BIOMECHANICAL EVALUATION OF EFFECTS OF ESTROGEN, SELECTIVE ESTROGEN RECEPTOR MODULATOR DRUGS AND VITAMIN K2 ON OSTEOPOROTIC BONE

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

BIOMECHANICAL EVALUATION OF EFFECTS OF ESTROGEN, SELECTIVE ESTROGEN RECEPTOR MODULATOR DRUGS AND VITAMIN K2 ON OSTEOPOROTIC BONE

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In this study different bioactive agents were used to investigate their single and combined effects on biomechanical properties of osteoporotic bone.
Estrogen, the most common hormon replacement therapy (HRT) agent, was used in single and combined with raloxifen, a well known osteoporosis drug. Despite their high clinical uses, they have not been tried before, in combination. They act as agonist of each other in bone and antagonist of each other in uterus and mammary glands. Hence it was expected to prevent HRT side effects by using combinations while enhancing the healing on osteoporotic bone. So, the study was designed to see the interaction effects of these two agents on bone and uterus, to observe the mechanical behaviour upto fracture, and to investigate the bone mechanical properties by strain gauges and bending theory with ovariectomized rat model.

Second approach to osteoporosis treatment, VitK2 was chosen to be used alone or in combination with raloxifen in same model. Although recent studies mentioned the effects of VitK2 on bone, its rebuilding or repair effect was not

completely established. So, VitK2-bone relation was aimed to be clarified with the project.VitK2 raloxifen combination was also a new study, that has not been carried out so far.

As a result of mechanical tests, it was found that E+R combination is the most effective treatment. All treatment's were resulted in numerically (though not statistically significant) higher values on femur mechanical properties, and significantly better on tibia compared to the untreated controls. VitK2 performs well in energy absorption upto fracture, but worse in others (PL, YL etc.) compared to other treatments indicating that it plays a specific role in modifying bone structure thus, rendering bone stronger under high stress. However, similar to estrogen case, its combination with raloxifen performs better than its individual administration. With combinations it was aimed to reduce the adverse effects of estrogen on uterus and mammary glands by using raloxifen. This idea appears to be achieved with better histological results of uterus in combinations than estrogen groups. Additionally it was observed that direct strain data obtained by strain gauge experiments can be more informative than theoretical model in calculating modulus of elasticity, and shown that shear contribution can be neglected if depth/span ratio and set up dimensions properly chosen.

Biochemical analysis of the blood showed an increment in bone formation (ALP activity) compared to both controls. ALP activity was the highest in R group, which was lower in combinations. Thus existence of a different mechanism in osteoporotic bone repair in combinations was suggested .

Keywords: Osteoporosis, Bone Biomechanics, Raloxifen, Estrogen Therapy, Vitamin K2, Mechanical Strain

ÖZ

ÖSTROJEN VE SEÇİCİ ÖSTROJEN RESEPTÖRÜ MODÜLATÖR İLAÇLARIN VE K2 VİTAMİNİNİN OSTEOPOROTİK KEMİĞE ETKİLERİNİN BİYOMEKANİK ÖZELLİKLER YÖNÜNDEN DEĞERLENDİRİLMESİ

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Bu projede, biyoaktif ajanların tek ve kombine kullanımları sonucunda osteoporotik kemikteki biyomekanik özelliklere etkileri araştırılmaktadır. En yaygın hormon replasman tedavisi (HRT) ajanlarından biri olan östrojen, tek başına ve osteoporoz tedavisinde yaygın olarak kullanılan raloksifen ile beraber kullanılmıştır. Klinikte çok tercih edilmelerine rağmen bu iki ilacın birlikte etkisi klinik veya deneysel olarak daha önce araştırılmamıştır. İki ilacın kemikte agonist göğüs ve rahimde ise antagonist etkide oldukları bilinmektedir. Bu nedenle HRT'de rastlanan yan etkileri engellemek ve osteoporotik kemikte iyileşmeyi arttırmak amacıyla bu ilaçlar kombine olarak uygulanmıştır. İlaçların kemik ve rahimdeki etkileşimlerinin sonuçlarını görmek, kemiğin çatlağa kadarki mekanik davranışını gözlemlemek, birim deformasyon ölçme uniteleri ve eğilme teorisi yardımıyla kemiğin mekanik özelliklerini araştırmak üzere sıçan ovariektomi modeli ile osteoporoz çalışması düzenlenmiştir.

Osteoporoz tedavisine diğer bir yeni uygulama olarak, VitK2'nin tek başına ve yine raloksifenle kombine kullanımı aynı modelle araştırılmıştır.

Yakın zamanda VitK2'nin osteoporoz ve kemik yapısına etkileri konusunda pek çok araştırmalar yapılmış olmasına rağmen, bu vitaminin kemikte yeniden yapılanma ve onarım özellikleri tamamen belirlenememiştir. Projeyle, bu

kesinleşmemiş konu hakkında araştırmalara katkıda bulunmak amaçlanmıştır. VitK2 ve raloksifenin birlikte kullanımı da henüz çalışılmamış yeni bir araştırmadır.

