at higher torsional loads in comparison to saline rats, although this difference was not significant (19.5%, p=0.15).
The differential impact of lithium on the old versus new matrix was surprising. Whether it is lithium or the synthetic molecule 603281-31-8, GSK-3B inhibitors act to increase the bone-forming osteoblastic activity. In the OVX state, lithium has been shown to restore the lost balance between adipogenesis and osteoblastogenesis leading to an increase in bone mass. Thus, the improved volume and mineral content in simple fractured OVX rats aligns with the impact of lithium. However, the fact that lithium did not augment the properties of new tissue may be due to the composition of the callus present. The fracture callus may not have been conducive for lithium, which acts on committed cells of osteoblastic lineage during the callus transformation stage. Micro-CT images showed radiolucent small calluses that were mineralized only at the surface ends. Moreover, the gap remained devoid of the periosteal layer. Periosteam restoration occurs during the initial phase of the repair process and it then serves as an important source of pre-osteoblasts during callus transformation. Thus, the thinned and unerestored periosteam may have failed to provide the substrate for lithium to act upon. Overall, the poor biological response may have been interlinked with an unstable mechanical environment in these simple fractures, which together prevented osteoblastic progression in the callus.

**Challenges in simple fracture healing**

The failure of the optimized lithium treatment in these OVX rats may have been related to the simple fracture pattern, as other experiments using simple fractures have demonstrated similar impairments in healing. Butezloff et al (2015) published their findings related to the effects of vibrational therapy for fracture healing in OVX rats. Their OVX model was similar to that used in our study, however they created the fractures by osteotomy. In their paper, \(\mu\)CT images at 28 days post fracture showed periosteal callus around but not at the gap, matching the structure and mineralization pattern obtained in our OVX rats. In fact, numerous such studies that create cortical injury by osteotomy have shown healing femurs that are similar in callus structure to our study. Based on the studies that evaluate later time points, the fracture lines and poor bridging are likely to be visible even at 6 and 8 weeks post fracture.

In hindsight, two healthy rats in the verification stage (one in each treatment group) had simple rather than fragmentary fractures. Both these rats were found to have unhealed fracture gaps that appeared similar on \(\mu\)CT images to the OVX simple fractures. The lack of callus bridging at the plane of the fracture gap in all these rats indicates an underlying common factor regardless of the
health status. It is known that mechanical loading is an important factor for bony union and that the forces realized by a simple two-part fragmented bone is considerably different from fractures involving more fragmentation. While soft-tissue injury and greater bone damage may lead to slightly longer healing duration, these conditions may actually be advantageous for fracture repair. Nikolaou et al (2009) suggested that despite a shorter healing time and comparatively less care required for simple fractures, they might have greater association with unpredictability of achieving union. In a simple fracture, there is no interdigitation of the two parts that can resist displacement when loaded. Their end surfaces have negligible bone-to-bone contact, which under weight bearing are free to slide or move. This in turn signals the callus to remain cartilaginous to allow for elongation and deformation to prevent complete failure. In contrast, comminuted bones have multiple gaps amongst which the load gets distributed and their overlapping fragments interdigitate to help resist rotational and translational motion.

Several animal studies have evaluated the impact of mechanical forces on fracture healing. Finite element modeling and controlled loading experiments in animals have shown that healing is significantly impacted under torsion, especially in the early stages, and is most accommodating for compression. This becomes critical for a completely reduced simple fracture in which small amounts of motion (translation or rotation) at the fracture line could result in high strains. To compensate for it, there is greater resorption to increase the gap, a process that is radiographically seen at ~2 weeks after fracture. However, if high strain continues to be exerted, the healing may not progress to mineralization, ultimately leading to delayed or even non-union.

