THE EFFECTS OF IRRADIATION ON BONE FRACTURE HEALING: CAN IT PROMOTE MINERALIZATION AT LOW DOSES?

by

Nurettin Heybeli

M.D., Istanbul University (1992)

Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Engineering

Boğaziçi University
February 2010

THE EFFECTS OF IRRADIATION ON BONE FRACTURE HEALING: CAN IT PROMOTE MINERALIZATION AT LOW DOSES?

APPROVED BY:

Assoc. Prof. Dr. Halil Özcan Gülçür .....
(Thesis Advisor)

Prof. Dr. Fahri Erdoğan .....

Assoc. Prof. Dr. Can A. Yücesoy .....

DATE OF APPROVAL: February 26, 2010

ABSTRACT

THE EFFECTS OF IRRADIATION ON BONE FRACTURE HEALING: CAN IT PROMOTE MINERALIZATION AT LOW DOSES?

Non-union, or delayed union of a bone fracture poses a major burden both to the individual and society. This experimental study investigated the hypothesis that low dose irradiation can enhance fracture healing and mineralization. Standardized transverse femur fractures were created and intramedullary fixed with an open technique to forty young adult, male Sprague-Dawley rats and randomized to RT (irradiation with 1 Gy) and C (controls, sham treatment) groups. At third and sixth week after fracture, high resolution Bone Mineral Density (BMD) analysis, bone scintigraphy and radiographic examination with a mammography device were performed to subgroups (RT3, C3, ve RT6, C6) and rats were sacrificed for histopathological examinations. Statistically significant differences were found at sixth week; as BMD index was found to be higher in RT group ($p = 0.006$) and BMD value was found lower in the non-fractured regions of the irradiated femurs ($p = 0.005$). No statistically significant differences were found between groups for other parameters. Lamellar bone formation was disorganized at group RT6 when compared with controls by histopathological examinations. The results showed increased mineralization at the fracture site only when compared with irradiated non-fractured bone region, which cannot be regarded as a basis for clinical practice. However, when applications like heterotopic ossification prophylaxis are considered, the issue remains to be solved by molecular techniques, specifically for doses between 1 and 5 Gy.

Keywords: irradiation, fracture healing, bone mineral density, bone scintigraphy.

ÖZET

KIRIK İYİLEŞMESİ ÜZERİNE RADYASYONUN ETKİLERİ: DÜŞÜK DOZLAR MİNERALİZASYONU ARTTIRABİLİR Mİ?

Kırığın geç kaynama ya da kaynamama sorunları hem bireye hem de topluma ciddi bir sorun oluşturmaktadır. Bu deneysel çalışma düşük doz radyasyonun kırık iyileşme ve mineralleşmesini arttırabilme hipotezini araştırmıştır. Kırk Sprague-Dawley genç erkek rat standart açık yöntemle transvers femur kırığı ve K-teli ile intramedüller çivileme uygulandıktan sonra RT (1 Gy radyoterapi) ve C (kontrol, sham radyoterapi) gruplarına ayrıldı. Kırık sonrası üçüncü ve altıncı hafta alt gruplarında (RT3, C3, ve RT6, C6) yüksek çözünürlüklü Kemik Mineral Yoğunluğu (Bone Mineral Density, BMD), kemik sintigrafisi ve mammografi cihazı ile radyolojik incelemeler sonrası histopatoloji için sakrifikasyon uygulandı. İstatistiksel olarak anlamlı sonuçlar, sadece altıncı haftada görüldü; ışınlanan ratlarda kontrole göre BMD indeksinde yükseklik ($p=0.006$) ve ışınlanmış kemiğin kırık olmayan bölgesinde BMD düşüklüğü ($p=0.005$) saptandı. Diğer parametrelerde anlamlı fark yoktu. Histopatolojik incelemelerde RT6 grubunda kontroller ile karşılaştırıldığında lamellar kemik oluşumu organize değildi. Sonuçlar, düşük doz radyasyonun sadece ışınlanmış komşu kemik ile karşılaştırıldığında kırık bölgesinde mineralizasyon artışına neden olabildiğini göstermiştir. Bu durum klinik uygulamalara temel oluşturabilecek yönde değildir. Ancak heterotopik ossifikasyon profilaksisi gibi ilgili uygulamalar dikkate alınınca konunun moleküler açıdan ve 1 - 5 Gy aralığında incelenmesinde yarar görünmektedir.

Anahtar Sözcükler: radyoterapi, kırık iyileşmesi, kemik mineral yoğunluğu, kemik sintigrafisi.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BMP	Bone Morphogenic Protein
DEXA	Dual-Energy X-ray Absorptiometry
FGF	Fibroblast Growth Factor
GDF	Growth Differentiation Factor
HIF	Hypoxia Inducible Factor
IGF	Insulin-like Growth Factor
IL-1	Interleukin 1
IL-6	Interleukin 6
LIPU	Low Intensity Pulsed Ultrasonography
MDP	Methylene Diphosphonate
MSC	Mesenchymal Stem Cell
PDGF	Platelet Derived Growth Factor
PEMF	Pulsed Electromagnetic Field
TNF-α	Tumour Necrosis Factor Alpha
TGF-β	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor

1. INTRODUCTION

Bone has unique characteristics. It is a mineralized connective tissue providing the structural support for the body and is storage for essential ions. Extracellular matrix proteins which can mineralize under the surveillance of cells, maintain the structural integrity of the bone while responding to the metabolic requirements of the body. Bone is a composite material with outstanding properties. By the help of its unique structure and composition, it has excellent resistance to failure, which is bone fracture in the clinical setting, while retaining low mass. This capacity allows it to mechanically optimize its structural role and its role in mineral homeostasis [1].

Bone is not an inert material. It is a dynamic tissue that has cellular sensory and response systems. Bone tissue is capable of monitoring the local environment and, in case can organize its mass to accommodate functional demand. It has significant ability for self-renewal, therefore can be specified as ingenious among tissues to heal without scarring.

An understanding of the principles of bone healing is needed for the treatment of fractures, non-unions, bone defects, correction of bone deformities, and healing of osteotomies and arthrodeses. Bone formation during a bone fracture healing process is influenced by various factors which can be delayed or even arrested. Bone formation cascade consists of interlaced steps; formation of a hematoma and inflammatory response of the body, angiogenesis and cartilage formation, cartilage calcification and removal, bone formation and finally bone remodeling [2]. Fracture healing can occasionally be complicated with problems like delayed unions and non-unions. The condition of non-union is one of the major devastating complications in trauma care. Nonunion has been defined as no demonstrated change in healing on serial radiographs over a 3-month period. Delayed union is defined as a speed of fracture healing that is slower than anticipated, with no implied expectancy of either eventual healing or eventual nonunion [3].

Non-union, or delayed union of a fracture poses a major burden both to the individual and society. The incidence of a long bone non-union is reported to be about 5 - 10% of all fractures [4]. For the year 1996, In the United States of America 18 million cases of limb injuries have been reported [5]. In 1999, approximately 6 million extremity fractures occurred [6]. For the year 2000, the number increased to approximately 7.9 million fractures [7].

The economic costs have been identified for different types of treatment of long bone non-unions. Assuming an average cost in lost wages and additional medical treatment for each of these cases of 10000 US Dollars, the annual economic loss is 3 to 6 billion US Dollars [8]. In a best-case scenario of a study from United Kingdom, an uncomplicated course of a standard fracture and patient with ordinary activities and demands in terms of function, the cost of common long bone non-unions is over 15.000 Pounds [9].

1.1 Aim of This Study

Combined basic and clinical research resulted in definitive solutions to many problems in orthopedic surgery, regrettably non-unions is not one of these problems. Although many adjunctive treatment options to stimulate normal fracture healing, delayed unions and non-unions have been developed, the problem still have not been solved completely.

It is evident that radiation can profoundly affect the normal biological process of bone repair. Since irradiation is quite likely to be in the armamentarium of physicians as a method of treatment or prophylaxis for problems of musculoskeletal system, it is important to know the dose-effect relation of this highly beneficial and at the same time potentially hazardous modality. Growing cartilage and bone is relatively radiosensitive compared to mature bone and cartilage. Rapidly reproducing bone marrow cells are highly radiosensitive. Irradiation alters DNA transcription arresting osteoid formation. It prevents differentiation of pluripotent mesenchymal stem cells

(MSCs) into osteoblasts [10]. Therefore, we can conclude that in clinical conditions of high metabolic and cellular activity like fracture healing, bone is more susceptible to irradiation.

Very recently, Zhou et al. [11] claimed that low dose irradiation promoted mineralization at the stage of hard callus formation in a rat fracture model. This study was criticized because of inappropriate methods and analysis of the results by Heybeli et al. [12]. Biological systems can have different dose response relationships. Therefore, trying to find out the effects of low dose irradiation on bone fracture healing by using appropriate methods is essential. In this study, we tried to find out if low dose irradiation exerts beneficial effects that can be used as an adjunctive therapy in fracture healing.

2. BONE FORMATION

Bone formation is a very complex and unique biological process. Unlike the other parts of the musculoskeletal system, formed bone tissue after a fracture is identical to the original. Stages of fracture healing are events with a distinct sequence. Starting with the formation of a hematoma and inflammatory response of the body, angiogenesis and cartilage formation are followed by cartilage calcification, removal, bone formation and eventually bone remodeling. Bone formation after a fracture resembles the stages of embryonic bone development. Therefore we can name this process of bone formation as "regeneration", not "reparation". Molecular, mechanical, cellular, local and systemic variables can affect bone healing and formation. These many variables that are crucial to bone formation must be studied in detail for the formulation of a treatment plan for a fracture.

During development there are two paths of bone formation; intramembranous and endochondral bone formation [1]. Intramembranous bone formation occurs independent of a preexisting model while endochondral bone formation occurs by replacement of a cartilaginous structure. During endochondral bone formation, cartilage proceeds through stages of hypertrophy and calcification. The product is woven bone. Woven bone is composed of a poorly organized matrix which scaffolds deposited hydroxyapatite. Osteoclasts remove woven bone to replace by lamellar bone. The main characteristic of lamellar bone is its highly organized mineralized matrix and the Haversian canal system [13, 1].

Another way to analyze bone formation is to classify the process as; primary (direct) or secondary (indirect). Primary bone formation occurs mainly when the apposed fractured areas can be directly approximated and this can only be accomplished if anatomic reduction and rigid fixation is employed, by surgical open reduction and internal fixation with plates and screws, or rigid nails. Then, the main remodeling unit, "cutting cone" can efficiently act. Therefore, osteoclasts form a pathway for

osteoblasts to form bone. In this type of bone formation, minimal interfragmantary strain and distance (less than 200 - 500 micrometers) is required. In secondary bone healing, a combination of intramembranous and endochondral bone healing can be seen. Relative stability (or instability) at the fracture site leads to callus formation, showing that all anatomical structures around the fracture participates to the healing process.

2.1 Regulation of Bone Formation

Fracture repair proceeds through the coordination of multiple steps such as migration, differentiation, and activation of multiple cell types and tissues [14]. The essential elements for bone formation are as follows:

1. Active cell population (Osteogenity)
2. Powerful signaling molecules to induce and maintain the healing cascade (Osteoinduction)
3. Appropriate scaffold (Osteoconduction)
4. Mechanical stability

2.1.1 Osteogenity

Osteogenesis needs bone forming cells. Osteogenity is the generation of bone-forming cells. The mesenchymal stem cells are the precursor cells of osteoblastic lineage. Multipotential precursor cells locating in the bone marrow are capable of differentiating into fat, cartilage and bone [15]. These cells constitute the main source of pro-osteogenic cells during the fracture repair process. Periosteum is the primary reservoir of MSCs [16]. Interleukin 1 (IL-1), interleukin 6 (IL-6) and Tumour Necrosis Factor Alpha (TNF-α) secreted by inflammatory cells recruit MSCs. Platelet-derived

Growth Factor (PDGF) and Transforming Growth Factor Beta (TGF-β) from activated platelets induce MSC migration, activation and proliferation [17]. In primary bone healing MSCs derive from the cortical bone, the adjacent periosteum and the bone marrow within the fracture. In this form of healing, the periosteal reaction is minimal. Vascular endothelial cells and perivascular mesenchymal cells supply the osteoprogenitor cells [17, 16]. However, as mentioned before secondary bone healing is subject to both of the processes of intramembranous and endochondral bone formation. In intramembranous bone formation, MSCs from adjacent periosteum differentiates to form bone and the cartilage while the intermediate step is skipped. Differentiation starts just after trauma, having a peak at first week to 10 days and decreases by day 14, although does not come to an end [18]. Endochondral ossification takes places simultaneously with intramembranous ossification in areas with relative stability. Recruitment of bone progenitor cells occurs from surrounding periosteum and this process is enhanced by the soft tissues around the fracture. Proliferation of MSCs begins on the third day after fracture. Differentiation and proliferation to chondrocytes takes place starting at the first week through third week. Then, process progresses to the soft callus stage. In this stage, chondrocytes secrete type II collagen and proteoglycans which provide "the construct" relative stability. Consequently, hypertrophy and mineralization followed by vascular invasion to cartilage leads to removal of chondrocytes and woven bone formation [18, 19]. New progenitors of osteoblasts mobilize from perivascular cells of the blood vessels. The cambium layer of the periosteum and MSCs from marrow stroma constitutes the primary supply for osteogenic cells for fracture healing. It is known that abundant amounts of osteogenic cells are present in the post-injury hematoma [16]. The human fracture hematoma, present after injury but not has potent angiogenic activity that appears to be predominantly due to VEGF [20]. Therefore, for a successful treatment of a bone fracture, every orthopaedic surgeon should do his best to protect the early hematoma formed after a fracture during operative interventions. Additionally, we must not forget the influence of extrinsic factors which are as important as local factors. For instance, impaired fracture healing in the elderly may be related to age, malnutrition, anemia, osteoporosis, use of steroidal or non-steroidal anti-inflammatory drugs.

2.1.2 Osteoinduction

Osteoinduction is the ability to modulate the differentiation of stem cells and progenitor cells along an osteoblastic pathway. It is the process that supports the mitogenesis of undifferentiated mesenchymal cells to progenitor cells that can form new bone. According to their function in the bone healing cascade, molecules that promote osteoinduction can be divided in to three subgroups.

1. Pro-inflammatory molecules that initiate the repair cascade: IL-1, IL-6 and TNF-α [19, 21].
2. Molecules that interfere with growth and differentiation such as the TGF-β superfamily and PDGF.
3. Molecules such as metalloproteinases and angiogenetic factors that promote angiogenesis.

The pro-inflammatory molecules (IL-1, IL-6, TNF-α derive from inflammatory cells like macrophages, and mesenchymal origin cells, like periosteum cells. They exert chemotactic effects, stimulate extracellular matrix formation, angiogenesis, recruit endogenous fibrogenic cells to the injury site and at later stages enhance bone resorption [22].

The TGF-β family includes the bone morphogenic proteins (BMPs), TGF-β, the growth differentiation factors (GDFs), activins, inhibins and the Mullerian inhibiting substance [23]. Out of the 14 different BMPs that have been studied, BMP-2, BMP-4 and BMP-6 are more potent in their ability to promote osteoblast differentiation of mesenchymal progenitor cells. They are produced from osteopreogenitor cells, mesenchymal cells, osteoblasts and chondrocytes within the extracellular matrix and act on osteoblasts and mesenchymal osteoprogenitor cells [24]. Although BMPs are interrelated functionally, they show a distinct temporal expression pattern during the fracture repair. Their main activity is on the differentiation of undifferentiated mesenchymal

cells into osteoblasts and chondroblasts and the differentiation of osteoprogenitor cells into osteoblasts [25].

Bone morphogenic proteins also stimulate synthesis and secretion of other growth factors like insulin-like growth factor (IGF) and vascular-endothelial growth factor (VEGF). They also can directly activate endothelial cells to stimulate angiogenesis [26].

Transforming Growth Factor Beta is primarily released by platelets in the very early stages of the fracture healing process. It is believed to be an initiator of callus formation [27, 28]. It is also secreted by endothelial cells, extracellular matrix, chondrocytes and osteoblasts and acts as a mitogenic and chemotactic agent for MSCs, osteoblasts and macrophages; however it is believed to be a weak osteoinductive factor [29].

Platelet derived growth factor is released by platelets during the early stages of fracture healing. It is a potent chemotactic stimulator for inflammatory cells. It also exerts a major proliferative and migratory stimulus for MSCs and osteoblasts. Although it is mitogenic for mesenchymal cells and osteoblasts, as well as chemotactic for inflammatory and mesenchymal cells, its therapeutic potential needs further investigations [29]. Fibroblast Growth Factor (FGF) is mostly related to angiogenesis. Insulin-like Growth Factor promotes bone matrix (IGF-I) and stimulates type I collagen formation (IGF-II) [30].

Angiogenesis temporally precedes osteogenesis as indicated by microscopical and angiographic analysis in bone chamber models [31]. The potential synergism between potent angiogenic factors, such as VEGF and osteoinductive factors such as BMPs suggest that combination therapies might produce better results, non-unions. Within the context of this study, VEGF as an angiogenic and potentially osteogenic factor will be discussed in detail.

Biologic methods of bone regeneration have and will continue to have increasing role in the treatment of fractures. Knowledge of the pathologic changes in the fracture repair process that lead to delayed union or nonunion is important [32].

2.1.3 Osteoconduction

Osteoconduction is the ability to provide the scaffold on which new bone can be formed. It refers to the matrix substance that supports the attachment of bone forming cells for subsequent bone formation. In bone grafting and skeletal reconstruction, wide use of three-dimensional porous scaffolds is employed. The main function of these implants is to provide a surface and structure that facilitates the attachment, migration, proliferation, differentiation, and survival of osteogenic stem and progenitor cells throughout the implant site [2].

The presence of an adequate scaffold for the healing cascade to occur is a prerequisite for bone formation. Microstructural features can influence fluid flow and diffusion of oxygen and other nutrients through the scaffold. A macropore size between 150 and 1,000 μm is optimal, where bone ingrowth is needed to depths of 3 to 5 mm. If primary bone healing is concerned, the event takes place between the opposed fracture fragments, i.e. the cortical bone. However, in secondary bone healing callus which was formed by the extracellular matrix is considered. It then defines the orientation and facilitation of blood vessels and the creation of Haversian systems into the bone scaffold [33].

2.1.4 Mechanical Stability

Mechanical stability is another important component. More bone is produced under electronegative potentials and resorbed under electropositive potentials [34], which can explain bone healing response to applied axial load.

Goodship and Kenwright [35] experimented on two groups of sheep with tibial diaphysial fractures stabilized by external fixators. In one group rigid fixation was maintained while in the other group controlled axial micromovement, was applied. The authors noticed a significant improvement in healing with the application of controlled micromovement. Recent studies on mechanical stability argue that the prerequisites are the fracture's being at early stages and having adequate vascularity. According to Jagodzinski and Krettek, perfusion is the main stimulus for cell proliferation and micromotion at this stage enhances cell differentiation. Micromotion should be avoided in later stages of fracture healing especially when the soft callus is calcifying to produce hard callus [36].

2.2 Irradiation and Bone Formation

Mature bone and cartilage is relatively radioresistant. Growing cartilage and bone in a child is very sensitive to irradiation when compared with an adult. When irradiation is required for a malignant tumor control, very low fractions of doses (1.8 Gy and less) are advised for children [37]. When irradiation is applied for childhood tumors, cranial and total body irradiations can cause hormonal deficiencies, which may affect both the stature and the bone density [38].

Bone remodeling and wound healing is known to be altered by irradiation, which may lead to osteoradionecrosis. It has been attributed to radiation fibrosis of small blood vessels. After irradiation therapy for carcinoma of the head and neck, it occurs in 3% to 10% of patients [39]. Stress fractures after radiation therapy are observed even in patients without a history of bone reconstruction [40].

2.2.1 Irradiation for Heterotopic Ossification Prophylaxis

Heterotopic ossification is a disorder of spatial regulation of the bone formation which is characterized by ectopic normal bone at soft tissue. It is believed to occur when

pluripotent mesenchymal cells inappropriately differentiate into osteoblastic stem cells. Main clinical causes of heterotopic ossification are trauma, burns, infections, neoplasia, seronegative spondyloarthropaties, neurological diseases, post surgical trauma, chronic venous insufficiency and heritable disease [41].

Many pharmacological and physical modalities have been proposed in the treatment and prevention of heterotopic ossification as diphosphonates [42], nonsteroidal anti-inflammatory drugs [43], physiotherapy, surgical resection [44] and irradiation. Among these listed, the main treatment options appear to be use of indomethacin and irradiation. Single 7 Gy dose has been applied with success.

The risk is significantly high after open reduction and internal fixation of acetabular fractures affecting half of the cases who did not have any prophylaxis. In a recent review to compare indomethacin with irradiation after acetabular fractures, the authors found five appropriate prospective studies, describing 384 patients. They concluded that until further information is available, the evidence supports radiation therapy as the preferred method for preventing heterotopic ossification [45].