**Conclusion**

Simple fractures were obtained in osteoporotic rats using drop loads that were just sufficient to induce bicortical breaks in the femoral shaft without causing additional bone damage. Contrary to expectations, lithium treatment was not able to improve healing by four weeks based on the result for primary outcome measure. An evident fracture line with no mineralized callus at the fracture gap may have resulted from a lack of required stability. It is difficult to predict if the healing was delayed or if simple fractures are inherently associated with such a progression wherein callus bridging occurs at a later time point. Lithium use in the context of simple fractures requires further understanding of the healing of simple fracture type in order to better evaluate the optimal period for lithium treatment.
4.6 Results: Comminuted Fractures

Drop loads were varied depending on the weight of the rat in order to achieve comminuted fractures without shattering the femur. Post fracture, any rat with severe comminution or fragment displacement on the radiographic evaluation was sacrificed; 4 rats were sacrificed for this reason. Three rats were excluded from analysis due to metaphyseal fragmentation. Metaphyses include trabecular bone and the load distribution in this region is different from diaphysis; hence, its fragmentation may heal differently in comparison with a pure diaphyseal fracture. One rat was excluded since its healing femur broke at the femoral neck during torsion load testing as a result of slippage in the potted cement. Another rat could not be evaluated since the intramedullary pin could not be removed from its canal at the time of harvestation. Finally, one rat was excluded since its medullary canal was visibly wider than other OVX rats. The diameter of the intramedullary pin was smaller for a canal of such a width, leading to inadequate fracture stabilization and greater pin motion during the healing period. Thus, the resultant fracture healing in this rat would be different in comparison to other OVX rats. The group size for comminuted fracture analysis was 11 and 13 for the lithium and saline treatment groups, respectively.

4.6.1 Qualitative Healing

μCT images of the healing femurs did not reveal any intergroup differences in callus structure or quality. The periosteal layer had restored at most of the gaps with poorly mineralized periosteal callus bridging the fragment ends (Figure 18). OVX calluses were smaller and less radiopaque in comparison with calluses of healthy rats (verification phase) despite the similar extent of comminution. The periosteal layer appeared thinner in all of the OVX femurs compared to healthy rat femurs.

![Figure 18: Representative sections of μCT images from healing femurs of (right) lithium-treated OVX rat and (left) saline-treated OVX rat.](image-url)
4.6.2 Stereology

**Fractured Femurs:** Average bone volume fraction and bone mineral density were lower for the lithium group (9.9% and 17.6%, respectively). No differences were found for other stereologic parameters (Table 6).

**Contralateral Femurs:** The average bone volume fraction was slightly lower for the lithium group, with the difference trending toward significance (p=0.08). All other parameters were similar.

Table 6: Stereologic analysis for fractured and contralateral femurs of fragmentary femurs in OVX rats.

<table>
<thead>
<tr>
<th></th>
<th>Bone Volume (mm³)</th>
<th>Total Volume (mm³)</th>
<th>BV/TV (%)</th>
<th>BMD (mgHA/ccm)</th>
<th>TMD (mgHA/ccm)</th>
<th>BMC (mgHA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fractured Femur</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>279.61 (30.07)</td>
<td>627.22 (112.98)</td>
<td>45.15 (5.82)</td>
<td>428.32 (97.28)</td>
<td>1183.84 (228.94)</td>
<td>332.23 (76.11)</td>
</tr>
<tr>
<td>Saline</td>
<td>285.10 (39.24)</td>
<td>589.67 (128.35)</td>
<td>49.26 (5.66)</td>
<td>513.26 (90.44)</td>
<td>1198.69 (266.01)</td>
<td>339.84 (78.24)</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.71</td>
<td>0.44</td>
<td>0.09</td>
<td><strong>0.05</strong></td>
<td>0.89</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Contralateral Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>199.38 (23.46)</td>
<td>333.18 (39.12)</td>
<td>59.85 (3.20)</td>
<td>745.57 (114.96)</td>
<td>1359.73 (321.89)</td>
<td>270.09 (64.45)</td>
</tr>
<tr>
<td>Saline</td>
<td>201.92 (17.11)</td>
<td>327.87 (33.44)</td>
<td>61.76 (3.06)</td>
<td>760.49 (133.77)</td>
<td>1275.31 (267.85)</td>
<td>257.38 (58.00)</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.80</td>
<td>0.74</td>
<td>0.16</td>
<td>0.79</td>
<td>0.54</td>
<td>0.66</td>
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</tbody>
</table>
4.6.3 Biomechanical Testing

**Fractured Femurs:** The lithium group demonstrated a weak trend towards higher yield torque (234.0 N-mm vs 206.2 N-mm, p=0.10) and torsional stiffness (21.5 vs 16.4 N-mm/°, p=0.12). The differences between the two groups were 13.5% for torque and 31.1% for stiffness. Twist angle at failure did not vary between them (Table 7).