Irradiation affects by altering DNA transcription and arresting the initial step in osteoid formation, preventing differentiation of pluripotent mesenchymal cells into osteoblasts [46]. Early studies advocated the use of relatively higher doses like 2000 cGy [47] while consequent studies showed high success rate with lower doses like 600 cGy [48]. The rate of trochanteric non-union was reported as 25% after 10 Gy radiation therapy to prevent heterotopic ossification [10]. Therefore, the risk of the radiation induced malignant disease and the risk of side effects of irradiation has pressed the physicians to find the minimal dose with efficiency.

2.2.2 Irradiation for Other Benign Musculoskeletal Pathologies

Therapeutic use of irradiation on benign disease for musculoskeletal system pathologies is not limited to heterotopic ossification. Radiotherapy of non-malignant

diseases is not a worldwide practice. However, it has long been used in Germany although a low acceptance rate and practice have been observed in Anglo-American countries. Of the many benign diseases listed, some of them are musculoskeletal problems. Arthritis, bursitis, synovitis, insertion tendinitis have been reported to be treated with single doses of 0.2 - 1 Gy and a total dose of 0.6 to 12 given in fractions. The highest irradiation amount recommended have been for "Dupuytren's disease" by a single dose of 2.0 - 4.0 Gy and a total dose of 20 - 40 Gy [49]. Adamietz et al. [50] reported good results in the treatment of shoulder impingement with radiotherapy applied as a total dose of 6Gy by fractions The author very recently published their criteria to obtain favorable results for the treatment of calcifying tendinitis [51].

Gonarthrosis (osteoarthrosis of the knee joints), a very common problem have also been reported to be treated with success by radiotherapy [52]. Another common problem that is treated with irradiation in Germany is plantar fasciitis (heel spurs). By the help of a standardized structured questionnaire, it was found out of the 146 institutions, (79.3%) that returned the questionnaire, 136 (93.2%) have been employing radiotherapy for refractory heel spur patients, the total dose ranging between 2.5 and 18.75 Gy (median 6), and single fractions between 0.3 and 1.5 Gy (median 1) [53].

3. ENHANCEMENT OF BONE HEALING

A new era in the treatment of clinical problems on bone regeneration commenced with the better understanding of bone fracture healing by histological and molecular means. Promotion of bone healing may be required in many situations such as fractures that delayed healing is expected (osteoporotic fractures, open and high energy fractures, segmental fractures or fractures with bone defects) or fractures that non-union have already developed.

Enhancement of bone healing can be promoted through several means. These are categorized into two main groups. In the first group enhancement is attempted through physical methods whilst in the second group enhancement is achieved through biological means.

3.1 Physical Modalities

The first report on the use of a physical modality for a fracture non-union is quite historical. Hartshorne reported the treatment of a non-union with the application of electric "shocks" in 1841 [54]. More than 100 years later, electrical stimulation of bone regained attention clinically when "piezoelectric potentials" generated by mechanical stress on the crystalline structure of bone was described [55].

Physical forces can be applied as direct current, capacitive coupling, pulsed electromagnetic field and combined magnetic field. Ultrasound is another physical means that has been shown to enhance bone formation [56].

Increased intracellular calcium has been accepted as one of the main mechanisms for physical forces. The common effect of the physical forces appears to be an increase in intracellular calcium by a variety of cellular mechanisms and pathways resulting in

increased intracellular calcium thereby leading to increase in osteoblastic function in cells capable of bone formation [8].

We should not forget that mentioned modalities are an adjunct to standard fracture care, not a replacement for them and there are contraindications, especially for electrical stimulation. These are segmental bone loss, congenital, infected and synovial non-unions, and lack of mechanical stability of the fracture site which should be obtained by revision surgery.

Meta-analysis of randomized controlled trials are the mainstay of evidence based medicine. In a recent systematic review and meta-analysis of randomized controlled trials to evaluate the effect of electromagnetic stimulation on long-bone fracture-healing, the authors could find only 11 studies out of 2546 citations in their search of the literature between 1980 to April 2008. They concluded that the impact of electromagnetic stimulation on fracture-healing is uncertain because of methodological limitations and high between-study heterogeneity [57]. The following year, in 2009, another systematic review of randomized controlled trials was published, investigating the efficacy of low intensity pulsed ultrasonography for healing of fractures. The authors could find 13 randomized trials, of which five assessed outcomes of importance to patients. The authors concluded that the evidence for the effect of low intensity pulsed ultrasonography on healing of fractures is moderate to very low in quality and provides conflicting results [58]. In summary, although physical modalities have long been employed in the treatment of non-unions, still both experimental and clinical level I studies are needed to clarify their efficiency.

3.1.1 Direct Current

Direct current generates a high pH and a low pO2 which was thought to be favorable to bone formation as low pO2 was found at the bone-cartilage junction of the growth plate and in newly formed bone and cartilage in fracture callus [59]. Direct current also promotes proteoglycan and collagen synthesis inducing osteogenesis [60].

A more recent study investigating on mouse calvarial organ cultures to explain the osteogenic effects of direct electrical stimulation has shown that a faradic reaction at the cathode lower oxygen concentration, increase pH, and produce hydrogen peroxide [61], where these conditions have been shown to increase osteoblastic activity [62, 63].

Direct current techniques involve an implanted cathode placed in the area of expected bone stimulation and a battery-based anode placed subcutaneously delivering a constant 20 μA (5 to 100 μA) direct current [8]. In the original technique, the anode used to be placed on the skin, with a battery pack worn at the waist which was quite cumbersome. With the development of implantable batteries patient compliance has started not to be a problem as they can be applied as a concomitant procedure while a surgery for internal fixation or bone grafting is applied. The Food and Drug Administration approved the application of direct current treatment for established non-unions in 1979 [64].

3.1.2 Capacitive Coupling

Capacitive coupling is a noninvasive technique to stimulate bone formation. It involves placing two electrodes on the skin which are positioned so that the fracture fragments are between them. An alternating current is used to create an electrical field within the fracture site. Potentials of 1 - 10 V at frequencies of 20 - 200 kHz are applied to the electrodes, developing electric fields of 1 - 100 mV/cm at the fracture site [65].

The acting mechanism of capacitive coupling to cause proliferation and osteogenesis has some explanations [66]. Bone cell proliferation resulting from capacitive coupling is accompanied by an increase in intracellular calcium concentration. The drug verapamil which is a voltage-gated calcium channel blocker was found to halt the bone cell proliferation [67], which may lead to a conclusion that the proliferative response of bone cells to capacitive coupling is mediated by calcium translocation via voltage-gated calcium channels. An alternative mechanism by which capacitive cou-

pling influences osteogenesis is the up-regulation of the mRNA expression for BMPs-2, -3, -4, -5, -6, -7, and -8. These growth factors are important for the proliferation and differentiation of osteoblastic cells [68].

3.1.3 Pulsed Electromagnetic Field

This technique is also referred as "inductive coupling". In this application, Pulsed Electromagnetic Field (PEMF) device is placed on the skin over the fracture site. The PEMF is generated by the help of a wire coil through which a current is passed. Therefore, the magnetic field induces an electrical field across the fracture fragments. The PEMF signal was developed to induce electrical fields in bone similar in magnitude and time course to the endogenous electrical fields produced in response to strain. These fields are thought to underlie the ability of bone to respond to a changing mechanical environment, as described by Wolff's law. The signal consists of 4.5 msec long bursts of twenty 220 μ sec 18 G pulses repeated at 15 Hz. This results in a time-varying extracellular and intracellular electrical field. Use magnetic coils that receive a specific pulsed electrical current that results in a magnetic flux density 0.1 to 18 G (gauss) in the form of a pulse train with a 15 Hz or sinusoidal 76 Hz frequency. A pulse train is a rapid sequence, typically of twenty 220 μ sec repeating spikes. A gauss (G) is a unit of electromagnetic flux; to give a comparison the earth's geomagnetic field is approximately 0.6 G. [8].

Pulsed electromagnetic fields modulate the cellular activity of osteochondral progenitor cells, chondrocytes and osteoblasts by affecting the synthesis of TGF-β and BMPs [8]. Capacitive coupling stimulates bone cell proliferation whereas combined electromagnetic fields stimulate signal transduction pathways and growth factor production [69].

3.1.4 Low-Intensity Pulsed Ultrasound

Ultrasound induced osteogenesis is not a novel idea in orthopaedic trauma surgery dating back to1950s. Ultrasound has thermal as well as non-thermal effects such as acoustic streaming and cavitation on tissues and cells [70]. There is evidence to suggest that ultrasound may influence the inflammatory, the soft callus formation [71] and the reparative phases of fracture healing [72]. Very recently a study on the mechanism of Low-intensity Pulsed Ultrasound (LIPU) demonstrated that LIPU does not increase osteogenic cell presence. The authors suggested the mechanism is "likely" by affecting osteogenic cell differentiation [73].

In a very recent meta-analysis, the authors concluded that the evidence for the positive effect of LIPU on healing of fractures is moderate to very low in quality with conflicting results. Although overall results are promising, the authors concluded that establishing the role of LIPU in the management of fractures requires large, blinded trials, directly addressing patient important outcomes such as return to function [58]. In this study the device used in 12 of the 13 eligible studies was the Sonic Accelerated Fracture Healing System (SAFHS) (Exogen, Piscataway, NJ) (Fig. 3.1). The trials that used this device required their treatment groups to receive daily 20 minute sessions with an ultrasound signal composed of a burst width of 200 μs (SD 10%) containing 1.5MHz (SD 5%) sine waves, with a repetition rate of 1 kHz (SD 10%) and a spatial average temporal intensity of 30 mW/cm^2 (SD 30%).

In 1994, the Food and Drug Administration approved the use of LIPU for accelerating conservatively managed fresh fracture healing, and in the year 2000 approved its use for the treatment non-unions [72].

3.2 Biological Modalities

Numerous types of grafts and promoting agents have been used so far and those currently in use include autograft, allograft, demineralized bone matrix, hydroxyapatite

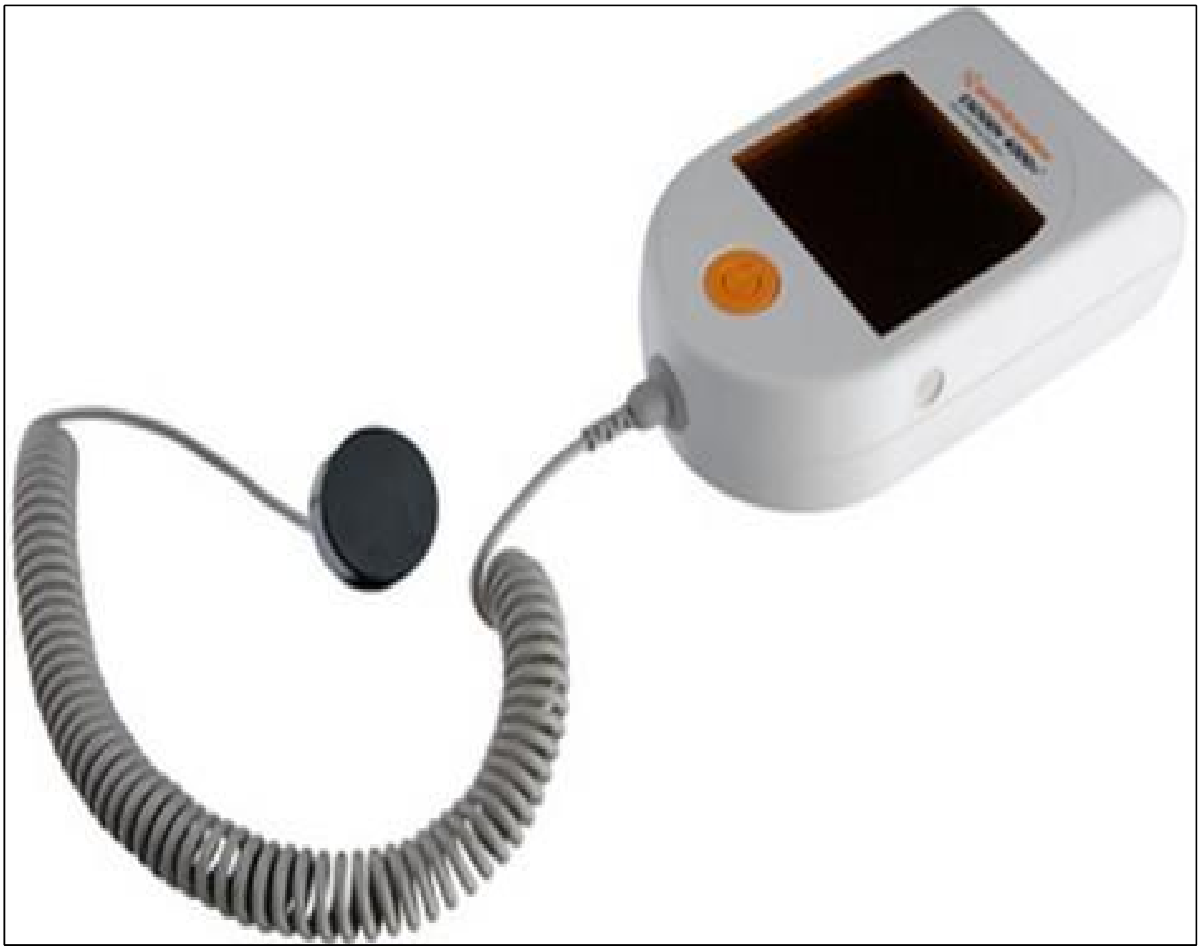

Figure 3.1 An example of a commercially available ultrasound device: The Sonic Accelerated Fracture Healing System (www.exogen.com).

calcium phosphate, autogenous bone marrow aspirates (bone marrow injections), BMPs and many other related growth factors (VEGF, PDGF, etc.). Autologous bone grafting was introduced as a valuable surgical technique in the clinical setting more than 100 years ago, the autologous iliac crest graft being reported to be superior to other sites like distal radius.

Autologous bone graft can be used to fill bone defects due to tumor or infection in weightbearing and non-weightbearing bones, to assist arthrodesis of joints and osteotomies, to work as a structural support to implanted devices, and finally and notably to enhance bone healing in fracture non-unions [74].

A number of issues have been identified as potential problems about the usefulness of autologous bone grafts, which include the disadvantage of a second intervention requiring a second skin incision, which may lead to numerous local complications as formation of a hematoma, iatrogenic nerve injury, fracture, infection, and chronic pain. Prolonged operation time, lengthening of hospital stay, and an increase of the costs as well as its limited availability are other important problems [75]. Because of these potential problems, development of artificial bone substitutes preferably acting as active biological substances are being researched, as means of alternatives.

Bone morphogenetic proteins are a group of signaling molecules, acting as active biological substances for bone fracture healing [76]. They exert their effects by binding to specific membrane receptors on different cell types, mainly MSCs, osteoblasts, and osteoclasts [77]. In humans, two BMP molecules have been particularly well described, BMP-2 and BMP -7, that is also named as OP-1 [78]. Early experimental studies on BMPs date back to the first years of our decade. In a study by Salkeld et al. [79] an improvement of bone healing in segmental defects created in dogs was achieved by adding BMP-7 to allograft, and even better results were obtained compared with autogenous grafts. The promising results obtained with BMP-7 were also seen using BMP-2, the most remarkable one being repair of fractures of allograft in rats, showing BMPs capacity to initiate a biological response in the allograft tissue [80].

Despite all the advance in the isolation, sequencing, and manufacturing using recombinant DNA techniques of BMPs, we should be aware of the fact that the autologous bone grafts possesses all three important properties for bone regeneration: osteogenicity, osteoinductivity and osteoconductivity, whilst, BMPs pose only one property, osteoinductivity [74].

4. MATERIALS AND METHODS

4.1 Experimental Procedure: Animals

Forty young male Sprague Dawley rats (body weight 280±30 g) were obtained from the Institutional Experimental Research and Animal Unit. The rats were subjected to 14:10 hours light:dark cycle and were maintained at standardized temperature (22±2°C) and humidity conditions (%55±5). All animal experiments were conducted adhering to the guidelines from Institutional Animal Ethics and Radiation Safety Committees. The animals had free access to the sterile water and food, allowed normal weight bearing as tolerated and they were housed in a polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment. In the follow up time all rats were subjected to veterinary care. The animals were randomized into irradiation (RT) and sham control groups (C) and further subdivided to third week sacrification (RT3, C3) and sixth week sacrification (RT6, C6) groups. The rats were sacrificed at a time interval of postoperative third and sixth weeks after imaging studies for histopathological studies (Fig. 4.1).

All experimental procedures were performed on anesthetized rats; during irradiation and scintigraphy, anesthesia was maintained with ketamine (Ketalar, Pfizer İlaçları Limited Şirketi, Istanbul, Turkey) with a dose of 100 mg/kg body weight and xylazine (Rompun, Bayer Türk Kimya Sanayi Limited Şirketi, Istanbul, Turkey) with a dose of 3.9 mg/kg body weight ip.

4.2 Experimental Procedure: Osteotomy Technique

Left mid shaft transevers fractures were created by open technique using a standard protocol [81]. After shaving and sterilization of the rats under anesthesia, a lateral longitudinal incision was made to split vastus lateralis muscle (Fig. 4.2).

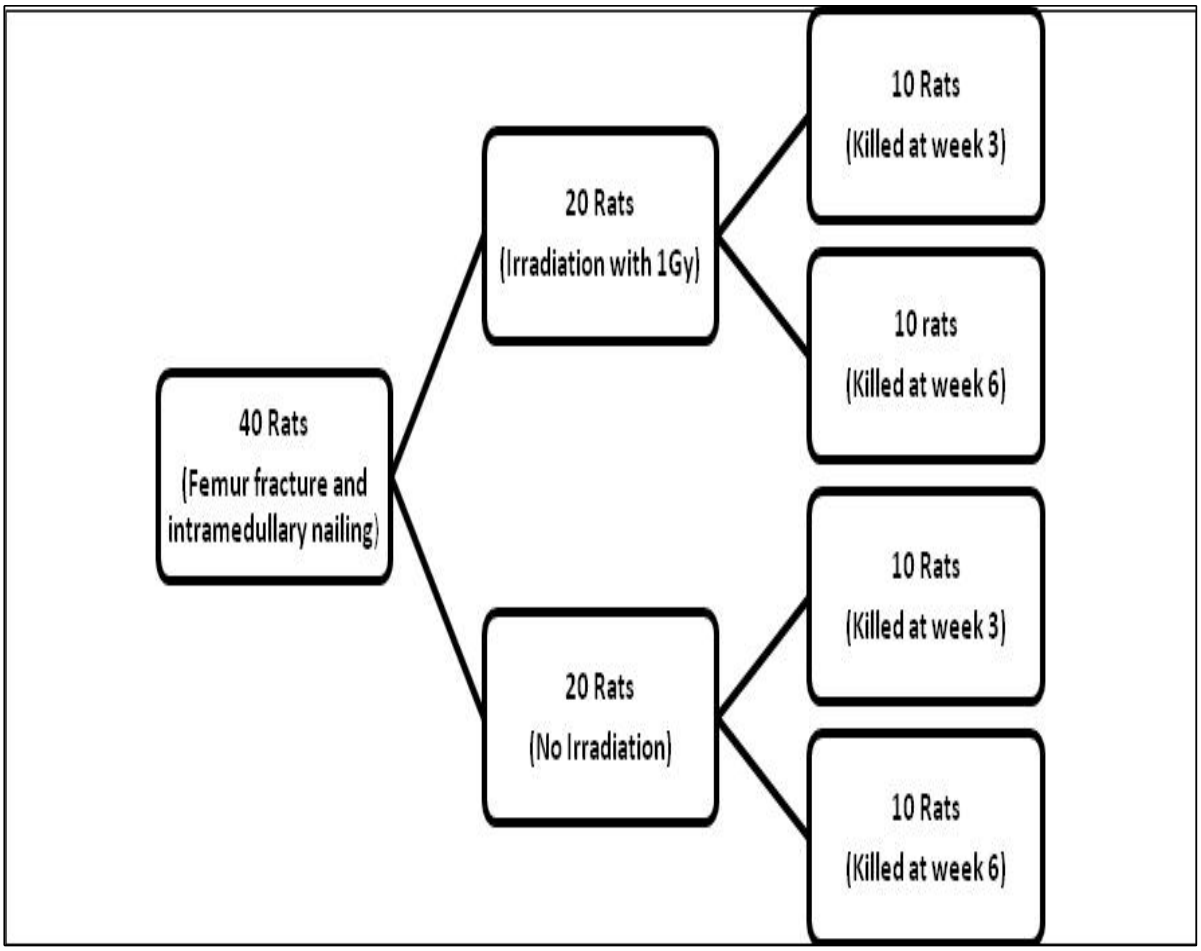

Figure 4.1 The scheme for the planning of the experiment.

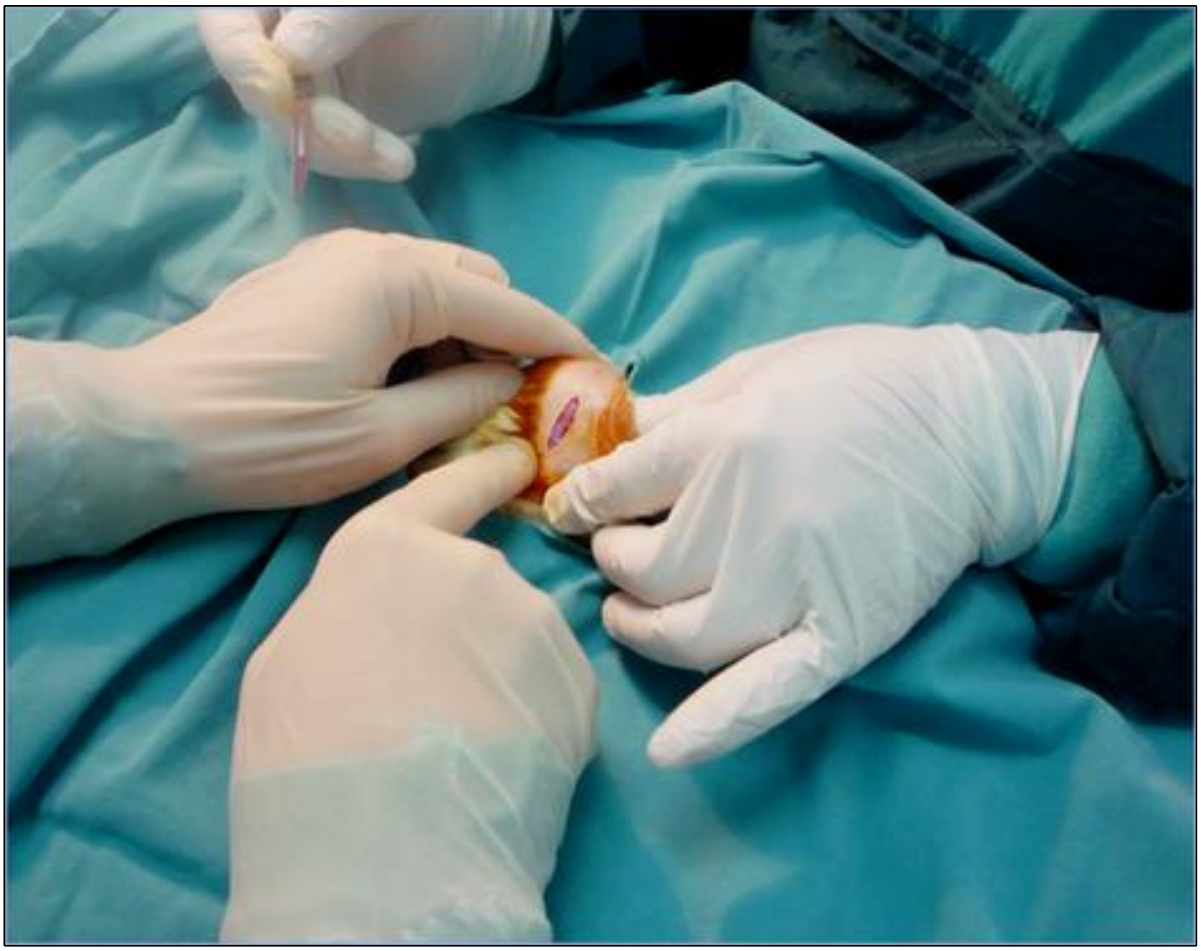

Figure 4.2 Approach: Longitudinal incision to expose the femur.

A transvers osteotomy was made with surgical instruments to exposed femur and internally fixed with 1.2 mm Kirschner wire using retrograde nailing technique (Fig. 4.3).

4.3 Irradiation

The rats in the Group 1 and 3 were irradiated individually with a single dose of 1 Gy defined at a depth of 1.25 cm through an anterior 4 by 4 cm single portal (with a 0.5 cm bolus) covering the right femur in its entirety with a Cobalt 60 treatment unit (Cirus, cis-Bio Int., Gif Sur Yvette, France) at a source skin distance of 80 cm. The dose rate was approximately 1.07 Gy/min. The rats were anesthetized and then

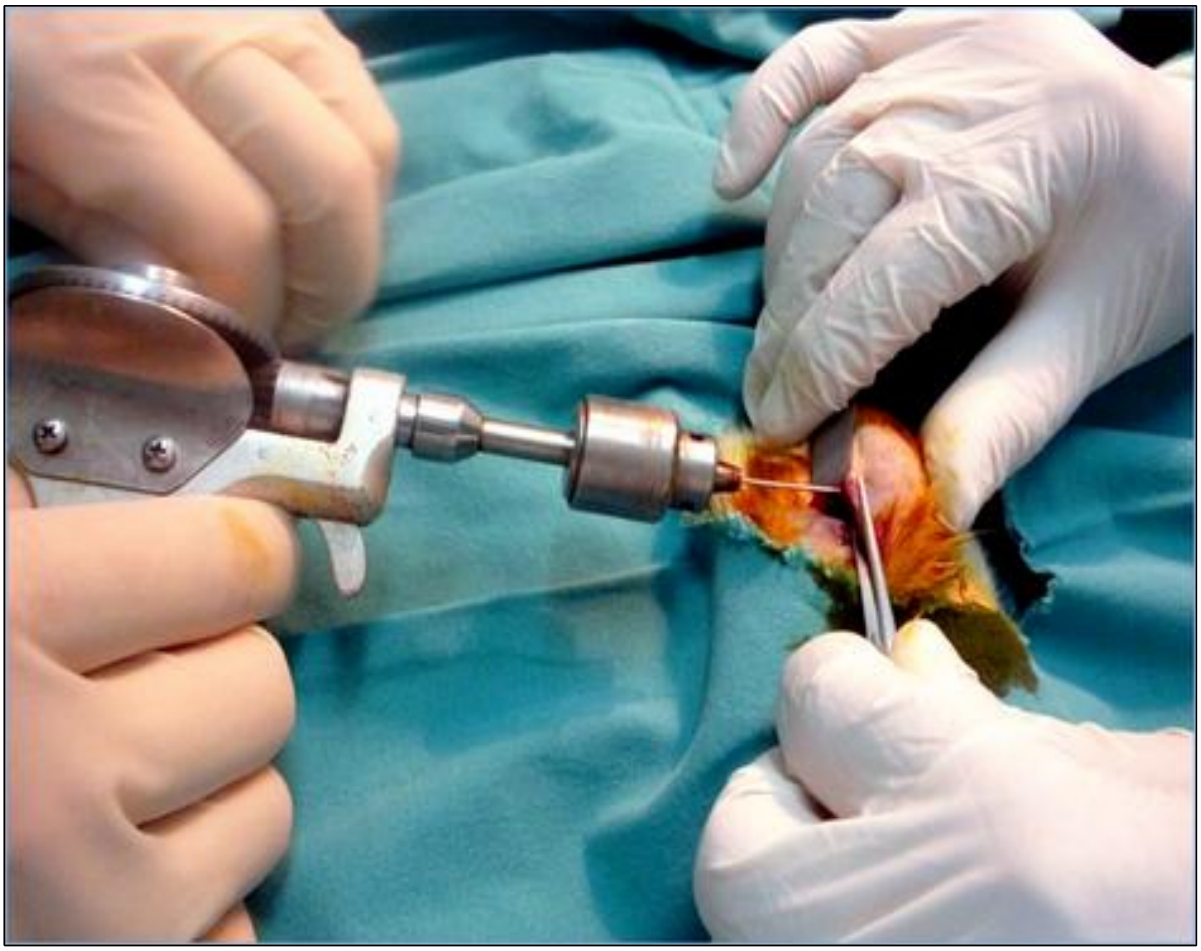

Figure 4.3 Intramedullary nailing of the femur for internal fixation of the fracture.

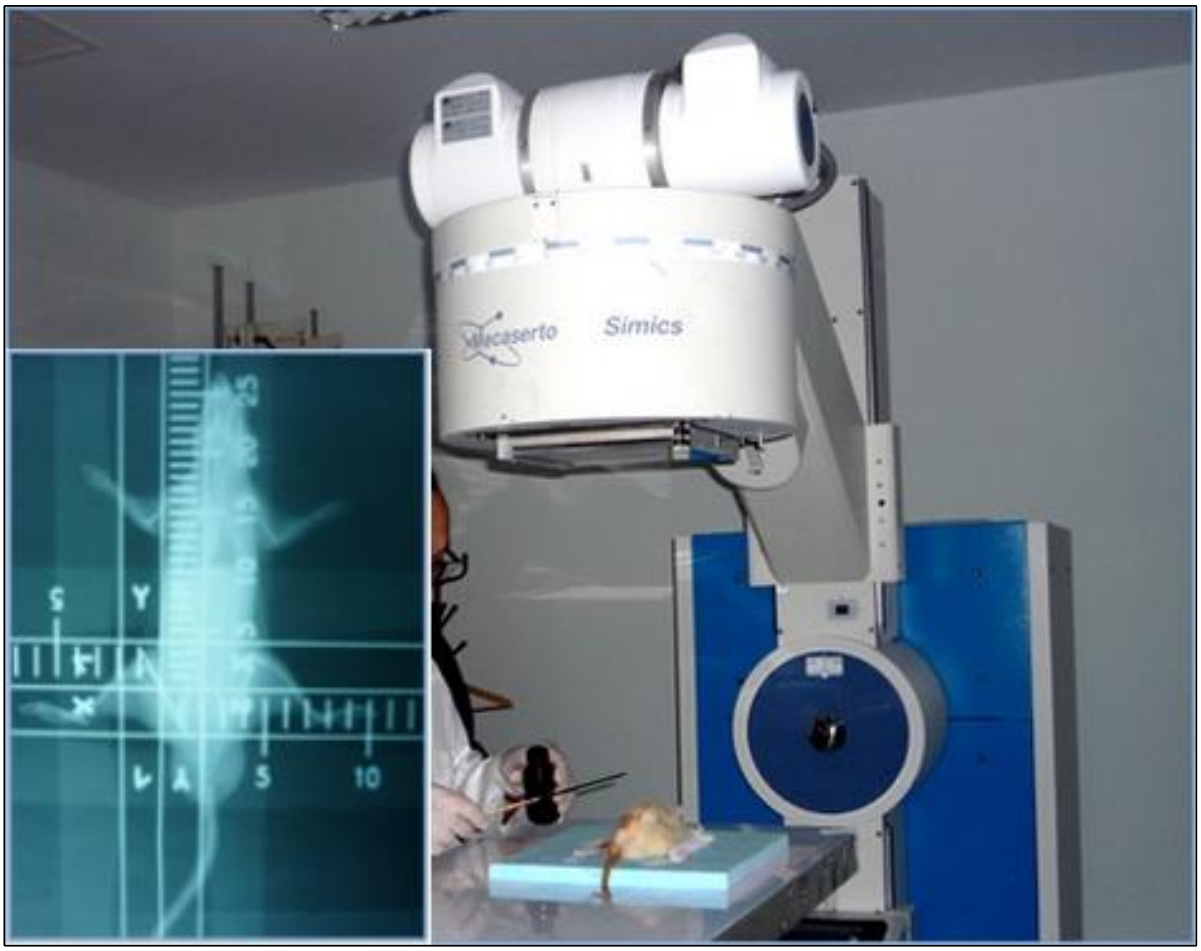

Figure 4.4 During irradiation the correct positioning of the fields was controlled for each individual rat using a therapy simulator. The monitor view can be seen at the insert.

fixed on a 20 by 30 cm blue Styrofoam treatment couch (Med-Tec, Orange City, IA) in a prone position. Correct positioning of the fields was controlled for each individual rat using a therapy simulator (Mecaserto-Simics, Paris, France) (Fig. 4.4). Special dosimetry was done for the irregular fields. The dose homogeneity across the field was 5%. After irradiation, the animals were closely observed until recovery from anesthesia. The control groups received equal field sham irradiation.

4.4 Nuclear Medicine

Bone mineral analysis by by Dual-Energy X-ray Absorptiometry (DEXA) and bone scintigraphy were performed.

4.4.1 Bone Mineral Analysis

The animals were scanned by DEXA, (Hologic QDR 4500, Hologic, Waltham, MA) equipped with a rat high-resolution software. The scan field size was 2 by 4 cm, resolution was 0.002 by 0.001 cm and scan speed was 1 mm/sec. Data output for Bone Mineral Content (g), two-dimensional projected area (cm^2), and Bone Mineral Density (g/cm^2) (BMD) have been recorded. Fracture region and non-fractured neighboring bone were found BMD Index was calculated using the equation

$$BMD..Index = \frac{R1}{R2} \tag{4.1}$$

where R1 is bone mineral density at the fracture region and R2, bone mineral density at the fractured neighboring bone.

4.4.2 Bone Scintigraphy

Intravenous access was accomplished by using a 24F catheter via tail-vein of the rats. Bone scintigraphy was obtained 3 hours after intravenous administration of 3 - 5 mCi of 99m Tc-labelled methylene diphosphonate (99mTc-MDP). The gamma camera (Siemens E Cam, Siemens Medical systems, Hoffman Estates, IL, USA) equipped with high resolution collimator was used. In order to quantitate the bone scan, regions of interest on each limb and background was selected. The count of radionuclide uptake was obtained in mid-shaft of the femur, both for fractured and non fractured parts and

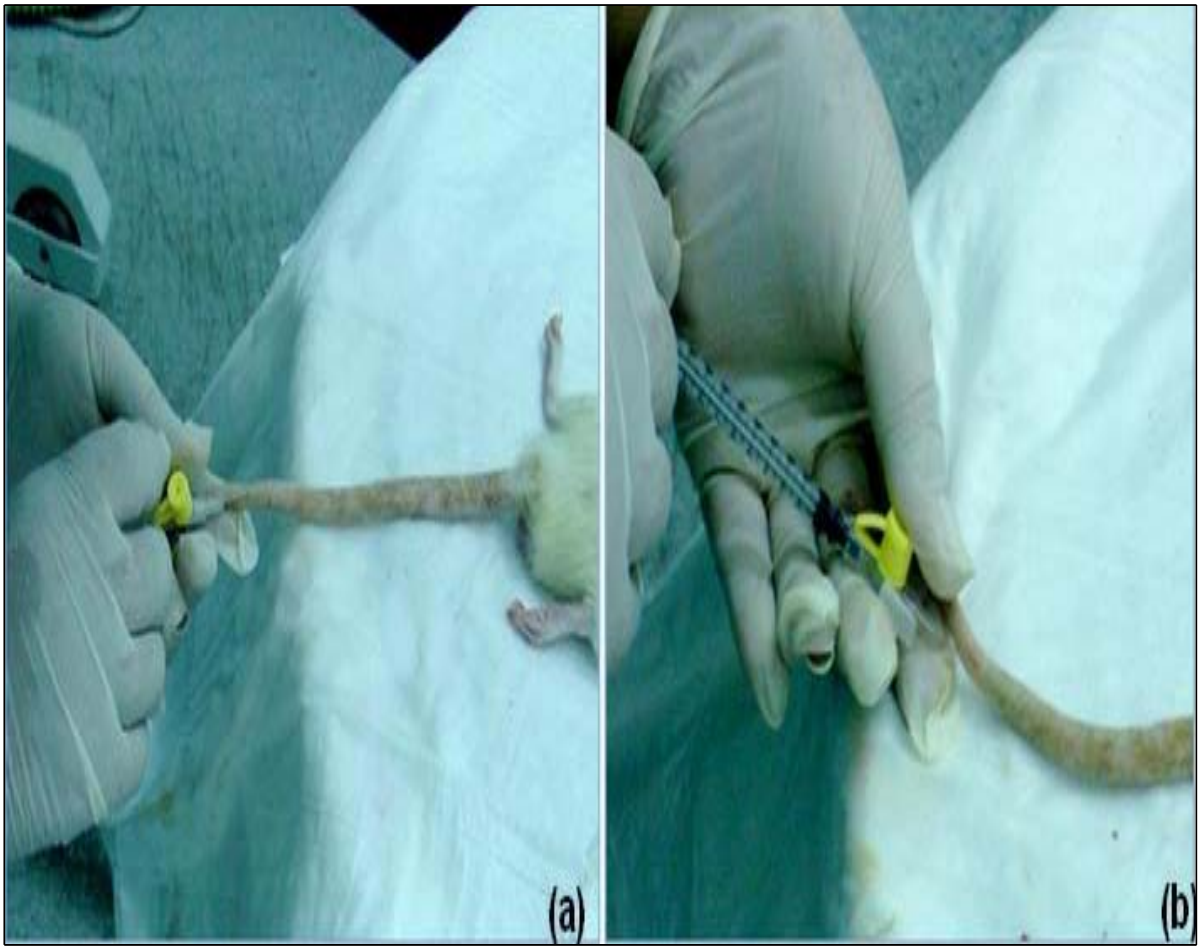

Figure 4.5 Intravenous access was accomplished by using a 24F catheter via tail-vein of the rats (a), and 99m Tc-labelled methylene diphosphonate was injected by this route (b).

soft tissue area. The average pixel counts of regions were obtained and MDP Uptake Ratio were calculated using the equation

$$MDP.Uptake.Ratio = \frac{R1 - BG}{R2 - BG} \tag{4.2}$$

where R1 is scintigraphic activity at the fracture region, R2, scintigraphic activity at the fractured neighboring bone and BG, basal ground scintigraphic activity.

4.5 Radiodiagnostics

Mineralized tissue formation was assessed by 2 D Full Field Digital Mammography. Radiography was performed in our mammography unit (Selenia Dimensions; Hologic) for monitoring the process of fracture healing at the end of the third and sixth week of the osteotomies. Before all radiologic examinations, animals were anesthetized. The femurs were positioned for both anteroposterior and lateral radiographs. The X-ray output voltage was set at 39 kV, 7 mA. Image analysis was performed using SecurView diagnostic workstation.

A blinded radiologist, unaware of the femur fracture group, scored the amount of visible bone formation within the fracture site using a previously described method [82]. A graded scoring system (0=no evidence of healing; 1=callus formation evident but fracture gap not yet bridged; 2=callus formation evident with possible bridging of the fracture gap; and 3=fracture union) was used by observer to grade each femur.

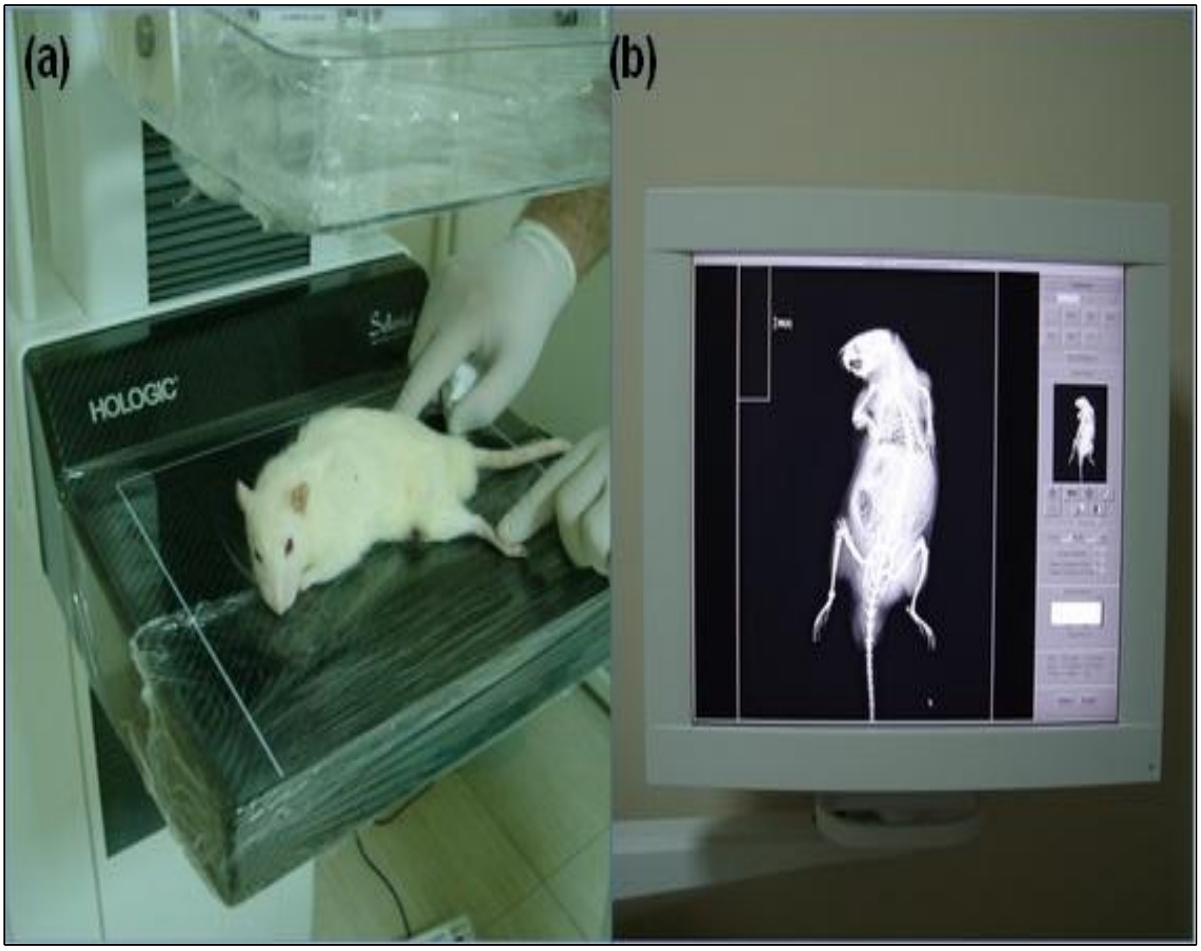

Figure 4.6 Before all radiologic examinations animals were anesthetized (a), image analysis was performed using SecurView diagnostic workstation (b).