**Contralateral Femurs:** No differences were found between the groups for any measure of torsional strength (Table 7).

Table 7: Results from torsional load testing of fractured and contralateral femurs of OVX rats with fragmentary fractures.

<table>
<thead>
<tr>
<th></th>
<th>Max Torque (N-mm)</th>
<th>Angle at Failure (°)</th>
<th>Torsional Stiffness (N-mm/°)</th>
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<tr>
<td><strong>Fractured Femur</strong></td>
<td></td>
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<tr>
<td>Lithium</td>
<td>233.96 (49.63)</td>
<td>13.68 (9.02)</td>
<td>21.49 (7.99)</td>
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<td>Saline</td>
<td>206.16 (37.85)</td>
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<tr>
<td>P-value</td>
<td>0.10</td>
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<td>0.12</td>
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<td><strong>Contralateral Femur</strong></td>
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<tr>
<td>Lithium</td>
<td>387.20 (91.72)</td>
<td>9.95 (3.29)</td>
<td>41.39 (7.13)</td>
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<td>Saline</td>
<td>348.20 (95.02)</td>
<td>9.85 (3.42)</td>
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<tr>
<td>P-value</td>
<td>0.413</td>
<td>0.950</td>
<td>0.293</td>
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4.6.4 Correlation Analysis

A significant correlation was found only between torsional stiffness and bone mineral content (r=-0.42, p=0.04). Inverse relations between stiffness and total volume (r=-0.36), and twist angle at failure and bone mineral content (r=0.39) trended towards significance.
Table 8: Pearson correlation analysis for healing OVX femurs with fragmentary fractures.

<table>
<thead>
<tr>
<th></th>
<th>Bone Volume</th>
<th>Total Volume</th>
<th>BV/TV</th>
<th>BMD</th>
<th>TMD</th>
<th>BMC</th>
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<td><strong>Yield Torque</strong></td>
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<td>.123</td>
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<td></td>
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<td>.565</td>
<td>.745</td>
<td>.575</td>
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<tr>
<td><strong>Angle at Failure</strong></td>
<td>Pearson (r)</td>
<td>.321</td>
<td>-.271</td>
<td>-.342</td>
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<td>.387</td>
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<td></td>
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<td>.126</td>
<td>.200</td>
<td>.102</td>
<td>0.232</td>
<td>0.062</td>
</tr>
<tr>
<td><strong>Torsional Stiffness</strong></td>
<td>Pearson (r)</td>
<td>-.261</td>
<td>.304</td>
<td>.232</td>
<td>-0.332</td>
<td>-.422</td>
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<tr>
<td></td>
<td>P-value</td>
<td>.218</td>
<td>.149</td>
<td>.276</td>
<td>.113</td>
<td>.040</td>
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</tbody>
</table>
4.7 Discussion: Fragmentary Fractures

Comminution in elderly fracture patients can occur even under low impact trauma. Fragmented long bones remain a challenge as their surgical management is affected by the diminished health of the underlying bone and the weakened cortices. Current fracture epidemiology is significantly higher amongst the elderly population today in comparison to the rate in past decades possibly due to the increased level of physical activity. This study aimed to evaluate if the optimized lithium dosing regimen developed for otherwise healthy healing femoral fractures was also capable of rescuing the impaired healing process in case of osteoporotic bone. It was found that the lithium regimen did not improve fracture healing in the OVX rats at four weeks post fracture.

Imaging Analysis

Reconstructed μCT scans of the harvested femurs revealed less prominent callus in OVX rats compared to the callus seen in healthy rats (from the verification stage) at the same timepoint. Also, the callus appeared less mineralized, which was confirmed by the lower average TMD of OVX versus healthy rats. Even though the rats are not age-matched (OVX: 6 months old and healthy: 3 months old at the time of fracture), they had sexually mature bone that primarily differed in only the estrogen status. After four weeks, healing progression was not the same in the two models, which may have impacted the overall efficacy of the lithium treatment.

The poor healing observed in the OVX rats is not surprising. Bone health is well tied with endocrine function. Estrogen receptors, alpha and beta, are expressed on osteoblasts and osteoclasts in a differential manner and they direct estrogen signaling for cell proliferation and/or osteogenic gene expression. When the estrogen level plummets, the healing ability, at least via the default repair pathways, is affected. Batra et al (2003) found that the callus biopsies from older patients, taken 10-15 days after fracture, had fewer mesenchymal cells expressing the beta isoform and that the older women also had reduced number of osteoblasts. Chow et al (2014) similarly reported slow recruitment and activity of progenitor cells in fractured OVX rats compared to sham controls. Clearly, the biological response to fracture injury is significantly affected by estrogen deficiency. This may explain why the callus would develop slowly and may suggest a weaker response to anabolic treatments. Findings demonstrating similar impairment in vivo were reported by Namkung-Matthai and colleagues in their 2001 paper. They found smaller,
less mineralized calluses (cross-sectional area lower by 40%) in OVX rats compared to sham-operated rats at 21 days after fracture. Therefore, the healing response in our rats was likely subdued or perhaps delayed, as is the case with OVX animals.

**Stereological and Biomechanical Testing Analyses**

\(\mu\)CT-based quantitative analysis showed that the OVX lithium group had lower bone volume fraction (-10%) and BMD (-17.6%) in comparison with the saline group. These calluses did not differ in their overall mineral content, which implied that the lithium-treated femurs had greater callus volume rather than a lower level of mineralization. This was supported by the higher total volume (albeit not significant) for the lithium group. From a biomechanical standpoint, while large calluses are expected to impart higher structural rigidity, the lack of increased overall mineralization of the callus may negate any such effect.

In contrast to the healthy model, lithium treatment in OVX rats did not significantly improve the torsional strength of the healing femurs. All the OVX femurs twisted to a similar degree prior to elastic deformation and failed through the callus. While the lithium group had a higher average yield torque (233.9 vs 206.1 N-mm) and torsional stiffness (21.5 vs 16.4 N-mm/°), the differences were not significant. Considering that yield torque is the primary outcome measure study, the minimum required improvement in torque for clinical significance is 20%. Thus, the 13.5% higher torque is not sufficient to support the evaluated lithium treatment even if the difference was statistically significant. With respect to torsional stiffness, a 31.5% difference was found; however, this difference was also not significant and the high variability within the saline group limited an intergroup comparison. Thus, the lithium regimen seemed to have positively impacted healing but not substantially in comparison to saline treatment.

High variation was found in this study for some outcome measures including total volume, BMD, TMD, BMC and torsional stiffness. While it is not uncommon for biological experiments to have variability, additional factors specific to this study may have further contributed to it. The most important of these factors may have been the combined impact of the variable degree of femur comminution and the uncontrollable degree of injury to the surrounding tissues. While the femur was positioned under the guillotine blade in a consistent manner, the resulting fractures were heterogeneous. This is attributed to the fracture method where factors such as soft tissue...
coverage and energy dissipation through the bone ultimately dictate the level of comminution. The number and arrangement of bone fragments affected their interaction under load, which likely dictated the quality and distribution of callus. Simultaneously, if the trauma involved severe damage to the periosteum, the healing was likely to be negatively impacted. Several studies have shown a greater contribution to fracture healing from surrounding muscles when they are damaged. These muscles supplement the fracture milieu with stem cells and growth factors, thereby aiding rather than hindering the healing process. However, extensive soft tissue damage is detrimental to fracture healing. Thus, the variable degree of load-inflicted trauma in the rats may also have impacted the evident callus. Other factors such as physiological response to injury especially under impaired bone conditions and the amount of weight bearing in the initial period after fracture may have further contributed to the variability seen in this study.