Table 4.1
The Numerical Scoring Scheme of Bone Union for Histologic Evaluation.

Score	Finding at the Fracture Site
1	Fibrous tissue
2	Mainly fibrous tissue
3	Equal amounts of fibrous tissue and cartilage
4	Mainly cartilage, little fibrous tissue
5	Cartilage
6	Mainly cartilage, little immature bone tissue
7	Equal amounts of cartilage and immature bone tissue
8	Mainly immature bone tissue, little cartilage
9	Fracture healing with immature bone tissue
10	Fracture healing with mature bone tissue

4.6 Histopathology

The explanted bone specimens were fixed in 10% formol solution before decalcification in a 10% formic acid bath for one week. After decalcification, transverse sections were made to fracture region and neighboring bone and soft tissue structures. Histological sections were studied by staining with Haematoxylin-Eosine and scored according to Huo et al [83].

4.7 Statistical Analysis

The Kolmogorov-Smirnov test was used to assess the normality of numeric variables. For the numeric variables that were normally distributed, comparison between two groups was made by the independent sample t test and results were expressed as mean $\pm$ standard deviation. For the numeric variables that were non-normally distributed and the score variables, comparison between two groups was made by the Mann-Whitney U test and results were expressed as median and interquartile range. Significance was defined as $p<0.05$.

5. RESULTS

One rat from Group RT3 and one from group C6 died during the intravenous administration of the drug during scintigraphy. However, radiography, bone mineral analysis and histopathological examinations could be performed in these rats but scintigraphic examination was completed with 9 rats in these two groups.

5.1 Nuclear Medicine

The results for bone mineral analysis and bone scintgraphy are as follows.

5.1.1 Bone Mineral Analysis

There was not any statistically significant difference between irradiated rats and controls at week 3 (Table 5.1). Bone Mineral Density Index was higher in non-irradiated rats, 1.20 (SD 0.22) vs. 1.15 (SD 0.30), though not significant (p=679).

There was not any statistically significant difference between irradiated rats and controls at week 6 for Global BMD and R1 BMD values (Table 5.2). However for BMD Index and BMD values at R2 region, the differences were statistically significant.

Table 5.1
Bone Mineral Density Values of the Irradiated Rats and Control Rats, Third Week After Fracture.

	Group RT3			**Group C3**			p
	Mean±SD	**Min**	**Max**	**Mean±SD**	**Min**	**Max**	
HR DEXA BMD GLB	0.21 ± 0.01	0.198	0.231	0.22 ± 0.01	0.204	0.236	0.168
HR DEXA BMD R1	0.24 ± 0.04	0.175	0.314	0.25 ± 0.04	0.196	0.304	0.622
BMD Index (R1/R2)	1.15 ± 0.30	0.72	1.80	1.20 ± 0.22	0.68	1.51	0.679
	Median (25-75%)	Min	Max	Median (25-75%)	Min	Max	
HR DEXA BMD R2	0.22 (0.19-0.24)	0.174	0.249	0.21 (0.20-0.22)	0.179	0.289	0.579

Table 5.2
Bone Mineral Density Values of the Irradiated Rats and Control Rats, Sixth Week After Fracture.

	Group RT6			**Group C6**			
	Mean±SD	**Min**	**Max**	**Mean±SD**	**Min**	**Max**	p
HR DEXA BMD GLB	0.22 ± 0.02	0.175	0.247	0.23 ± 0.01	0.217	0.245	0.284
HR DEXA BMD R1	0.27 ± 0.05	0.196	0.340	0.25 ± 0.03	0.175	0.291	0.522
BMD Index (R1/R2)	1.35 ± 0.18	1.10	1.60	1.06 ± 0.23	0.66	1.38	0.006
	Median (25-75%)	Min	Max	Median (25-75%)	Min	Max	
HR DEXA BMD R2	0.20 (0.19 - 0.21)	0.131	0.232	0.24 (0.21 - 0.28)	0.202	0.324	0.005

Table 5.3
Bone Scintigraphy Values of the Irradiated Rats (RT3), Third Week After Fracture.

	N	Min	Max	Median	25 - 75 Percentile
R1	9	388.8	1242.3	901.7	776.5 - 1065.6
R2	9	85.0	260.0	154.5	107.8 - 242.0
Soft Tissue (ST)	9	31.3	169.4	60.300	44.350 - 82.950
R1-ST	9	347.3	1073.9	854.5	721.5 - 1016.4
R2-ST	9	45.9	187.6	78.4	52.850 - 141.0

Bone Mineral Density Index was higher in irradiated rats compared to controls (1.35 vs. 1.06), and the difference was statistically significant (p=0.006) (Fig. 5.1). Mean BMD at R2 region, which is neighboring non-fractured bone, was lower in irradiated rats compared to controls (0.20 g/cm^2 vs. 0.24 g/cm^2), and the difference was also statistically significant (p=0.005) (Fig. 5.2).

5.1.2 Bone Scintigraphy

In order to find out the MDP uptake ratio (scintigraphic index) values for groups, the average pixel counts of regions were obtained (Fig. 5.3). Activity rates for R1 (fracture region), R2 (non-fractured neighboring bone), BG (basal ground of soft tissue) were found (Table 5.3) through Table 5.6). Then using the equation 4.2, MDP Uptake Ratios were calculated (Table 5.7).

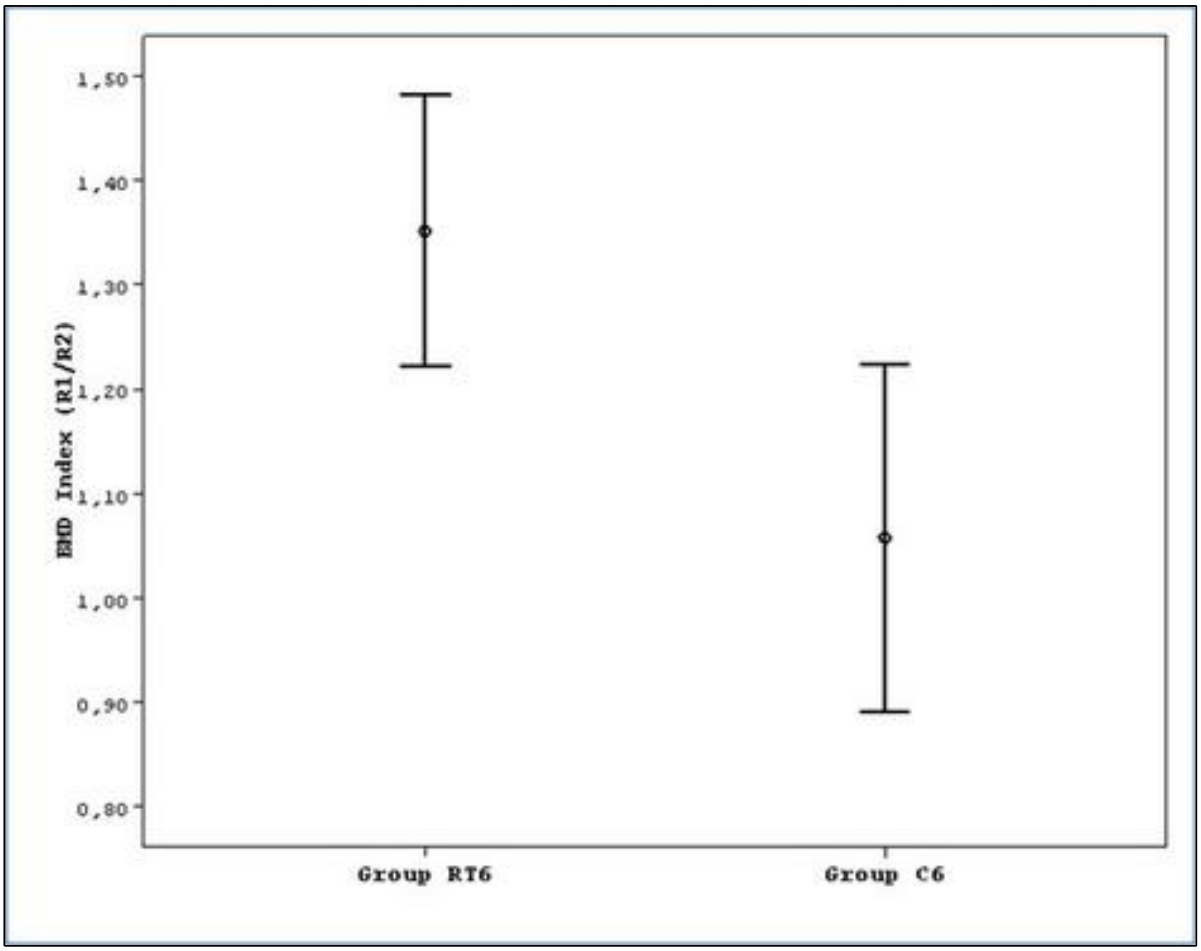

Figure 5.1 The distribution of bone mineral density index values for groups RT6 and C6.

Table 5.4
Bone scintigraphy Values of the Control Rats (C3), Third Week After Fracture.

	N	Min	Max	Median	25 - 75 Percentile
R1	10	85.7	338.9	297.1	164.0 - 337.0
R2	10	14.4	106.0	41.6	29.7 - 56.0
Soft Tissue (ST)	10	5.3	37.7	19.8	6.9 - 26.4
R1 - ST	10	80.4	317.6	278.2	148.7 - 302.9
R2 -ST	10	8.6	68.3	21.8	14.8 - 37.1

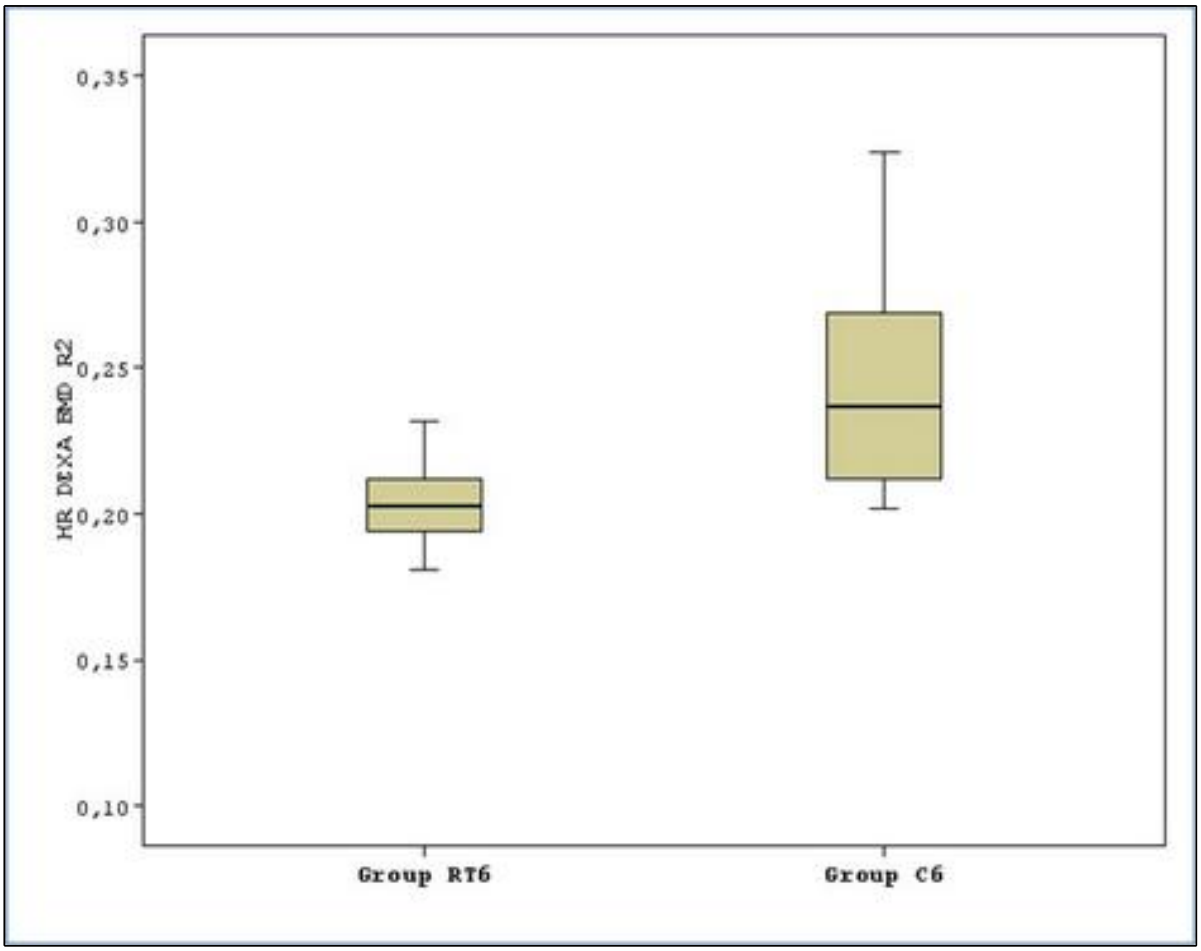

Figure 5.2 The distribution of the bone mineral density values at R2 for groups RT6 and C6.

Table 5.5
Bone Scintigraphy Values of the Irradiated Rats (RT6), Sixth Week After Fracture.

	N	Min	Max	Median	25 - 75 Percentile
R1	9	86.2	1363.7	610.7	280.8 - 1185.9
R2	9	17.1	289.1	106.6	40.5 - 196.9
Soft Tissue (ST)	9	5.7	110.5	34.2	16.9 - 95.4
R1-ST	9	80.5	1325.3	549.6	261.9 - 1110.8
R2-ST	9	11.4	184.3	77.0	23.6 - 118.8

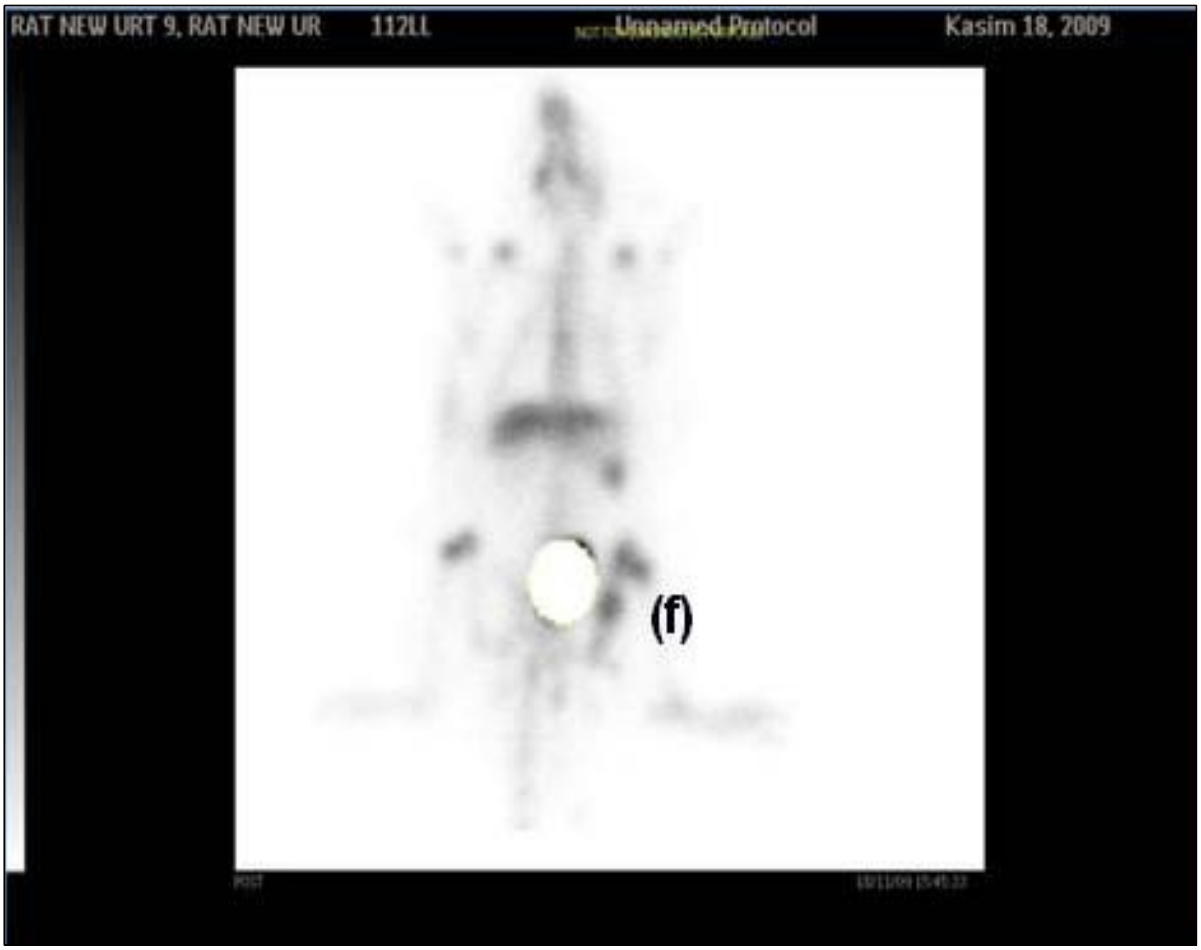

Figure 5.3 An example of the scintigraphic examination process. The average pixel counts of regions were obtained. Fracture region is denoted with the letter (f).

Table 5.6
Bone Scintigraphy Values of the Control Rats (C6), Sixth Week After Fracture.

	N	Min	Max	Median	25 - 75 Percentile
R1	9	31.7	298.1	142.5	94.8 - 271.3
R2	9	6.0	57.1	30.8	26.7 - 46.8
Soft Tissue (ST)	9	1.4	20.5	12.3	8.0 - 17.2
R1-ST	9	30.3	2832	131.3	86.9 - 253.9
R2-ST	9	4.6	36.6	18.7	18.5 - 32.2

Table 5.7
Values of MDP Uptake Ratios Obtained by Bone Scintigraphy and Comparison Between Groups for the Third and Sixth Week After Fracture.

Group	N	Mean	S.D.	Min	Max	p
Group RT3	9	10.50	5.52	4.43	17.88	0.2872
Group C3	10	10.91	5.64	2.70	19.94	
Group RT6	10	10.33	6.83	3.34	25.69	0.179
Group C6	9	7.06	2.22	4.64	11.45	

When the MDP uptake ratio results of Group RT3 and Group C3 with bone scintigraphy were compared, we did not find statistically significant difference for third ($p=0.872$) or sixth weeks ($p=0.179$) after fracture.

5.2 Radiodiagnostics

At week 3, for RT group none of the rats was scored as 0, while 3 rats were scored as 1, 3 were scored as 2 and 4 were scored as 3. In the control group for the same week; 1 rat was scored as 0 (Fig. 5.4), 1 was scored as 1, 3 was scored as 2 and 5 were scored as 3 (Fig. 5.5). At week 6, for RT group; 2 of the rats was scored as 0, another 2 were scored as 1, 1 was scored as 2 and 5 were scored as 3. The results for controls were exactly the same at the same time period (Fig. 5.6). There was not any statistically significant difference between the groups ($p>0.05$).