Contralateral femora of the lithium and saline groups were similar in their torsional strength. However, the lithium group had 4% lower bone volume fraction. Interestingly, this is similar to the 4.3% difference noted in the healthy model. Moreover, in both the healthy and OVX models, bone volume fraction was the only outcome measure that differed between the treatment groups in the contralateral limbs. Thus, lithium’s off-target effect was similar irrespective of the bone health in case of rats with fragmentary fractures. This was possibly due to the similar physiological response to the traumatic injury. However, for the OVX group with simple fractures, the contralateral femurs exhibited different set of changes following lithium treatment. The volume of the femur and its bone mineral content were increased rather than changes in the bone volume fraction.

Despite of the identical lithium treatment, the evident impact of the treatment on fracture healing was different in the osteoporotic versus healthy rats at 4 weeks post fracture. This may have resulted from differences related to their age as well as bone status. Healthy rats were three months old at the time of fracture, while the OVX rats were six months old. In rats, maximal bone growth occurs in the first three months of their life, which then slows down but not halt completely, in the period of 3-6 months of age. Govindarajan et al (2014) performed a study to understand tibial fracture healing in young and osteoporotic rats. They showed that the tibial torsional stiffness in 2.5 months old healthy rats was lower compared to 5.5 months old sham-operated rats. Also, the 5.5 month old ovariectomized rats had similar stiffness as the sham
operated 5.5 months old rats. In addition to the age difference, the bones of osteoporotic rats were diminished in quality at the time of fracture. Thus, the subsequent healing response especially due to the absence of estrogen may have negatively affected the extent of recovery. Osteoporotic rats have been consistently shown to heal their fractures slower than sham-operated animals, implying that the osteoporotic rats were likely at a slightly earlier stage in the healing process at the four week time point. Thus, these physiological differences may have ultimately affected the impact of the identical lithium treatment.

**Conclusion**

The lithium regimen that was efficacious in healthy animals failed to augment fracture healing in ovariectomized osteoporotic rats. No differences in torsional strength (primary outcome measure) of the healing femurs were found between the treatment and the control group. A sub-analysis based on the fracture type also did not find any significant benefit of lithium for either simple or fragmentary fractures. Hence, further optimization and evaluation of the lithium administration parameters is required in osteoporotic rats to truly understand lithium’s potency for osteoporotic bone fractures.
4.8 Potential Shortcomings of the Lithium Regimen in OVX Model

Fractures in both the healthy and OVX model were induced in a similar traumatic fashion, fixed with an intramedullary pin and rats were treated identically with respect to feed and drug regimen. Lithium treatment was efficacious for healthy bone fracture healing and improved the primary outcome measure of biomechanical strength. However, in case of OVX bone fractures, lithium failed to impact the quality of the healing tissue or its mechanical strength at week four post fracture. This suggests a need for further optimization of the administration parameters in consideration of the physiological impairment associated with estrogen deficiency and osteoporotic bone.

In the screening stage of this study, a significant inverse relation was found between maximum yield torque and treatment onset time. Any fracture that undergoes endochondral healing, whether in a healthy or an impaired bone, will go through a transition phase from soft to hard callus. This transition period is the target point for lithium. In the OVX model, adjusting the key parameter of treatment onset time may be required due to the delay in the healing progression.

Namkung-Matthai and colleagues (2001) were the first to show that estrogen depletion leads to a delay in the early stages of fracture healing. Based on their results, this would represent the reparative phase of callus development. It is during this phase that angiogenesis occurs and the tightly regulated differentiation of osteochondral cells ensues. Both these processes are affected directly and indirectly by the lack of estrogen. Estrogen mediates neovascularization and, in fact, several potential therapeutics (for instance, vascular endothelial growth factor and statins) aim to improve angiogenic response during healing to induce bone formation. Treatment with these agents has led to higher BMD and increased expression of osteoblastic biomarkers in the healing callus.

In addition to the indirect implication of estrogen depletion, ovariectomized rats have reduced osteoinductive ability as a direct effect of the lack of osteoblastic signaling through estrogen receptors. These missing components cannot be compensated by loading-stimulated bone growth (i.e. weight bearing). Considering that lithium only augments healing once the mesenchymal cells become committed to osteochondral cells and that successful chondrocytic tissue has formed, the ideal treatment onset time likely will be delayed in OVX rats. As such, delaying the
onset of lithium administration (from day 7 to day 10 or 14) may be beneficial in OVX rats. A two-week treatment from this point should capture the duration in which osteogenic activity is maximal during the healing process.

Another factor to consider is the lithium dose delivered to the OVX rats. Poor renal function occurs in ovariectomized animals (similar to that seen in in elderly patients). Estrogen is believed to play a protective role in normal kidney function. Nielson et al. (2003) were the first to evaluate the impact of menopause on lithium clearance by the renal system in middle-aged Wistar rats. They showed that ovariectomized animals had significantly reduced lithium fractional excretion in comparison with sham-operated controls, and that the lithium clearance rate did not increase as much with maximal stimulation by glycine infusion. Despite the older age of these rats, additional treatment groups (OVX+ progesterone and/or estrogen supplementation) demonstrated that age as well as estrogen depletion induced a reduction in renal function, which was restored through supplementation. These results are in line with findings from other studies that implicate a link between estrogen, its receptors and kidney function (Rogers et al., 2007). As such, in our study, the serum lithium levels in OVX rats may have been higher than desired due to lower rates of renal clearance. In the screening stage of our study, higher lithium doses were associated with poorer healing in comparison to no treatment controls.

Apart from the administration parameters, the time point of evaluation may have influenced the lack of evident healing. Femurs evaluated at 28 days post fracture may have been too early to evaluate the potential benefits of lithium in osteoporotic bone healing. Healthy rats are expected to achieve union in femoral shaft fractures after 6-8 weeks. Numerous studies have collectively demonstrated that OVX animals experience slower healing. While the exact timeline of the delayed process is not established, Chow et al. (2011) found that the healing in OVX rats was delayed at the 2-4 weeks time point, based on molecular and histomorphometric analyses. This is the period when the callus undergoes mineralization. This may explain the inefficacy of lithium administration from weeks one to three to target and improve mineralization by week four in the OVX rats. Evaluation of the healing OVX femurs between week 5 and 6 post fracture may be more suitable to assess the impact of lithium therapy.
5. Future Directions and Conclusion

This thesis confirmed that a dose of 20mg/kg lithium chloride given daily beginning 7 days post fracture for 2 weeks will significantly improve the biomechanical strength of healing shaft fractures in otherwise healthy bone. With respect to future clinical translation, this regimen is attractive as it involves a low dose of an inexpensive drug that needs to be orally ingested for only 2 weeks. Moreover, lithium intake needs to begin a few days after injury, giving sufficient time to the patient to obtain the drug and begin administration. Thus, with the successful determination of the effective regimen in the healthy rodent model, it is worthwhile to continue pursuing lithium as a potential candidate for augmentation of fracture repair. However, additional work needs to be done toward evaluating and optimizing lithium administration in the scenario of impaired bone healing.

5.1 Evaluation of Lithium Treatment in OVX Rats

Despite the inefficacy of the optimized lithium regimen in osteoporotic rats, lithium cannot be ruled out as ineffective for treating fractures in impaired bone. The delay in the healing process and a reduction in lithium clearance rate (occurs in osteoporosis) may have contributed to this result. While the precise stage of delay in the healing process is debated, the process has been shown to be evidently delayed at the 2 week time point. Thus, if the callus mineralization does begin at ~ day 7, then the lithium treatment onset used may be too early and require extension. Moreover, older and/or osteoporotic rats have impaired renal clearance. Thus, it is possible that the osteoporotic rats had serum lithium that was significantly higher than in healthy rats, suggesting further optimization of lithium dosing.