5.3 Histopathology

At week 3, necrotic bone lamellae and fibrinous materials, inflammation with abscess formations were seen around the Kirschner wire and fracture region for group RT3 (Fig. 5.7). The amount of necrotic bone lamellae were less at controls. When compared with the irradiation group also the inflammation was less extensive. Proliferating chondrocytes were more abundant in the controls. Woven bone formation was

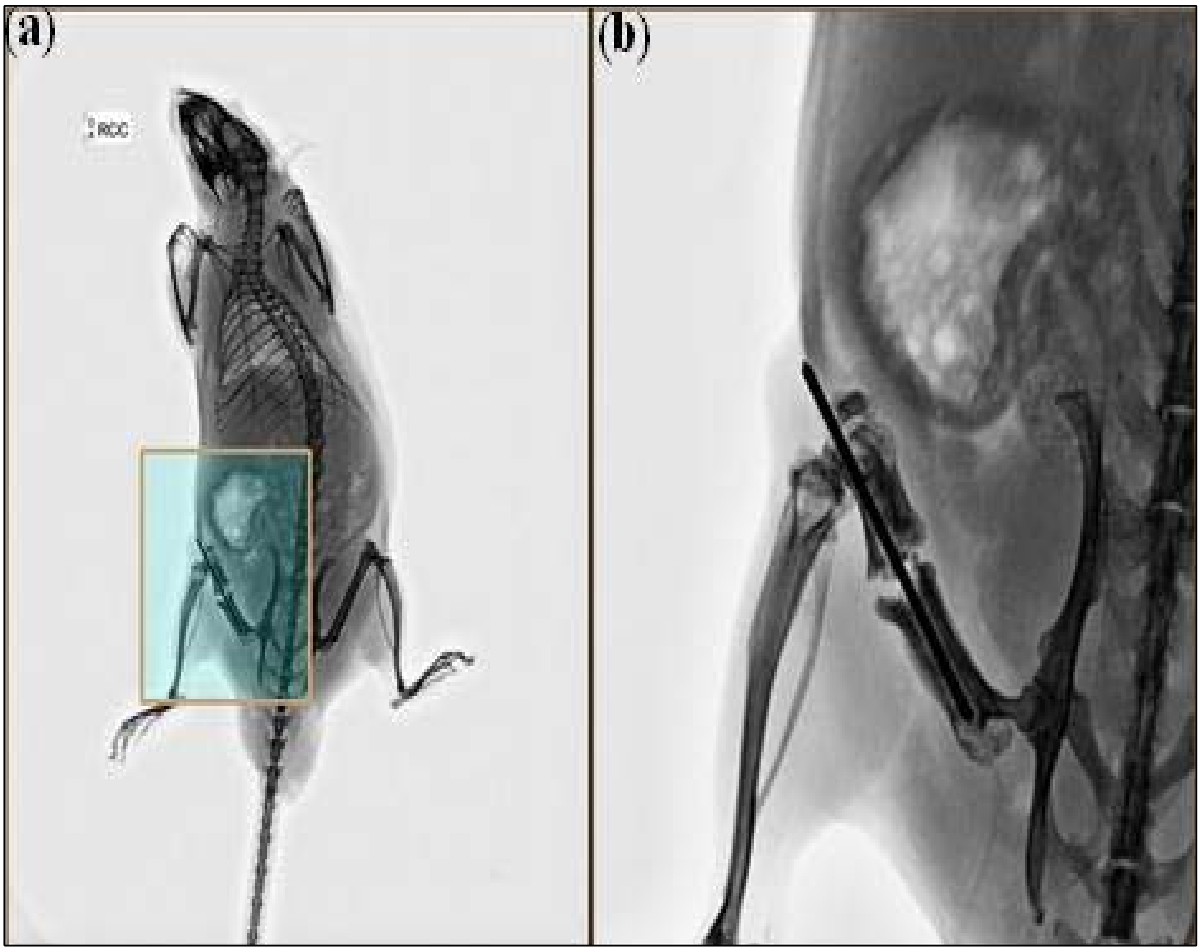

Figure 5.4 An example of the radiographic examination. This rat was scored as zero because of the non-union. Note the intramedullary nail.

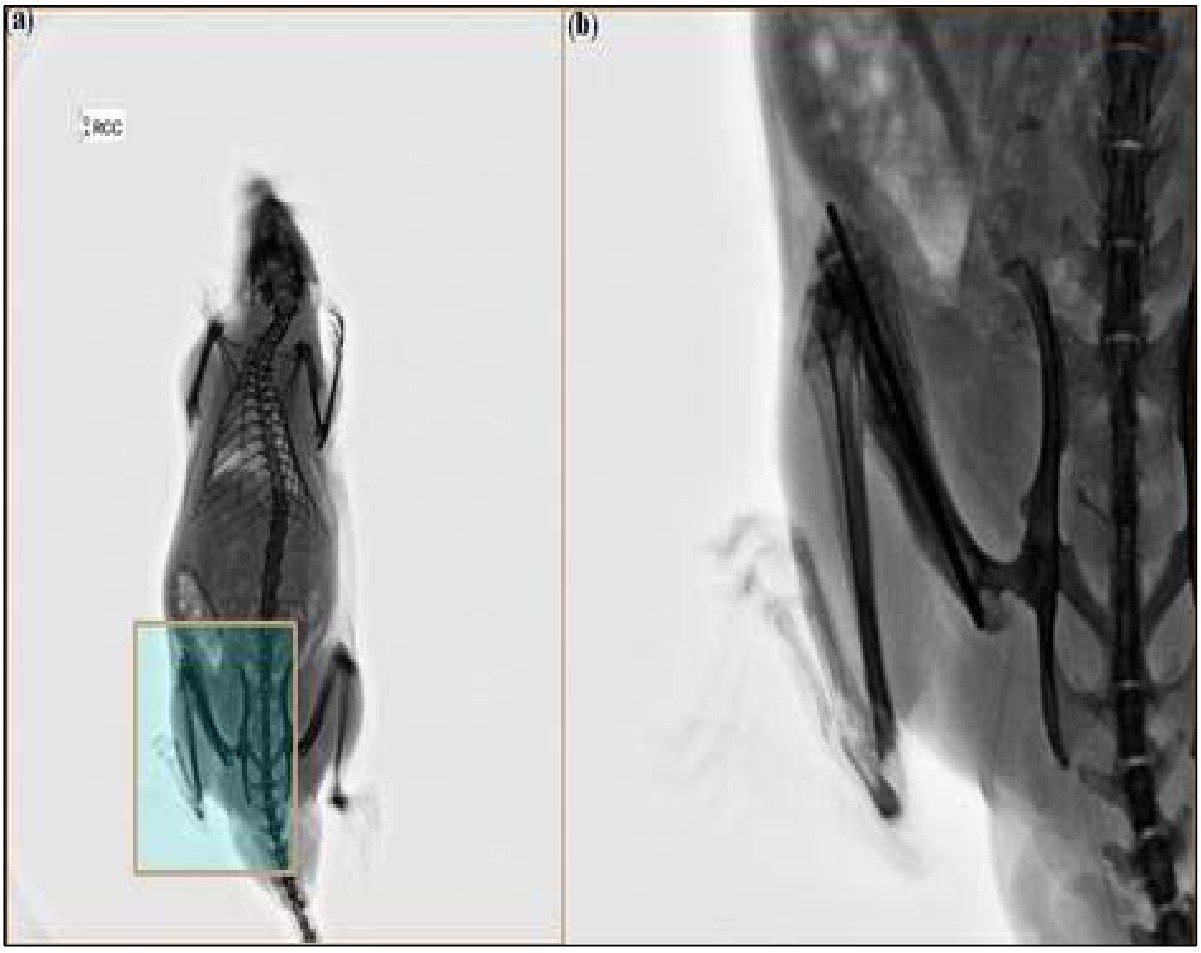

Figure 5.5 An example of the radiographic examination. This rat was scored as three because of the complete healing of the fracture.

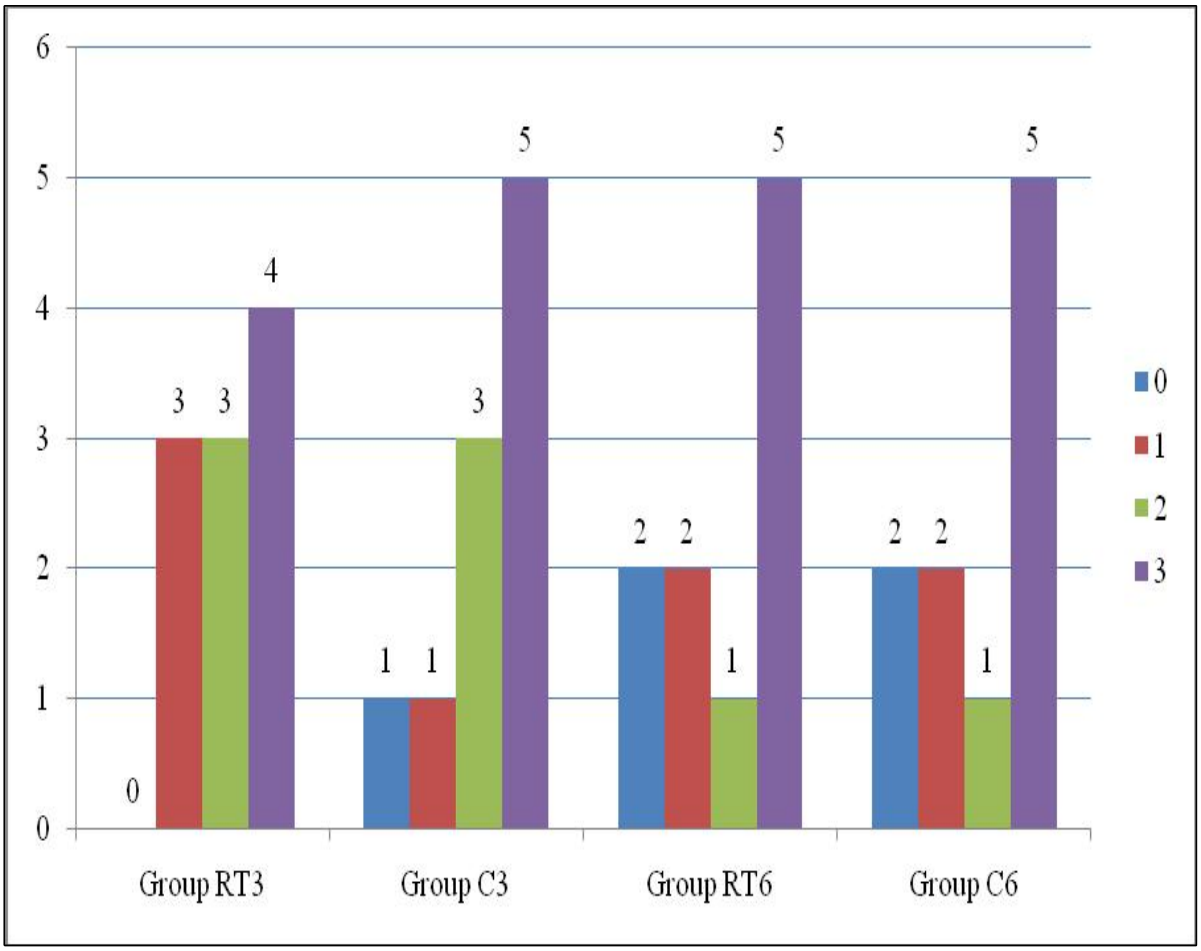

Figure 5.6 Number of rats-radiographic scoring graph for week 3 and week 6.

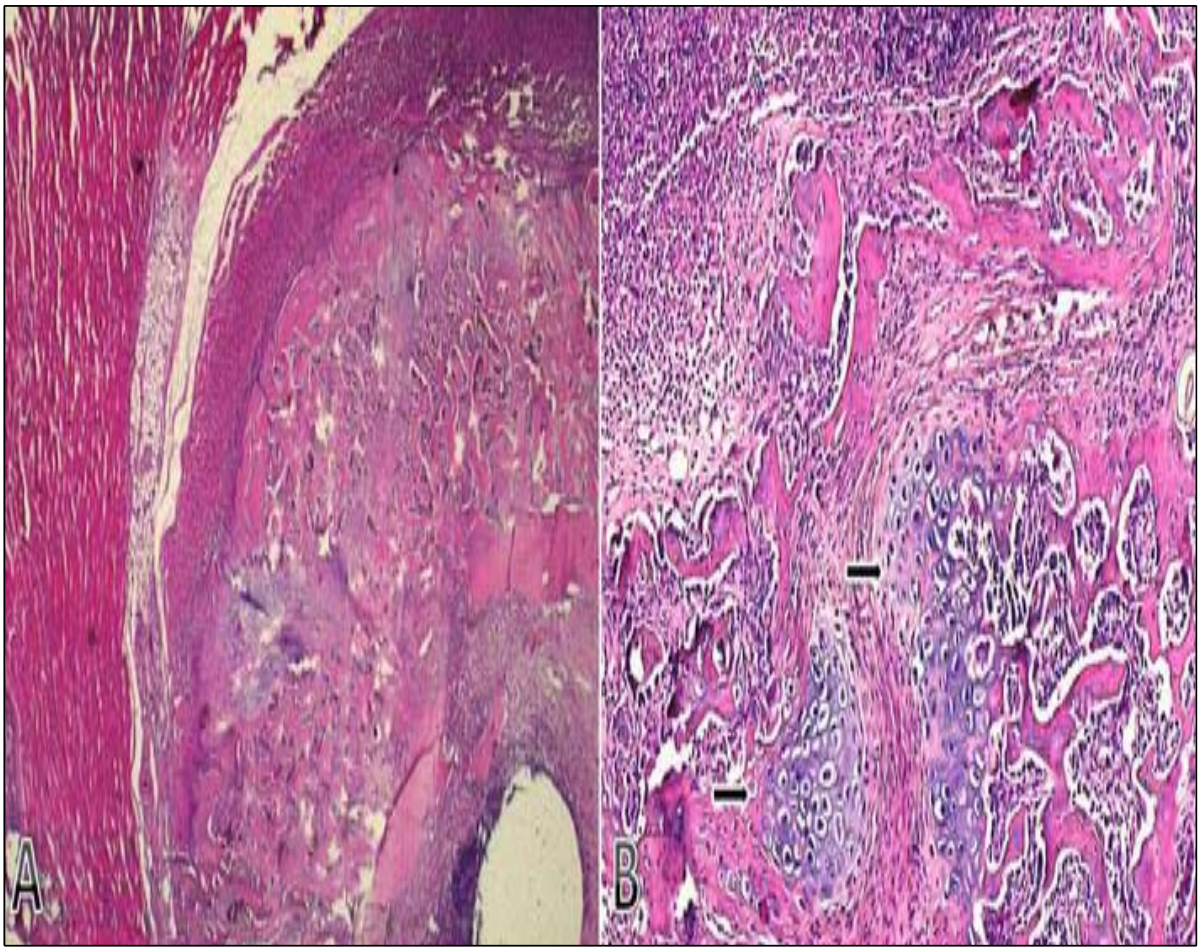

Figure 5.7 (a) Enchondral ossification with woven bone formation in the irradiation group at week 3. (b) Inflammation with abscess formation around the Kirschner wire of the irradiated rats on the left corner, whereas chondrocyte proliferation was detected on the right corner of the figure (arrows) (a; H&E x 12.5 b; H&E x 50).

seen on both groups; however the woven bone in irradiated rats was less organized than the controls. Woven bone formation was more pronounced at group C3 compared to RT3 (Fig. 5.8). Lamellar bone formation was more common in control rats than the irradiated rats. In irradiated rats, woven bone formation was also seen, however more disorganized features were dominant in irradiated rats (Fig. 5.9). At week 6, necrosis was not detected in control rats and only was found in one rat from irradiation group. The degree of the inflammation was reduced in both of the groups. Because of the maturation of the lamellar bone, proliferating chondrocytes and woven bone amount were less. Lamellar bone formation was seen in both groups however it was disorganized at the irradiated rats.

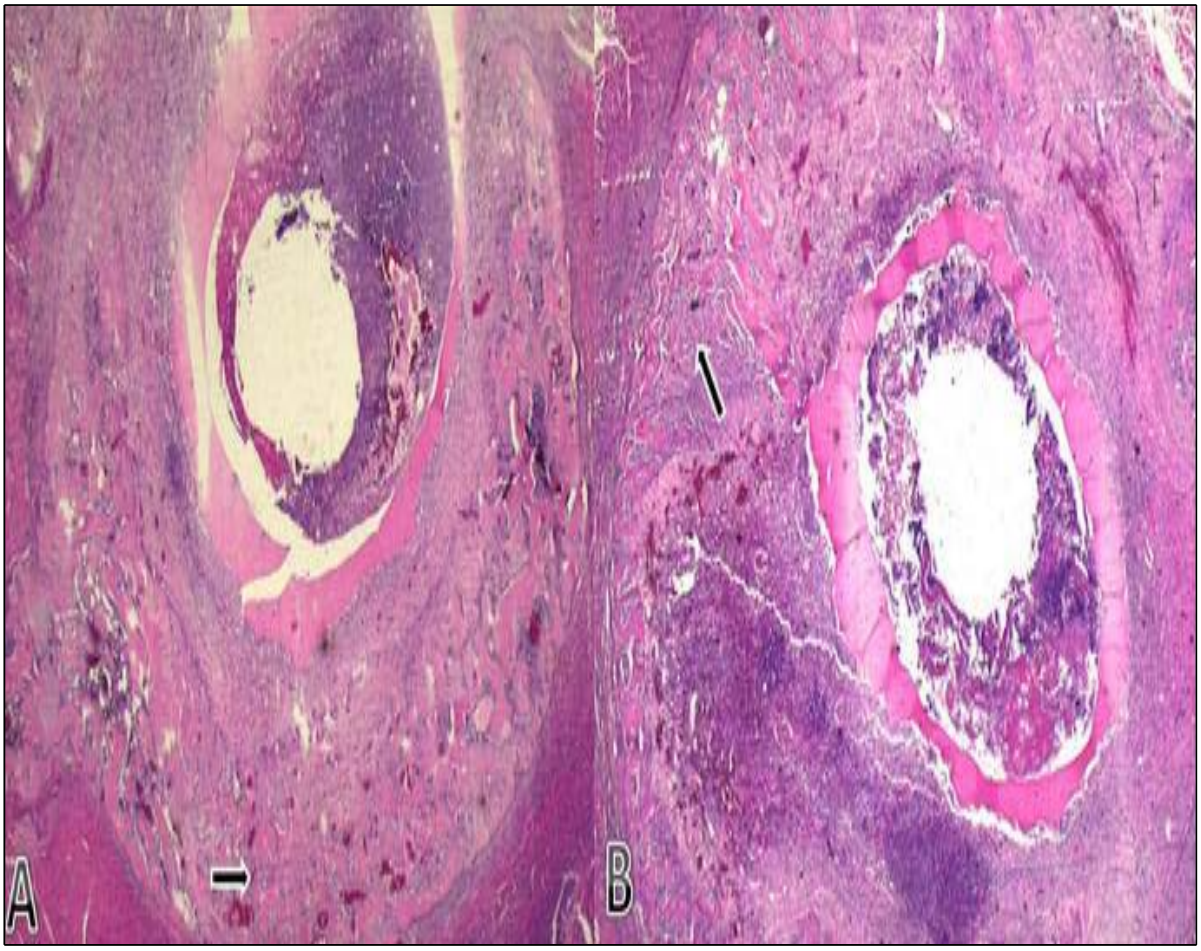

Figure 5.8 Rats in the control group showed distinct woven bone formation (arrow) than the irradiation group at week 3 (a; control, b; irradiation H&E x 12.5).

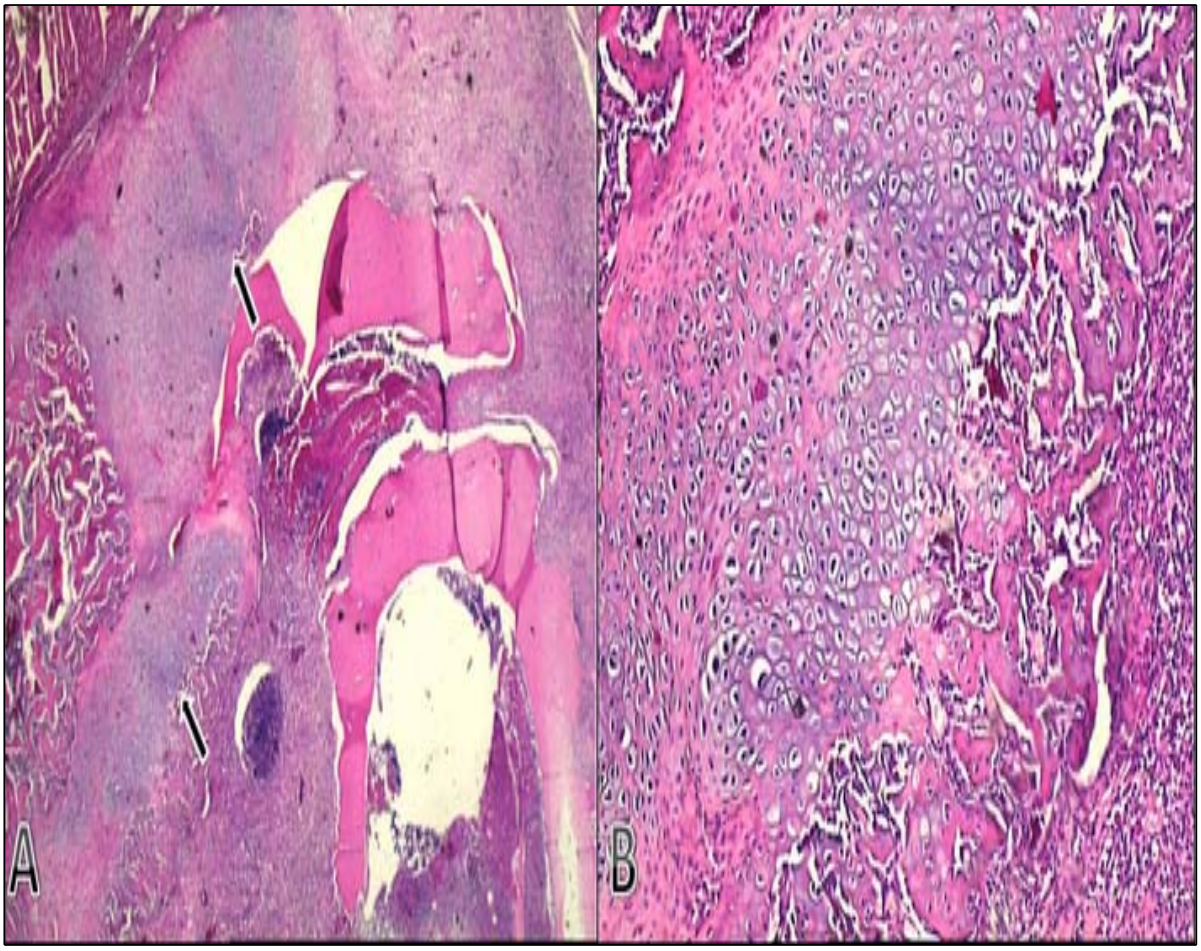

Figure 5.9 (a,b) Disorganized woven bone formation with proliferating chondroctyes at 3 weeks (arrows) (a; H&E x 12.5 b; H&E x 50).

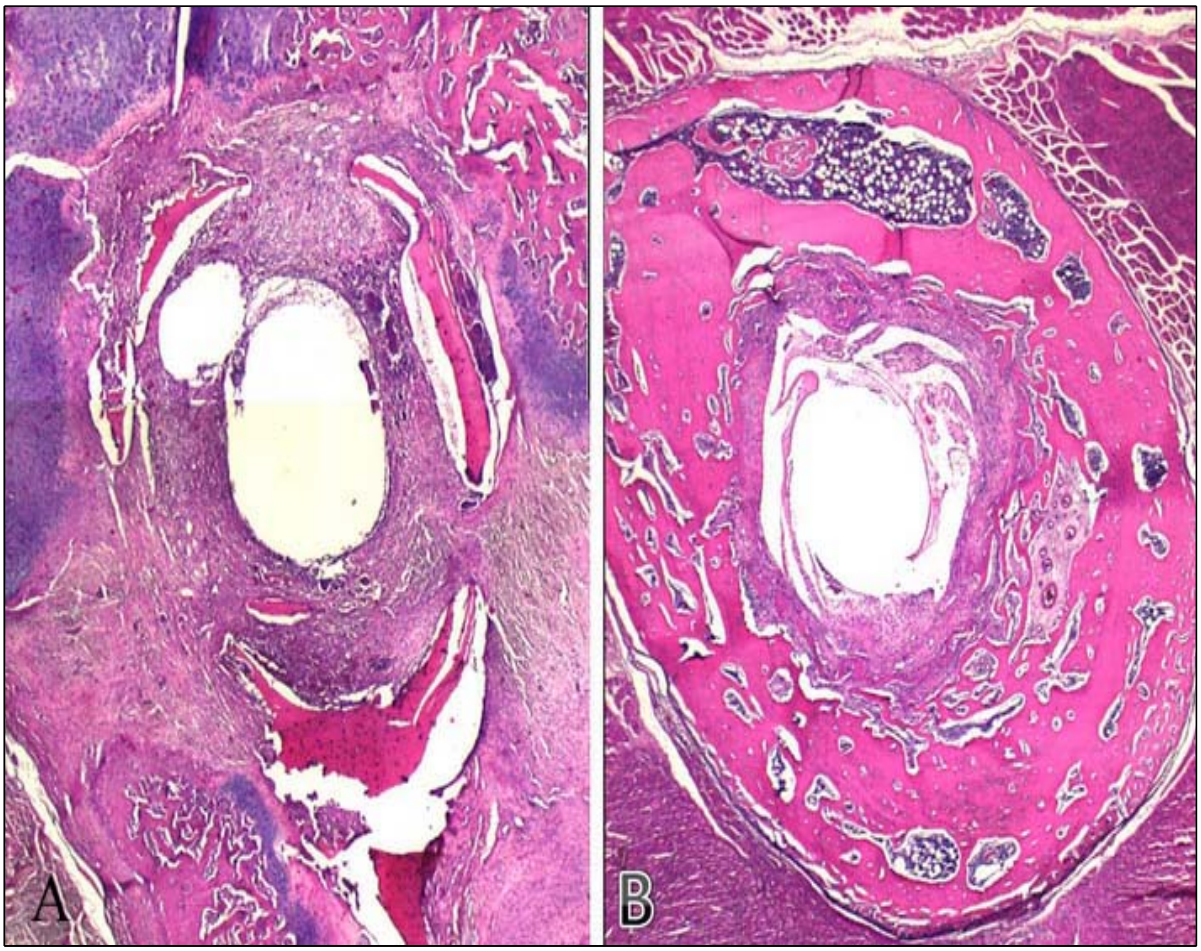

Figure 5.10 The diameter of the lamellar bone of the irradiation rats at 6 weeks was greater than the controls. Also note the disorganization of the bone formation in the irradiated rats (a; irradiation b; control H&E x 12.5).

6. DISCUSSION

This experimental study was designed to test the hypothesis that irradiation at low doses may show beneficial effects on fracture healing cascade promoting mineralization. Using a standard fracture model evaluation was made by using DEXA to find out the BMD values, bone scintigraphy to find out MDP uptake ratio, radiography to score the fracture healing and finally histopathology to observe the microscopic features as the lamellar bone formation. Statistical analysis to find out significant differences resulted in positive results in only 2 parameters. At week 6 after fracture; a promotive effect of irradiation which was evidenced by increased "BMD Index" in RT6 group compared to C6 (1.35 vs. 1.06, p=0.006) and a decreased BMD R2 in RT6 group compared to C6 (0.20 vs 0.24, p=0.005).

The other results were statistically insignificant, however although not significant, a difference in MDP index at week 6 was noted (10.3 vs 7.0, p=0.179). At this time point, The MDP index was lower at controls (Group C6). The histological findings were noteworthy and usual finding was the retarded maturation in the irradiated rats. Despite the significant increase in bone mineral density in irradiated rats at week 6, this bone was not found to be maturated to form lamellar bone.

One of the methods employed in this study to evaluate bone fracture healing was nuclear medicine techniques. Both static (bone mineral density measurements) [84] and dynamic (radionuclide bone imaging, bone scintigraphy) [85] measurements which have already been shown to correlate well with fracture healing by previous studies were done.

Radionuclide bone imaging has long been used in the diagnosis of musculoskeletal problems. Detection of occult fractures or localization of the anatomic lesions for systemic diseases that cannot be seen by radiographic techniques has been the major fields. Starting with 1970s, reports on the use of radionuclide bone imaging for the eval-

uation of fracture healing has started [86]. Clinical consequences have been the early differentiation of delayed healing and non-union patients [85]. The main agent for bone scanning in clinical use present is 99mTc MDP which is a phosphate analogue. Methylene diphosphonate circulates in the vascular system short after intravenous injection, and then equilibrates to the extravascular space. Subsequent accumulation of MDP in bone is rapid. Residual MDP is excreted via the urine. Approximately half of the administered dose is eliminated within 4 hours, producing a high bone-to-background ratio of activity [87]. Two to four hours after injection whole body imaging takes place. Therefore, we have performed the imaging 3-hours after injection of MDP. Some soft tissue MDP uptake is normally expected. In order to eliminate the soft tissue uptake effect, we used the MDP index and calculated according to the formula given in the materials and methods section to find out the actual activity levels.

The radionuclide bone scan is a sensitive technique which is applicable in a wide variety of pathologic and physiologic conditions of the musculoskeletal system. Determination of the fracture healing is one of them. Combining radionuclide bone scan with DEXA enabled us to evaluate fracture healing with improved techniques.

Dual-energy X-ray absorptiometry is the method of choice for defining bone mineral content accurately. Bone mineral analysis with bone densitometry, DEXA gives important information on bone mineralization, and was shown to correlate well with the biomechanical status of bone [84]. Determining the density of healing callus has traditionally been performed by direct radiographs. Plain radiographs can be used to determine the stiffness index of healing bone. The results showed the cortex to callus ratio -the thickness of the cortex, including the periosteal callus, normalized by the thickness of the cortex of the bone not surgically treated- correlated positively with the stiffness index of the bones [88]. The optical density of a radiograph is theoretically an indirect measure of bone mineral content however it has several limitations [89]. Therefore we tried to improve the accuracy of radiographic evaluation with the help of a scoring system [82]. Dual energy X-ray absorptiometry is accepted as a reliable measurement technique. It is more accurate than single or dual-photon absorptiometry to reveal material properties locally [90]. It has a high sensitivity and negative predictive

value which can contribute to the early detection of a healing problem [91].

Histology is another established method for evaluating fracture healing. Longitudinal and transverse sections through the fracture callus and the surrounding area are usually cut and stained. Common histological parameters, including callus formation, bone union, marrow changes and cortex remodeling, can be established. Therefore we preferred to work with histological features after the scarification of the animals.

Mechanical testing can be considered as a gold standard to monitor fracture healing however being an invasive technique; it cannot be used in the clinical setting. Therefore, attempts have been to find a noninvasive bone strength marker. Recently, the strength-strain index measured by peripheral quantitative computerized tomography was correlated with a biomechanical bone strength index using three-point bending test. The authors concluded that the strength-strain index measurement with peripheral quantitative computerized tomography is a valuable diagnostic tool not only in distraction osteogenesis but also in other techniques of bone healing [92]. Although very recent studies advocate the use of peripheral quantitative computed tomography, it was introduced in 1976. It has been more than 30-years since Rüegsegger et al. [93] introduced the use of this technique. It is quite obvious that still there is lack of consensus on the non invasive techniques to determine bone healing rate.

Despite the fact that the results of the experiment showed statistically significant increase in irradiated rats for BMD Index at week 6, higher BMD does not necessarily mean increased bone strength. One of the best examples of the correlation between bone quantity and bone quality is the use of bisphosphonates for the prevention and treatment of osteoporosis.

Since 1996, bisphosphonates are frequently prescribed for both the prevention and the treatment of osteoporosis. They act as bone resorption inhibitors. Spontaneous femur fractures in patients using alendronate which is a commonly used biphosphonate have been reported. It should be noted that these patients did not have lower BMD values that can cause an osteoporotic fracture on the contrary thicker cortices of the

femurs were on noted radiological examinations [94]. General practitioner's guidelines mention the use of bisphosphonates along with calcium and vitamin D as the preferred preventive medical treatment. Neviaser et al. collected data retrospectively on 70 femoral shaft fractures occurred after low energy impacts [95]. In this series, 25 female patients (23 Caucasian and 2 Asian) did use alendronate. The average period that alendronate was used was 6.2 years (range 1 to 10 years). Data analysis showed that 76% of the patients using alendronate had a specific fracture pattern which is a transverse fracture of the femur with a unicortical spike in an area of hypertrophy. This pattern was seen in only 2% of the patients who did not use alendronate. Furthermore, it was calculated that such a fracture pattern is 98% specific for alendronate-using patients. The same specific fracture pattern is also described by other authors. In a series of 17 patients with low-energy trauma subtrochanteric fractures, patients had received alendronate therapy for a mean of 4.4 years. Six of these patients were found to have a stress zone in the subtrochanteric region of their contra-lateral femur [96].

6.1 Irradiation Induced Injury

In the treatment of some malignant and metastatic lesions of the bone or some soft tissue tumors adjacent to the bone, the skeletal system may be exposed to irradiation. Another common application of irradiation to the musculoskeletal system is the prophylaxis of heterotopic ossification after total joint arthroplasty, particularly for the hip. Bone graft sterilization is another process that irradiation is used [97, 98].

Irradiation is associated with numerous side effects on the musculoskeletal system. Regeneration of bone during fracture healing is affected unfavorably, main features including decreasing number of osteocytes, suppression of the osteoblastic activity, and diminishing vascularity. Barnhard and Geyer [99] showed retardation of longitudinal bone growth; Riseborough et al. [100] showed side effects on spinal system with examples like scoliosis and Weatherby et al. showed [101] a very serious consequence of irradiation, sarcomatous degeneration. Osteonecrosis is another problem after irradiation that has long been appreciated [102]. This problem has started to be much more

Table 6.1
Types of Cellular Damage in Relation to Approximate Radiation Dose. Modified from [105].

Dose	Type of damage	Comments
0.01 - 0.05	Mutations (chromosomal aberrations and gene damage)	Irreversible chromosome breaks, may repair
1	Mitotic delay, impaired cell functions	Reversible
3	Permanent mitotic inhibition, impaired cell functions, activation and deactivation of cellular genes and oncogenes	Certain functions may repair; one or more divisions may occur
4 - 10	Interphase death	No division
500	Proteins coagulate, instant death	No division

frequent in the clinical setting after the more common use of irradiation for head and neck tumors [39].

Different tissues and cells have different susceptibilities to irradiation. Radiation can trigger effects that can lead to cancer or genetic damage. These effects can take years or generations to appear. The effects of the irradiation are because of its differential alterations in the cellular metabolism of different tissues. Generally speaking, an injury which has a high chance of repair can be considered as sublethal. If it can be repaired with treatment, it is potentially lethal, and which is permanent is lethal [103].

Among cell members, the nucleus is more radiosensitive than the cytoplasmic structures. Nuclear changes after radiation are swelling of the nuclear membrane and disruption of chromatin materials. Cytoplasmic changes include swelling, vacuolization, disintegration of mitochondria and endoplasmic reticulum, and reduction in the number of polysomes [104]. Depending on the dose of radiation and the subcellular changes, along with the previously described factors, the potential effects on the cell vary (Table 6.1).

After ionizing radiation exposure, cellular injury occurs in one of the following forms; division delay, reproductive failure, or interphase death [105]. Division delay is

seen after exposure to radiation in the range of 0.5 - 3 Gy. Delayed mitosis is observed however near normal restoration of mitotic activity is achieved following several generations. In reproductive failure, the failed mitotic activity is permanent and eventually results in cell death. This is observed in a linear fashion after exposure to irradiation more than 1.5 Gy. In interphase death, apoptosis, which is programmed cell death, is defined as a particular set of microscopic changes associated with cell death. Radiation induced apoptosis is highly related to the type of the involved cell. For instance, lymphocytes, are very sensitive to radiation by this mechanism [105, 106].

Irradiation produces severe antiangiogenic and fibrogenic effects [107]. Angiogenic factors can be produced to compensate for the radiation induced antiangiogenesis. This production is of sufficient quantity to produce systemic effects is not well known.

Hong et al. [108], on the basis that in situ nanostructural observations of bone in the transmission electron microscope provide insight into local changes in mineral and collagen under high energy irradiation of doses equivalent to those used clinically, aimed to investigate the effects of irradiation on the ultrastructure of thin plastic imbedded bone samples. The observations of irradiation induced collagen degradation and coarsening of bone mineral apatite indicates that the integrity and load carrying capacity of bones can be degraded through exposure to irradiation since coarsened apatite crystals deteriorate mechanical properties of bone, such as ductility. A change in the integrity of collagen fibrils and mineral apatite induced by radiation, without a change in bone mineral content or bone mass may lead to susceptibility to fracture after radiotherapy without a change in bone mineral content. In other words a change in bone quality develops [109].

In our study, we find out a statistically significant increase in rats that have undergone irradiation at week 6. However, the histopathological findings were not in agreement with the bone quality in favor of irradiation. Irradiation induced changes in collagenous structure of the bone, which may lead to susceptibility to a fracture despite the unchanged or even increased bone mineral density should be considered.

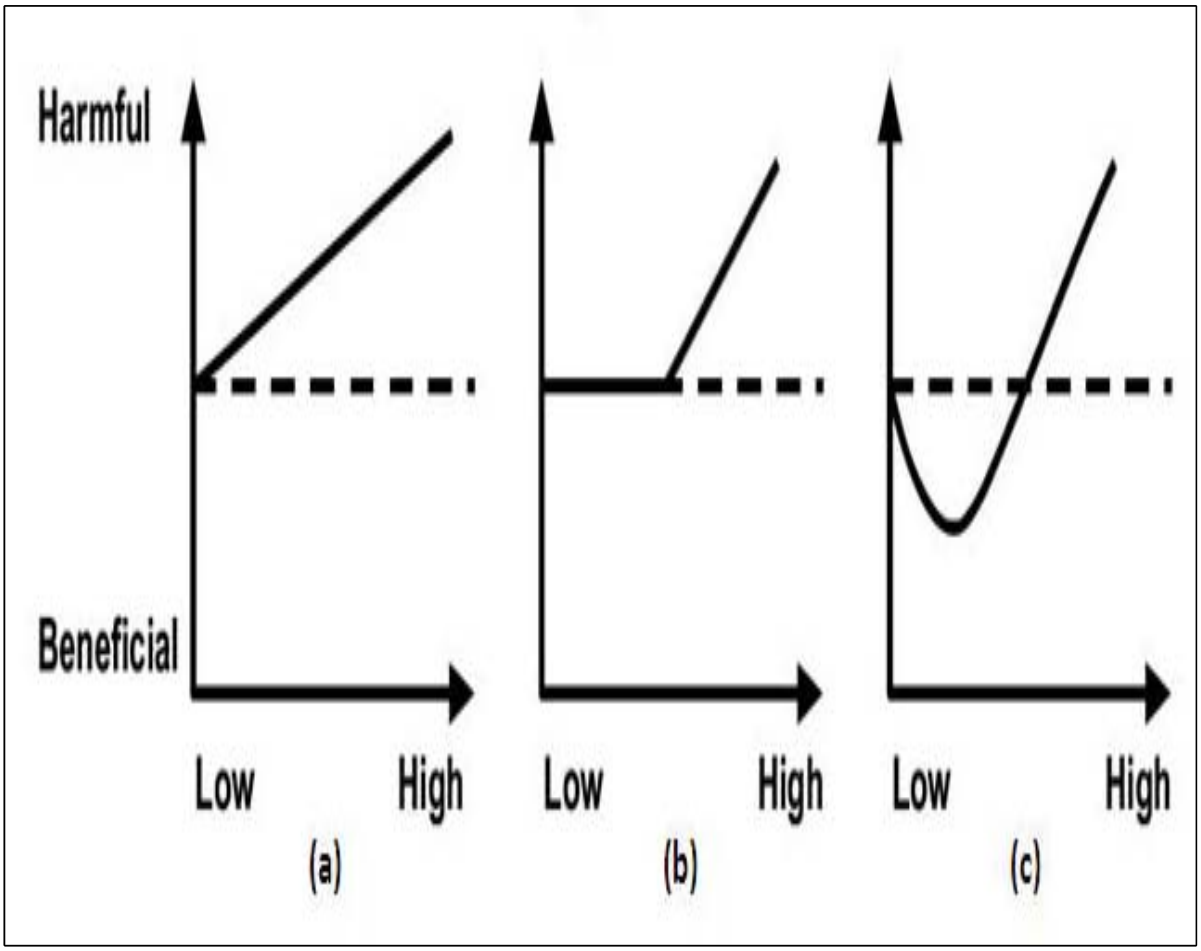

Figure 6.1 Comparison between three different dose-response models for irradiation: (a) Linear without a threshold, (b)Threshold and (c) Hormetic models. The dashed line represents health effects in the absence of radiation [110].

Dose-response models have been proposed to predict the relationship between the radiation dose and its effects (Fig. 6.1).

Main three assumptions are as follows:

1. Linear-No Threshold Model: This model assumes that any level of radiation is harmful. In addition, the risk increases linearly with increments of dose.

2. Threshold Model: This model assumes that the risk of radiation is linearly related to the dose after a certain threshold level. Below this threshold level, we do not expect any risks. The theory behind the threshold level is that some degree of

cellular damage should accumulate and produce cell damage.

3. Hormesis Model: In this model there is a bimodal effect of radiation. Below a certain threshold level radiation is protective, and when this threshold is exceeded, harmful effects can be seen. The rationale is that radiation at low levels induces protective cellular mechanisms which prevent DNA damage [110, 111, 112].