In consideration of these differences between healthy and osteoporotic rats, an additional set of 20 osteoporotic rats will undergo lithium or saline treatment, with the lithium treatment utilizing a day 10 onset point with a dose of 20mg/kg lithium chloride gavaged daily for 2 weeks. The calluses will be evaluated around 6 weeks instead of 4 due to the delayed nature of healing. The dose in this study will not be modified since dose parameter was not found to be significant.
during screening stage of the DOE model. However, lithium serum levels will be evaluated. This modified regimen is hypothesized to better target the osteoblastic phase of healing and result in significant improvement in biomechanical strength of the healing femurs compared to saline controls.

### 5.2 Clinical Translation of Lithium Regimen

A phase II trial was proposed based on the results of the pre-clinical study in the healthy femoral fracture model and it has recently received funding for a pilot scale study. This will be a single-center, double-blinded, placebo-controlled randomized trial to be conducted at Sunnybrook Health Sciences Centre in Toronto, Canada. Healthy patients presenting with fractures of the femur, tibia or humerus will be assessed preoperatively and will be given the option to participate in the trial if they meet the inclusion criteria. The patient must be between 18-50 years of age, be otherwise healthy with no pathological bone conditions, and have a closed diaphyseal fragmentary fracture with low soft tissue injury. It is of paramount importance that the patient has normal kidney function. About 20% of the lithium is cleared through the renal system, while the remaining is absorbed. In case of dysfunctional kidney, the serum lithium level could quickly rise to toxic concentrations. Current or past patients of psychiatric disorders or those on medications that may interfere with lithium will be excluded. Also, any patient with surgical intervention directed towards intramembranous ossification for healing will be excluded.

The primary healing outcome will be time to radiographic union defined as evident callus bridging in three of the four cortices in anteroposterior and mediolateral planes. The secondary measure will relate to clinical healing including functional assessment and pain-free weight bearing. The patient will be closely monitored by a clinical team for any adverse effects related to lithium drug.

The treatment will include a daily oral intake of ~250 mg/day beginning two weeks after fracture. It will be continued for 14 days. These administration values have been chosen based on the differences in rat and human physiology, and our understanding of endochondral bone healing.
Dose

Reagen Shaw and others (2007) provided detailed information for translating a dose from one species to another based on FDA guidelines. This approach is based on body surface area to better reflect basal metabolism rather than only body weight. According to their methodology, 20mg/kg in a rat translates to 3.24mg/kg in the human. For a 60-90 kg adult, the dose will be 195-290 mg/day. However, an important consideration specific to the lithium drug is lithium serum level. The upper limit of therapeutic range is 1.2mEq/L in serum, above which lithium’s side effects may be experienced. However the pharmacokinetics of lithium in the rat and human is very different. The half-life of lithium in rats is about 6 hours, while in humans it has shown to average 20 hours. Moreover, the pharmacointerval plays an important role in sustaining the serum concentrations. Thus, the translated dose is an estimation that may need to be optimized based on the results of the pilot trial study in patients.

Chmielnicka and Nasiadek (2003) orally administered 10 mg/kg or 20 mg/kg lithium to Wistar rats every day for five days. At two weeks, the serum levels averaged at about 4mg/L, meaning ~0.57 mEq/L in rats. The same serum levels may be achieved in humans with a 250-300 mg dose in humans, based on the work by Guelen et al (1992). Guelen and colleagues compared the bioavailability of lithium in adult males when it was administered as a tablet versus in the syrup form. The subjects, upon receiving a single dose of 250mg tablet, had peak serum concentration in an hour, which was 0.52 mEq/L. The level tapered off to 0.15 mEq/L after 12 hours.

Putting the proposed dose in perspective of the current standard treatment, it is about a third of the daily amount for maintenance therapy. The starting dose in bipolar disorder patients is anywhere from 900 to 1800 mg/kg depending on serum level response. Once the level is stabilized, the maintenance dose is 600 to 900 mg/day. Thus, the dosage for fracture treatment is not likely to manifest in undesirable, adverse side effects.