If hormesis model for the effects of irradiation on bone tissue is appropriate, then future studies should focus on how irradiation would be delivered to bone tissue. We believe that divided very low doses may be an alternative to single low dose, and this may be a subject of future research.

6.2 Vascularity and Bone Healing

Fracture healing is a well-characterized cascade of events that includes hematoma formation, inflammation, soft cartilaginous callus formation, neovascularization, osteoblastic callus mineralization, and osteoclastic remodeling of the hard callus back to mature lamellar bone [19]. Research on osteogenesis was primarily on the role and function of the osteoblasts, until experimental data revived interest in the functions of the vessels in osteogenesis. The vessels bring mainly cells and oxygen to tissues in the human body. However, starting with Trueta and Buhr [113], who proposed the presence of a factor at the fracture sites of the bones that stimulates bone formation, our insight of bone formation has changed dramatically.

Both intramembraneous and endochondral bone ossification occur concurrent with vascular growth. Invasion of capillaries into the mesenchymal area and differentiation of mesenchymal cells into mature osteoblasts is seen with intramembraneous ossification. Then osteoblasts start depositing bone matrix leading to the formation of bone spicules which fuses with others to form trabeculae. Woven bone and finally trabecular bone formation proceeds. Intramembraneous ossification occurs during embryonic development. Bones of load bearing joints form by endochondral formation

that uses the functional properties of cartilage and bone to provide a mechanism for the formation and growth of the skeleton. The rate of bone ossification that is formed by the coupling of chondrogenesis and osteogenesis is dependent on the amount of vascularization [114]. During this process VEGF isoforms are essential. They coordinate metaphyseal and epiphyseal vascularization, cartilage formation, and ossification during endochondral bone development [115].

Angiogenesis precedes osteogenesis and also plays a critical role in the process of endochondral ossification during bone formation [116]. Local vascularity of the fracture is a significant parameter for healing [117]. Blood supply during fracture healing is achieved by neovascularization and angiogenesis. Enhanced angiogenesis promotes fracture healing, whereas treatment with angiogenesis inhibitors blocks callus formation and produces atrophic non-unions [118, 119].

Irradiation produces severe antiangiogenic and fibrogenic effects. Angiogenic factors can be produced to compensate for the radiation induced antiangiogenesis. Okunieff et al. [107] found out increased Laser Doppler Flow in non-irradiated limbs of mice in an animal study and suggested that this effect can be a consequence of production of sufficient angiogenic factors showing some systemic effects.

One of the most important mediators of angiogenesis is VEGF. The VEGF family comprises at least seven members: VEGF/VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor. Endochondral ossification uses the functional properties of cartilage and bone to provide a mechanism for the formation and growth of the skeleton. The coupling of chondrogenesis and osteogenesis to determine the rate of bone ossification is dependent on the level of vascularization of the growth plate [114]. The VEGFs and their corresponding receptors are key regulators in a cascade of molecular and cellular events that ultimately lead to the development of the vascular system [120]. Treatment with exogenous VEGF was shown to promote angiogenesis and bone formation after injury by several studies [20]. The delivery of VEGF, to bony defects has been shown to significantly improve bone repair in irradiated sites [121].

The main theory supporting the beneficial effects of low dose irradiation is the determination of increased VEGF expression. Therefore, one can easily conclude that, the higher expressions of VEGF at the irradiated site can cause the formation of new vessels and, by the virtue of neoangiogenesis, osteogenesis is also stimulated. Previous studies have shown that endogenous VEGF is important for endochondral bone formation [20]. Dudziak et al. [122], in their experimental study, exposed osteoblast-like cells to ionizing radiation. This resulted in dose-dependent decreases in cellular proliferation and promoted cellular differentiation confirmed by increased alkaline phosphatase production. Dose-dependent decreases in total TGF-β 1 and VEGF protein production were found. Decreases in total TGF-β 1 production were found to be a consequence of a decrease in TGF-β1 production per cell. However, the authors found out that the decrease in total VEGF production was secondary to decreases in cellular proliferation, as the cellular production of VEGF by irradiated osteoblasts was moderately increased when VEGF production was corrected for cell number. When we correlate mentioned data with our experiment, we can speculate that although classic side effects of irradiation on tissues affect them, the tissue attempts to compensate by increasing the VEGF moderated neoangiogenesis pathway to increase osteoblastic proliferation. This results in increased BMD results at the late stages of fracture healing.

The reasons for irradiation as a causing factor for new vessel formation have explanations. Tissue hypoxia is believed to be critical for the initial signals for blood vessel invasion into bone and initiation of the angiogenic cascade [123]. The hypoxia-inducible factors (HIFs) activate genes encoding proteins that mediate adaptive responses to reduced oxygen availability [124], main adaptive response being angiogenesis. Hypoxia Inducible Factor-α (HIF-α) is a factor related with VEGF related angiogenesis and osteogenesis. It was proposed as being a significant factor in a pathway to couple angiogenesis with osteogenesis during long bone formation. This increase in bone formation is due to enhanced angiogenic activity, which is mediated by elevated levels of VEGF in HIF-α overexpressing osteoblasts [125]. However, we should not forget that bone mineralization itself is not enough for better fracture healing and because of the side effects of irradiation on collagen structures, despite the high levels of bone mineral density obtained, we may not have good quality bone finally.

Our results do not support low dose irradiation as a promoter of fracture healing that can be used as an adjunctive therapy. Although a promotive effect of low dose irradiation was not found totally, there is a great likelihood of enhancing bone formation represented by higher BMD with low doses of radiation. In a previous study by Markbreiter et al. [97], the authors applied 900 rad local irradiation 3 days after fracture, in a rat femoral closed fracture model. The authors using biomechanical parameters found out a delay of 4 weeks in fracture healing of the irradiated group when compared with controls. Interestingly, despite this lag, staging and stiffness approached normal controls within an 8-week period. We have also observed a delayed response in irradiated rats. This response was evident by increased BMD values at the fracture site. However, we should note that BMD Index values are affected by neighboring bone BMD values as well. Because of the decrease in BMD values of neighboring bone the difference was statistically significant. We can speculate that, because of the fracture and its consequences like VEGF related neoangiogenesis, new bone started to form at the fracture region compensating the effects of irradiation, but no such a compensation occurred at neighboring bone regions which are named as "R2" region.

There are not many studies in the current literature with very low doses of irradiation. Gal et al. [39] subjected mouse osteoblasts to irradiation doses of 0, 2, 4, or 6 Gy. The authors performed immunohistochemical analysis of TGF-β1 expression and collagen production and found out a distinct difference in collagen production between cells treated at 0 and 2 Gy when compared with those treated at 4 and 6 Gy. In this study, cells irradiated with 2 Gy demonstrated similar results with controls. Acutely at low doses ($<$2Gy) cells repair most sublethal damages successfully. An increase in TGF-β1 receptors was another noteworthy finding in irradiated cells. We should consider the contributing factors like hypoxia and effects of intercellular contacts in vivo when commenting on the effects of irradiation with in vitro experiments.

7. CONCLUSIONS

Bone formation and regeneration is a complex biological event. Despite remarkable advances in our understanding of the scientific basis of fracture healing with numerous experimental studies, and significant knowledge that up to date research has provided, the issue still remains unknown to a great extent. However, a complete understanding of the cascade of fracture healing process is of vital importance as we know that delayed unions and non-unions are not only health problems but have significant social impact and economical burdens. Therefore, further research is required both at the laboratory bench and the patient's bedside concomitantly in a faster and more efficient way. The study by Heissig et al. [126] was one of the rare studies combining the concepts on the effects of low dose irradiation and angiogenesis. The authors demonstrated the clinical potential of low-dose irradiation to induce neovascularization under ischemic conditions and identified mast cells as a source of VEGF. More recently, Potier et al. [127] studied on the effects of temporary hypoxia on angiogenic factor expression by MSCs. They found out that temporary hypoxia led to a 2-fold increase in VEGF expression at both the mRNA and protein levels. Other growth factors such as TGF-β1 and IL-8 were not affected by temporary exposure to hypoxia. Interesting finding was the down regulation of the osteoblastic markers for this study. Finally the authors suggested that that the exposure of MSCs transplanted in vivo to hypoxia may affect their bone forming potential. There is a potential for differing effects of radiation given in a single dose and fractionated doses. Fractionated doses which deliver a less concentrated level of radiation over a wider spectrum of time can show different biologic effects. Single doses should affect the particular biological events while fractionated approaches may help lowering magnitude of cellular injury. Using fractionated doses, the influence can be observed on a wider spectrum of the different ongoing, interconnected biological events of fracture repair. Therefore, one of the potential fields of low doses of irradiation can be using fractionated doses to find out the best harm-benefit combination. Bone strength is an important measure because it best describes the mechanical property and quality of bone [128]. In this study mechanical bone strength

was not evaluated which is one of the short-comings of this study. However, instead we tried to focus on histopathological properties on post mortem specimens. It is not usually possible to perform both of them as both are invasive techniques damaging to the explanted materials. While routine staining was performed with hematoxylin and eosin, immunohistochemical staining with an anti-vascular endothelial growth factor (VEGF) antibody could be added to the methods. Microangiography is also another technique that can be used to assess angiogenesis and its relationship with osteogenesis. There are numerous methods that can be useful to investigate bone formation, not only to find out the bone quantity but also bone quality. In a very recent study, high resolution transmission electron microscopy was used to investigate the effect of irradiation on collagen and mineral features of murine femoral lamellar bone. Damage to collagen and coarsening of apatite crystals which can deteriorate the strength and integrity of bone was found and the authors suggested this insight to patients who have undergone radiation therapy [108]. Despite our best efforts, there are some drawbacks of the experimental setting. We performed our study on young rats of the same age and characteristics. However, activity in young and healthy animals might not be predictive of that in older animals. Some growth factors are less active in older animals [20]. Therefore, we should not generate our results unless the same results can be obtained in older rats. Additionally, rat bones do not undergo normal haversian remodeling and they differ physiologically from human bone though they have been widely used in orthopaedic research [81]. Significant advances have been made in the understanding of fracture healing. However, delayed unions and non-unions are still challenging issues to treat. Despite the advances in biology, specifically introduction of therapeutic BMPs and other biologic methods for bone regeneration, physical methods are still needed to strengthen their effects. Low dose irradiation seems to be increasing bone mineral density however this increase was not supported with histological studies to form lamellar bone. Further studies are required, with different dose ranges, for instance 1 to 5 Gy and with different applications.

REFERENCES

1. Robey, P. G., *Normal bone formation structure*, pp. 3–12. Bone formation and Repair, Rosemont, Il: American Academy of Orthopaedic Surgeons, 1994.

2. Muschler, G. F., C. Nakamoto, and J. N. Grauer, *Bone healing and grafting*, pp. 29–38. American Academy of Orthopaedic Surgeons, Rosemont, Il: Springer, 2005.

3. Brighton, C. T., and S. R. Pollack, "Treatment of recalcitrant nonunion with a capacitively coupled electrical field: A preliminary report," *J Bone Joint Surg Am*, Vol. 67, pp. 577–585, 1985.

4. Brighton, C. T., P. Shaman, R. B. Heppenstall, J. L. E. Jr, S. R. Pollack, and Z. B. Friedenberg, "Tibial nonunion treated with direct current, capacitive coupling or bone graft," *Clin Orthop Relat Res*, Vol. 321, pp. 223–234, 1995.

5. Butcher, J. L., E. J. MacKenzie, B. Cushing, G. Jurkovich, J. Morris, A. Burgess, M. McAndrew, and M. Swiontkowski, "Longterm outcomes after lower extremity trauma," *J Trauma*, Vol. 41, no. 1, pp. 4–9, 1996.

6. Praemer, A., S. Furner, and D. P. Rice, *Musculoskeletal Conditions in the United States*, Rosemont, IL: American Academy of Orthopaedic Surgeons, 2nd ed, 1999.

7. "Musculoskeletal injuries report: Incidence, risk factors and prevention," tech. rep., American Academy of Orthopaedic Surgeons, Rosemont, Il, 2000.

8. Nelson, F. R., C. T. Brighton, J. Ryaby, B. J. Simon, J. H. Nielson, D. G. Lorich, M. Bolander, and J. Seelig, "Use of physical forces in bone healing," *J Am Acad Orthop Surg*, Vol. 11, no. 5, pp. 344–354, 2003.

9. Kanakaris, N. K., and P. V. Giannoudis, "The health economics of the treatment of long bone nonunions," *Injury*, Vol. 38, no. 2, pp. 77–84, 2007.

10. Ayers, D. C., C. M. Evarts, and J. R. Parkinson, "The prevention of heterotopic ossification in high risk patients by low dose radiation therapy after total hip arthroplasty," *J Bone Joint Surg Am*, Vol. 68, no. 9, pp. 1423–1430, 1986.

11. Zhou, X. Z., G. Zhang, Q. R. Dong, C. W. Chan, C. F. Liu, and L. Qin, "Low dose x irradiation promotes mineralization of fracture callus in a rat model," *Arch Orthop Trauma Surg*, Vol. 129, no. 1, pp. 125–132, 2009.

12. Heybeli, N., M. Caloglu, and V. Yurut-Caloglu, "Does low dose x irradiation promote mineralization of fracture callus in a rat model?," *Arch Orthop Trauma Surg*, Vol. 129, no. 7, pp. 999–1000, 2009.

13. Mundy, G. R., *Bone remodeling and its disorders*, London: Martin Dunitz, 1999.

14. Colnot, C., D. M. Romero, S. Huang, and J. A. Helms, "Mechanisms of action of demineralized bone matrix in the repair of cortical bone defect," *Clin Orthop Relat Res*, Vol. 435, pp. 69–78, 2005.

15. Friedenstein, A. J., R. K. Chailakhyan, and U. V. Gerasimov, "Bone marrow osteogenic stem cells in vitro cultivation and transplantation in diffusion chambers," *Cell Tissue Kinet*, Vol. 20, pp. 263–272, 1987.

16. Bielby, R., E. Jones, and D. McGonagle, "The role of mesenchymal stem cells in maintenance and repair of bone," *Injury*, Vol. 38, no. 1, pp. 26–32, 2007.

17. Dimitriou, R., E. Tsiridis, and P. V. Giannoudis, "Current concepts of molecular aspects of bone healing," *Injury*, Vol. 36, no. 12, pp. 1392–1404, 2005.

18. Barnes, G. L., P. J. Kostenuik, L. C. Gerstenfeld, and T. A. Einhorn, "Growth factor regulation of fracture repair," *J Bone Miner Res*, Vol. 14, no. 11, pp. 1805–1815, 1999.

19. Einhorn, T. A., "The cell and molecular biology of fracture healing," *Clin Orthop Relat Res*, Vol. 355, pp. 7–21, 1998.

20. Carano, R. A., and E. H. Filvaroff, "Angiogenesis and bone repair," *Drug Discov Today*, Vol. 8, pp. 980–989, 2003.

21. Gerstenfeld, L., T. Cho, T. Kon, T. Aizawa, A. Tsay, J. Fitch, G. Barnes, D. Graves, and T. Einhorn, "Impaired fracture healing in the absence of tnf-alpha signaling: the role of tnf-alpha in endochondral cartilage resorption," *J Bone Miner Res*, Vol. 18, no. 9, pp. 1584–1592, 2003.

22. Kon, T., T. J. Cho, T. Aizawa, M. Yamazaki, N. Nooh, D. Graves, L. C. Gerstenfeld, and T. A. Einhorn, "Expression of osteoprotegerin, receptor activator of nf-kappab ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing," *J Bone Miner Res*, Vol. 16, pp. 1004–1014, 2001.

23. ten Dijke, P., J. Fu, P. Schaap, and B. A. Roelen, "Signal transduction of bone morphogenetic proteins in osteoblast differentiation," *J Bone Joint Surg Am*, Vol. 85, pp. 34–38, 2003.

24. Tsiridis, E., N. Upadhyay, and P. Giannoudis, "Molecular aspects of fracture healing: which are the important molecules?," *Injury*, Vol. 38, no. Suppl 1, pp. 11–25, 2007.

25. Cheng, H., W. Jiang, F. M. Phillips, R. C. Haydon, Y. Peng, L. Zhou, H. H. Luu, N. An, B. Breyer, P. Vanichakarn, J. P. Szatkowski, J. Y. Park, and T. C. He, "Osteogenic activity of the fourteen types of human bone morphogenetic proteins," *J Bone Joint Surg Am*, Vol. 85, no. 8, pp. 1544–1552, 2003.

26. Deckers, M. M., R. L. van Bezooijen, G. van der Horst, J. Hoogendam, C. van Der Bent, S. E. Papapoulos, and C. W. Löwik, "Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor a," *Endocrinology*, Vol. 143, no. 4, pp. 1545–1553, 2002.

27. Bostrom, M. P., "Expression of bone morphogenetic proteins in fracture healing," *Clin Orthop Relat Res*, Vol. 355, pp. 116–123, 1998.

28. Bostrom, M. P., and P. Asnis, "Transforming growth factor beta in fracture repair," *Clin Orthop Relat Res*, Vol. 355, no. l, pp. 124–131, 1998.

29. Lieberman, J. R., A. Daluiski, and T. A. Einhorn, "The role of growth factors in the repair of bone biology and clinical applications," *J Bone Joint Surg Am*, Vol. 84, no. 6, pp. 1032–1044, 2002.

30. Prisell, P. T., D. Edwall, J. B. Lindblad, A. Levinovitz, and G. Norstedt, "Expression of insulin-like growth factors during bone induction in rat," *Calcif Tissue Int*, Vol. 53, no. 3, pp. 201–205, 1993.

31. Winet, H., J. Y. Bao, and R. Moffat, "A control model for tibial cortex neovascularization in the bone chamber," *Calcif Tissue Int*, Vol. 47, no. 1, pp. 24–34, 1990.

32. Kwong, F. N., and M. B. Harris, "Recent developments in the biology of fracture repair," *J Am Acad Orthop Surg*, Vol. 16, no. 11, pp. 619–625, 2008.

33. Jr, W. G. D. L., T. A. Einhorn, K. Koval, M. McKee, W. Smith, R. Sanders, and T. Watson, "Bone grafts and bone graft substitutes in orthopaedic trauma surgery a critical analysis," *J Bone Joint Surg Am*, Vol. 89, no. 3, pp. 649–658, 2007.

34. Rubinacci, A., J. Black, C. T. Brighton, and Z. B. Friedenberg, "Changes in bioelectric potentials on bone associated with direct current stimulation of osteogenesis," *J Orthop Res*, Vol. 6, pp. 335–345, 1988.

35. Goodship, A. E., and J. Kenwright, "The influence of induced micromovement upon the healing of experimental tibial fractures," *J Bone Joint Surg Br*, Vol. 67, no. 4, pp. 650–655, 1985.

36. Jagodzinski, M., and C. Krettek, "Effect of mechanical stability on fracture healing: an update," *Injury*, Vol. 38, no. 1, pp. 3–10, 2007.

37. Schechter, N. R., and V. O. Lewis, *Musculoskeletal system: the bone*, pp. 857–883. Radiation Oncology, Cox JD and KK Ang, eds., St Louis: Mosby, 2003.

38. Arikoski, P., J. Komulainen, R. Voutilainen, P. Riikonen, M. Parviainen, P. Tapanainen, M. Knip, and H. Kröger, "Reduced bone mineral density in long-term survivors of childhood acute lymphoblastic leukemia," *J Pediatr Hematol Oncol*, Vol. 20, no. 3, pp. 234–240, 1998.

39. Gal, T. J., T. Munoz-Antonia, C. A. Muro-Cacho, and D. W. Klotch, "Radiation effects on osteoblasts in vitro: a potential role in osteoradionecrosis," *Arch Otolaryngol Head Neck Surg*, Vol. 126, no. 9, pp. 1124–1128, 2000.

40. Bluemke, D. A., E. K. Fishman, and W. W. S. Jr, "Skeletal complications of radiation therapy," *Radiographics*, Vol. 14, pp. 111–121, 1994.

41. Hahn, G. V., and F. S. Kaplan, *Heterotopic ossification*, pp. 79–92. Bone Formation and Repair, Rosemont, IL: American Academy of Orthopaedic Surgeons, 1994.

42. Hu, H. P., W. Kuijpers, T. J. Slooff, J. R. van Horn, and D. H. Versleyen, "The effect of biphosphonate on induced heterotopic bone," *Clin Orthop Relat Res*, Vol. 272, pp. 259–267, 1991.

43. DiCesare, P. E., M. E. Nimni, L. Peng, M. Yazdi, and D. T. Cheung, "Effects of indomethacin on demineralized bone-induced heterotopic ossification in the rat," *J Orthop Res*, Vol. 9, no. 6, pp. 855–861, 1991.

44. Garland, D. E., "Surgical approaches for resection of heterotopic ossification in traumatic brain-injured adults," *Clin Orthop Relat Res*, Vol. 263, pp. 59–70, 1991.

45. Blokhuis, T. J., and J. P. Frölke, "Is radiation superior to indomethacin to prevent heterotopic ossification in acetabular fractures?: a systematic review," *Clin Orthop Relat Res*, Vol. 467, no. 2, pp. 526–530, 2009.

46. Nagi, O. N., M. S. Dhillon, and H. S. Batth, "Heterotopic ossification after total hip arthroplasty: A review of etiopathogenesis, risk factors and treatment modalities," *Indian Journal of Orthopedics*, Vol. 36, no. 4, pp. 225–233, 2002.

47. Coventry, M. B., and P. W. Scanlon, "The use of radiation to discourage ectopic bone a nine-year study in surgery about the hip," *J Bone Joint Surg Am*, Vol. 63, no. 2, pp. 201–208, 1981.

48. Hedley, A. K., L. P. Mead, and D. H. Hendren, "The prevention of heterotopic bone formation following total hip arthroplasty using 600 rad in a single dose," *J Arthroplasty*, Vol. 4, no. 4, pp. 319–325, 1989.

49. Seegenschmiedt, M., O. Micke, and N. Willich, "Radiation therapy for nonmalignant diseases in germany: Current concepts and future perspectives," *Strahlenther Onkol*, Vol. 180, no. 11, pp. 718–730, 2004.

50. Adamietz, B., R. Sauer, and L. Keilholz, "Radiotherapy for shoulder impingement," *Strahlenther Onkol*, Vol. 184, no. 5, pp. 245–250, 2008.

51. Adamietz, B., R. Schulz-Wendtland, S. Alibek, M. Uder, R. Sauer, O. Ott, and L. Keilholz, "Calcifying tendonitis of the shoulder joint: predictive value of pretreatment sonography for the response to low-dose radiotherapy," *Strahlenther Onkol*, Vol. 186, no. 1, pp. 18–23, 2010.

52. Mücke, R., M. H. Seegenschmiedt, R. Heyd, U. Schäfer, F. J. Prott, M. Glatzel, O. Micke, and the German Cooperative Group on Radiotherapy for Benign Diseases (GCG-BD), "Radiotherapy in painful gonarthrosis results of a national patterns-of-care study," *Strahlenther Onkol*, Vol. 186, no. 1, pp. 7–17, 2010.

53. Micke, O., M. H. Seegenschmiedt, and G. C. G. on Radiotherapy for Benign Diseases, "Radiotherapy in painful heel spurs (plantar fasciitis)–results of a national patterns of care study," *Int J Radiat Oncol Biol Phys*, Vol. 58, no. 3, pp. 828–843, 2004.

54. Hartshorne, E., "On the causes and treatment of pseudarthrosis and especially the form of it sometimes called supernumerary joint," *Am J Med*, Vol. 1, pp. 121–156, 1841.

55. Fukada, E., and I. Yasuda, "On the piezoelectric effect of bone," *J Phys Soc Japan*, Vol. 12, pp. 1158–1162, 1957.

56. Heybeli, N., A. Yesildag, O. Oyar, U. K. Gulsoy, M. A. Tekinsoy, and E. F. Mumcu, "Diagnostic ultrasound treatment increases the bone fracture-healing rate in an internally fixed rat femoral osteotomy model," *J Ultrasound Med*, Vol. 21, no. 12, pp. 1357–1363, 2002.

57. Mollon, B., V. da Silva, J. W. Busse, T. A. Einhorn, and M. Bhandari, "Electrical stimulation for long-bone fracture-healing: a meta-analysis of randomized controlled trials," *J Bone Joint Surg Am*, Vol. 90, no. 11, pp. 2322–2330, 2008.

58. Busse, J. W., J. Kaur, B. Mollon, M. Bhandari, P. Tornetta, H. J. Schünemann, and G. Guyatt, "Low intensity pulsed ultrasonography for fractures: systematic review of randomised controlled trials," *BMJ*, Vol. 27, pp. 338–351, 2009.

59. Brighton, C. T., J. Black, Z. B. Friedenberg, J. L. Esterhai, L. J. Day, and J. F. Connolly, "A multicenter study of the treatment of nonunion with constant direct current," *J Bone Joint Surg Am*, Vol. 63, pp. 2–13, 1981.

60. Brighton, C. T., and W. P. McCluskey, *Cellular response and mechanisms of action of electrically induced osteogenesis*, Vol. 898 of *Bone and Mineral Research/4*, pp. 213–254. Amsterdam: Elsevier Science Publishers BV, 1986.

61. Bodamyali, T., J. M. Kanczler, B. Simon, D. R. Blake, and C. R. Stevens, "Effect of faradic products on direct current stimulated calvarial organ culture calcium levels," *Biochem Biophys Res Commun*, Vol. 264, pp. 657–661, 1999.

62. Bushinsky, D. A., "Metabolic alkalosis decreases bone calcium efflux by suppressing osteoclasts and stimulating osteoblasts," *Am J Physiol*, Vol. 271, pp. 216–222, 1996.

63. Cho, M., T. K. Hunt, and M. Z. Hussain, "Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release," *Am J Physiol Heart Circ Physiol*, Vol. 280, pp. 2357–2363, 2001.

64. Kuzyk, P. R., and E. H. Schemitsch, "The science of electrical stimulation therapy for fracture healing," *Indian J Orthop*, Vol. 43, no. 2, pp. 127–131, 2009.

65. Aaron, R. K., D. Ciombor, and B. Simon, "Treatment of nonunions with electric and electromagnetic fields," *Clin Orthop Relat Res*, Vol. 419, pp. 21–29, 2004.

66. Aaron, R. K., B. D. Boyan, D. M. Ciombor, Z. Schwartz, and B. J. Simon, "Stimulation of growth factor synthesis by electric and electromagnetic fields," *Clin Orthop Relat Res*, Vol. 419, pp. 30–37, 2004.

67. Lorich, D. G., C. T. Brighton, R. Gupta, J. R. Corsetti, S. E. Levine, I. D. Gelb, R. Seldes, and S. R. Pollack, "Biochemical pathway mediating the response of bone cells to capacitive coupling," *Clin Orthop Relat Res*, Vol. 350, pp. 246–256, 1998.

68. Wang, Z., C. C. Clark, and C. T. Brighton, "Up-regulation of bone morphogenetic proteins in cultured murine bone cells with use of specific electric fields," *J Bone Joint Surg Am*, Vol. 88, pp. 1053–1065, 2006.

69. Fitzsimmons, R. J., J. T. Ryaby, F. P. Magee, and D. J. Baylink, "Combined magnetic fields increased net calcium flux in bone cells," *Calcif Tissue Int*, Vol. 55, no. 5, pp. 376–380, 1994.

70. Claes, L., and B. Willie, "The enhancement of bone regeneration by ultrasound," *Prog Biophys Mol Biol*, Vol. 93, no. 1–3, pp. 384–398, 2007.

71. Azuma, Y., M. Ito, Y. Harada, H. Takagi, T. Ohta, and S. Jingushi, "Low-intensity pulsed ultrasound accelerates rat femoral fracture healing by acting on the various cellular reactions in the fracture callus," *J Bone Miner Res*, Vol. 16, no. 4, pp. 671–680, 2001.

72. Rubin, C., M. Bolander, J. P. Ryaby, and M. Hadjiargyrou, "The use of low intensity ultrasound to accelerate the healing of fractures," *J Bone Joint Surg Am*, Vol. 83, pp. 259–270, 2001.

73. Rutten, S., P. A. Nolte, C. M. Korstjens, and J. Klein-Nulend, "Low-intensity pulsed ultrasound affects runx2 immunopositive osteogenic cells in delayed clinical fracture healing," *Bone*, Vol. 45, no. 5, pp. 862–869, 2009.

74. Giannoudis, P., "Fracture healing and bone regeneration: Autologous bone grafting or bmps?," *Injury, Int J Care Injured*, Vol. 40, pp. 1243–1244, 2009.

75. Goulet, J. A., L. E. Senunas, G. L. DeSilva, and M. L. Greenfield, "Autogenous iliac crest bone graft complications and functional assessment," *Clin Orthop Relat Res*, Vol. 339, pp. 76–81, 1997.

76. Davies, S. D., and M. W. Ochs, "Bone morphogenetic proteins in craniomaxillofacial surgery," *Oral Maxillofac Surg Clin North Am*, Vol. 22, no. 1, pp. 17–31, 2010.

77. Blokhuis, T. J., and T. Lindner, "Allograft and bone morphogenetic proteins: an overview," *Injury*, Vol. 39, no. 2, pp. 33–36, 2008.

78. Papathanasopoulos, A., and P. V. Giannoudis, "Biological considerations of mesenchymal stem cells and endothelial progenitor cells," *Injury*, Vol. 39, no. Suppl 2, pp. 21–32, 2008.

79. Salkeld, S. L., L. P. Patron, R. L. Barrack, and S. D. Cook, "The effect of osteogenic protein-1 on the healing of segmental bone defects treated with autograft or allograft bone," *J Bone Joint Surg Am*, Vol. 83, no. 6, pp. 803–816, 2001.

80. Lee, F. Y., S. Storer, E. J. Hazan, M. C. Gebhardt, and H. J. Mankin, "Repair of bone allograft fracture using bone morphogenetic protein-2," *Clin Orthop Relat Res*, Vol. 397, pp. 119–126, 2002.

81. An, Y. H., R. J. Friedman, and R. A. Draughn, *Animal models of bone fracture or osteotomy*, pp. 197–217. Animal Models in Orthopaedic Research, Boca Raton: CRC Press, 1999.

82. Warden, S. J., R. K. Fuchs, C. K. Kessler, K. G. Avin, R. E. Cardinal, and R. L. Stewart, "Ultrasound produced by a conventional therapeutic ultrasound unit accelerates fracture repair," *Phys Ther*, Vol. 86, pp. 1118–1127, 2006.

83. Huo, M. H., N. W. Troiano, R. R. Pelker, C. M. Gundberg, and G. E. Friedlaender, "The influence of ibuprofen on fracture repair: biomechanical, biochemical, histologic and histomorphometric parameters in rats," *J Orthop Res*, Vol. 9, pp. 383–390, 1991.

84. Link, T. M., S. Majumdar, W. Konermann, N. Meier, J. C. Lin, D. Newitt, X. Ouyang, P. E. Peters, and H. K. Genant, "Texture analysis of direct magnification radiographs of vertebral specimens: correlation with bone mineral density and biomechanical properties," *Acad Radiol*, Vol. 4, no. 3, pp. 167–176, 1997.

85. Gumerman, L. W., S. R. Fogel, M. A. Goodman, E. N. H. Jr, G. S. Kappakas, R. Rutkowski, and G. Levine, "Experimental fracture healing: evaluation using radionuclide bone imaging concise communication," *J Nucl Med*, Vol. 19, no. 12, pp. 1320–1323, 1978.

86. Muheim, G., "Assessment of fracture healing in man by serial 87m strontium-scintimetry," *Acta Orthop Scand*, Vol. 44, pp. 621–627, 1973.

87. Brooks, M. E., *The Skeletal System*, pp. 143–162. Practical Nuclear Medicine Third Edition, London: Springer-Verlag Ltd, 2005.

88. Sano, H., H. K. Uhthoff, D. S. Backman, and A. Yeadon, "Correlation of radiographic measurements with biomechanical test results," *Clin Orthop Relat Res*, Vol. 368, pp. 271–278, 1999.

89. Markel, M. D., and E. Y. Chao, "Noninvasive monitoring techniques for quantitative description of callus mineral content and mechanical properties," *Clin Orthop Relat Res*, Vol. 293, pp. 37–45, 1993.

90. Cullum, I. D., P. J. Ell, and J. P. Ryder, "X-ray dual-photon absorptiometry," *Br J Radiol*, Vol. 62, pp. 587–592, 1989.

91. Blokhuis, T. J., F. C. den Boer, J. A. Bramer, A. van Lingen, J. C. Roos, F. C. Bakker, P. Patka, and H. J. Haarman, "Evaluation of strength of healing fractures with dual energy xray absorptiometry," *Clin Orthop Relat Res*, Vol. 380, pp. 260–268, 2000.

92. Kokoroghiannis, C., I. Charopoulos, G. Lyritis, P. Raptou, T. Karachalios, and N. Papaioannou, "Correlation of pqct bone strength index with mechanical testing in distraction osteogenesis," *Bone*, Vol. 45, no. 3, pp. 512–516, 2009.

93. Rüegsegger, P., U. Elsasser, M. Anliker, H. Gnehm, H. Kind, and A. Prader, "Quantification of bone mineralization using computed tomography," *Radiology*, Vol. 121, no. 1, pp. 93–97, 1976.

94. Somford, M. P., G. F. Geurts, J. W. den Teuling, B. J. Thomassen, and W. F. Draijer, "Long term alendronate use not without consequences?," *Int J Rheumatol*, Vol. 2009, p. 253432, 2009.

95. Neviaser, A. S., J. M. Lane, B. A. Lenart, F. Edobor-Osula, and D. G. Lorich, "Low-energy femoral shaft fractures associated with alendronate use," *J Orthop Trauma*, Vol. 22, no. 5, pp. 346–350, 2008.

96. Kwek, E. B. K., S. K. Goh, J. S. B. Koh, M. A. Png, and T. S. Howe, "An emerging pattern of subtrochanteric stress fractures: a long-term complication of alendronate therapy?," *Injury*, Vol. 39, no. 2, pp. 224–231, 2008.

97. Markbreiter, L. A., R. R. Pelker, G. E. Friedlaender, R. Peschel, and M. M. Panjabi, "The effect of radiation on the fracture repair process a biomechanical evaluation of a closed fracture in a rat model," *J Orthop Res*, Vol. 7, no. 2, pp. 178–183, 1989.

98. Pelker, R. R., and G. E. Friedlaender, "The nicolas andry award-1995 fracture healing radiation induced alterations," *Clin Orthop Relat Res*, Vol. 341, pp. 267–282, 1997.

99. Barnhard, H. J., and R. W. Geyer, "Effects of x-radiation on growing bone a preliminary report," *Radiology*, Vol. 78, pp. 207–214, 1962.

100. Riseborough, E. J., S. L. Grabias, R. I. Burton, and N. Jaffe, "Skeletal alterations following irradiation for wilms' tumor: with particular reference to scoliosis and kyphosis," *J Bone Joint Surg Am*, Vol. 58, no. 4, pp. 526–536, 1976.

101. Weatherby, R. P., D. C. Dahlin, and J. C. Ivins, "Postradiation sarcoma of bone: review of 78 mayo clinic cases," *Mayo Clin Proc*, Vol. 56, no. 5, pp. 294–306, 1981.

102. Phemister, D. B., "Radiation necrosis of bone," *Am J Roentgenol*, Vol. 16, pp. 340–348, 1926.

103. Hu, W. W., B. B. Ward, Z. Wang, and P. H. Krebsbach, "Bone regeneration in defects compromised by radiotherapy," *J Dent Res*, Vol. 89, no. 1, pp. 77–81, 2010.

104. Ward, J. F., "Dna damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability," *Prog Nucl Acid Res Mol Biol*, Vol. 35, pp. 95–96, 1988.

105. Bolus, N. E., "Basic review of radiation biology and terminology," *J Nucl Med Technol*, Vol. 29, pp. 67–77, 2001.

106. Ernest, M., M. E. Freed, and A. J. Zametkin, "Health hazards of radiation exposure in the context of brain imaging research: special consideration for children," *J Nucl Med*, Vol. 39, pp. 689–698, 1996.

107. Okunieff, P., X. Wang, P. Rubin, J. N. Finkelstein, L. S. Constine, and I. Ding, "Radiation-induced changes in bone perfusion and angiogenesis," *Int J Radiat Oncol Biol Phys*, Vol. 42, no. 4, pp. 885–889, 1998.

108. Hong, S. I., S. K. Hong, J. M. Wallace, and D. H. Kohn, "Ultrastructural observation of electron irradiation damage of lamellar bone," *J Mater Sci Mater Med*, Vol. 20, pp. 959–965, 2009.

109. Hopewell, J. W., "Radiation-therapy effects on bone density," *Med Pediatr Oncol*, Vol. 41, no. 3, pp. 208–211, 2003.

110. Elgazzar, A. H., and N. Kazem, *Biological effects of ionizing radiation*, pp. 540–547. The Pathophysiologic Basis of Nuclear Medicine, Berlin: Springer Verlag, 2006.

111. Johansson, L., "Hormesis, an update of the present position," *Eur J Nucl Med Mol Imaging*, Vol. 30, pp. 921–933, 2003.

112. Feinendegen, L. E., "Evidence for beneficial low level radiation effects and radiation hormesis," *Br J Radiol*, Vol. 78, no. 925, pp. 3–7, 2005.

113. Trueta, J., and A. J. Buhr, "The vascular contribution to osteogenesis v the vasculature supplying the epiphyseal cartilage in rachitic rats," *J Bone Joint Surg Br*, Vol. 45, pp. 572–581, 1963.

114. Gerber, H., and N. Ferrara, "Angiogenesis and bone growth," *Trends Cardiovasc Med*, Vol. 10, no. 5, pp. 223–228, 2000.

115. Kanczler, J. M., and R. O. Oreffo, "Osteogenesis and angiogenesis the potential for engineering bone," *Eur Cell Mater*, Vol. 15, pp. 100–114, 2008.

116. Gerber, H. P., T. H. Vu, A. M. Ryan, J. Kowalski, Z. Werb, and N. Ferrara, "Vegf couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation," *Nat Med*, Vol. 5, pp. 623–628, 1999.

117. Keramaris, N. C., G. M. Calori, V. S. Nikolaou, E. H. Schemitsch, and P. V. Giannoudis, "Fracture vascularity and bone healing: a systematic review of the role of vegf," *Injury*, Vol. 39, no. S2, pp. S45–57, 2008.

118. Eckardt, H., M. Ding, M. Lind, E. S. Hansen, K. S. Christensen, and I. Hvid, "Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model," *J Bone Joint Surg Br*, Vol. 87, pp. 1434–1438, 2005.

119. Hausman, M. R., M. B. Schaffler, and R. J. Majeska, "Prevention of fracture healing in rats by an inhibitor of angiogenesis," *Bone*, Vol. 29, pp. 560–564, 2001.

120. Ferrara, N., and H. P. Gerber, "The role of vascular endothelial growth factor in angiogenesis," *Acta Haematol*, Vol. 106, pp. 148–156, 2001.

121. Kaigler, D., Z. Wang, K. Horger, D. J. Mooney, and P. H. Krebsbach, "Vegf scaffolds enhance angiogenesis and bone regeneration in irradiated osseous defects," *J Bone Miner Res*, Vol. 21, no. 5, pp. 735–744, 2006.

122. Dudziak, M. E., P. B. Saadeh, B. J. Mehrara, D. S. Steinbrech, J. A. Greenwald, G. K. Gittes, and M. T. Longaker, "The effects of ionizing radiation on osteoblast-like cells in vitro," *Plast Reconstr Surg*, Vol. 106, no. 5, pp. 1049–1061, 2000.

123. Steinbrech, D. S., B. J. Mehrara, P. B. Saadeh, J. A. Greenwald, J. A. Spector, G. K. Gittes, and M. T. Longaker, "Vegf expression in an osteoblast-like cell line is regulated by a hypoxia response mechanism," *Am J Physiol Cell Physiol*, Vol. 278, no. 4, pp. C853–860, 2000.

124. Semenza, G. L., "Hypoxia-inducible factor 1 : oxygen homeostasis and disease pathophysiology," *Trends Mol Med*, Vol. 7, pp. 345–350, 2001.

125. Wang, Y., C. Wan, L. Deng, X. Liu, X. Cao, S. R. Gilbert, M. L. Bouxsein, M. Faugere, R. E. Guldberg, L. C. Gerstenfeld, V. H. Haase, R. S. Johnson, E. Schipani, and T. L. Clemens, "The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development," *J Clin Invest*, Vol. 117, pp. 1616–1626, 2007.

126. Heissig, B., S. Rafii, H. Akiyama, Y. Ohki, Y. Sato, T. Rafael, Z. Zhu, D. Hicklin, K. Okumura, H. Ogawa, Z. Werb, and K. Hattori, "Low-dose irradiation promotes tissue revascularization through vegf release from mast cells and mmp-9-mediated progenitor cell mobilization," *J Exp Med*, Vol. 19, no. 6, pp. 739–750, 2005.

127. Potier, E., E. Ferreira, R. Andriamanalijaona, J. P. Pujol, K. Oudina, D. Logeart-Avramoglou, and H. Petite, "Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression," *Bone*, Vol. 40, pp. 1078–1087, 2007.

128. Turner, C. H., and D. B. Burr, "Basic biomechanical measurements of bone: a tutorial," *Bone*, Vol. 14, pp. 595–608, 1993.