Onset and duration

The onset time of 2 weeks is chosen to target the clinical time point when the soft cartilaginous callus begins to transform to a hard, mineralizing matrix. This is generally seen on radiographs approximately two weeks after fracture. A two week treatment duration should sufficiently up
regulate osteoblastic activity, and woven bone matrix is generally evident three to four weeks post fracture. Thus, continued treatment for longer than 2 weeks would not be necessary. From a clinical standpoint, for the therapeutic to be truly beneficial, it should be able to enhance bone formation by 4 weeks as fractured bone already has a fully mineralized callus at ~ week 6 even without adjunct treatment.
5.3 Conclusion

Fractures are complex injuries that require appropriate care and resources for the ultimate success of anatomic restoration of the bone. Improvements in surgical techniques and implant devices have helped to alleviate the overall burden of skeletal injuries; however, unpredictable failures in the healing process continue to pose a challenge. Impaired status of the underlying bone (i.e. due to osteoporosis) further complicates fracture healing. Current epidemiology estimates 200 million people with osteoporosis, of which more than 25% will experience an osteoporotic fracture.

Extensive research is underway to find effective, widely accessible solutions for reducing osteoporotic fracture risk. Current clinical approach involves administration of bisphosphonates, a group of drugs that impair bone resorption. Yet their long term efficacy remains questionable and their impact on resorption is detrimental to fracture healing. Many attractive pharmacologic or non-surgical therapies have emerged as promising options for aiding fracture healing. But their use is hindered by high costs, limited benefit in clinical trials or poor understanding of their long-term effects. New drug treatment options which may seem promising at the pre-clinical level, face a long testing and approval process for ultimate entry into clinical practice. As such, there is a need for an adjunct therapy that is economical, widely accessible and easily implementable for fracture repair.

Lithium is the gold standard for treating bipolar disorder, but it has been found to have an anabolic effect on bone. Despite this potential, it has remained largely unexplored for the purpose of augmenting fracture healing. To address this gap in our current knowledge, a preclinical study was undertaken in our laboratory in 2012, following the initial findings of Dr. Benjamin Alman’s laboratory in 2007. The overarching goal of the study was to delineate the precise administration conditions of lithium to improve healing in a rat femoral shaft fracture model. The first two stages of this DOE based study collectively proposed an optimal lithium regimen.

This thesis performed the verification stage of the DOE model in healthy rats. The results obtained in verification experiments well agreed with those from screening and optimization stages. The efficacy of the proposed optimal lithium regimen (7 day onset, 20 mg/kg dose and 2
week duration) was confirmed to yield a significant 1.5-fold increase in load bearing ability in comparison with controls. This marked the completion of the first aim of the thesis.

This optimal lithium regimen was then evaluated in osteoporotic rats to determine whether an impaired bone can benefit from this treatment. Lithium regimen was evaluated in two different fracture scenarios: one representing a low-trauma simple fracture more commonly present in osteoporotic patients, and the other representing a more traumatic injury with bone fragmentation. In the simple fractures both lithium and controls did not achieve cortical bridging at the fracture gap. Moreover, in contrast to the healthy rats, no significant improvement was conferred by lithium for fragmentary fracture healing in the osteoporotic rats. These results do not indicate a definitive lack of lithium’s potency for osteoporotic bone, but rather suggest the need for further optimization of the administration conditions under the impaired bone conditions.

In summary, this thesis confirmed the efficacy of the optimized lithium regimen for healing of healthy bone fractures. This regimen was not effective for fractures of osteoporotic bone. Thus, the findings in this thesis will guide further optimization studies related to lithium use in an osteoporotic bone. Meanwhile, the combined work from the DOE study strongly support the clinical evaluation of lithium drug among healthy fracture patients. Lithium is an inexpensive drug that has been in use for psychological treatment for over 60 years. Its therapeutic range and potential side effects are well understood. Lithium dose for fracture repair is expected to be only a third to a half of that currently administered to psychiatric patients, and is required for only a short duration. Thus, if proven effective in clinical trial, lithium can be easily incorporated as an adjunct therapy for fracture treatment and may ultimately represent a much needed accessible therapeutic for reducing the global burden of fractures.
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