Mekanik test sonuçlarına göre, Ö+R kombinasyonunun en etkin tedavi olduğu, ve bütün tedavilerin (femurda istatistiksel anlamlı olmamakla beraber) kontrollerden yüksek mekanik özellikleri olduğu görülmüştür. Aynı sonuçlar, tibiada istatistiksel olarak anlamlı elde edilmiştir. VitK2'nin kırılmaya kadarki enerji emiliminin yüksek, orantılı yük ve akma yükü gibi mekaniksel özelliklerde ise diğer gruplardan düşük olduğu gözlenmiştir. Bu sonuç VitK2 nin kemik yapısını değiştirmede özel bir rolü olduğunu ve kemiğin yüksek gerilme koşullarında daha güçlü hale geldiğini göstermektedir. Ancak östrojende olduğu gibi VitK2 ninde raloksifenle beraber kullanımında tek kullanımından daha iyi bir performans sergilediği görülmüştür. Projede ilaç kombinasyon çalışmalarıyla östrojenin rahimde olumsuz etkilerini azaltmak amacına kombinasyonla östrojene göre daha iyi histolojik verilerin elde edilmesiyle ulaşılmıştır. Buna ek olarak birim deformasyon uniteleriyle yapılan deformasyon ölçümünün elastisite modülü gibi bazı mekanik özelikleri hesaplamada teorik modelden daha bilgilendirici olduğu gözlenmiş, kesme etkisinin uygun derinlik/uzunluk ve düzenek boyutları seçilmesi durumunda ihmal edilebileceği gösterilmiştir. Kan örneklerinin biyokimyasal analizleri kemik oluşumunun (ALP aktivitesi) iki kontrolden de fazla olduğunu göstermiştir. Kombinasyonlarda düşük olan ALP aktivitesi en yüksek raloksifen grubunda bulunmuştur. Böylece kombinasyon gruplarında osteoporozdaki kemik onarımının farklı bir mekanizmayla gerçekleştiğini önermektedir.

Anahtar Kelimeler: Osteoporoz, Kemik Biyomekaniği, Raloksifen, Östrojen Tedavisi, K2 Vitamini, Mekanik Birim Deformasyon

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Rodin, “ Je suis fatigué, très fatigué “

Camille, “ Entrez… Vous y sentiriez reposé et rafraichi “

Entre, S’il vous plaît

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

LIST OF ABBREVIATIONS

BMD : Bone Mineral Density

DEXA : Dual Energy X-Ray Absorptiometry

E : Estrogen

HRT : Hormone Replacement Therapy

OVX : Ovariectomized

PBS : Phosphate Buffered Saline

R : Raloxifen

SC : Subcutanous injection

SERM : Selective Estrogen Receptor Modulator

VITK2 : VitaminK2

TPB : Three Point Bending

AMA : American Medical Association

WHI : Women Health Initiative

CHAPTER 1

INTRODUCTION

1.2. Definition of Osteoporosis

Osteoporosis is a greek word meaning porous bone. It is known as a silent disease since it has no symptoms while processing in the skeleton. This process consequently leads to low bone mass which makes the bone more fragile and more susceptible to fractures. Fractures are the results of osteoporosis. Fracture occurence is correlated with the degree of bone destruction progressed throughout the years from the disease has started.

While osteoporosis is mostly seen in women (80 %), it can occur at any age and gender depending on some risk factors. In America, there are 10 million diagnosed osteoporotic patients and other 18 milion is carrying the risk due to their low bone mass values. The annual number of cases resulting in an osteoporotic fracture is 1,5 milion. The treatment and care expenditures of these fractures reaches to 13,8 bilion dolars for each year. Half of the patients over 50 years of age experience osteoporotic fracture at least once during their life times (http://www.osteo.org/osteofastfact.html).

Osteoporosis can lead to chronic pain besides fractures, that may cause patients to avoid to join daily activities. Thus complicating the life of the patient with depression, loss of indepence and self-esteem owing to the social withdrawal (Green, 2001).

About 50 % of women having osteoporotic fracture require long term care. During this period patient has to be immobilized. This immobilization can lead to other metabolic illnesses like emboli formation. As a result, 20 % of the women having osteoporotic hip fracture die during this long term care.

Since it has no visible symtomps before serious fractures it is very important to determine the risk factors and diagnose the people who are under the risk of osteoporosis.

1.2. Pathophysiology of Osteoporosis

Bone formation is a complicated process that is interrelated with a wide range of factors from both inner body sources such as hormones, genetics, or some other diseases that patient may have and outer sources as mechanical damage. Healthy bone is continuously being absorped and rebuilt with so called 'remodelling' process. Bone tissue is under the exposure of excessive stress during daily activities. This makes the remodelling an important and necessary process (AMA, 2001). These stresses causing microcracks or microdamages in bone are demolished during absorption, and the damaged tissue is replaced with new healty bone tissue at the end of the rebuilding process. Specific types of bone cells are involved in remodelling: While osteoclasts carry out the absorping task, osteoblasts do the rebuilding. Excessive osteoclast or insufficient osteoblast number causes improper filling of resorption site and this leads to low bone mass.

Bone also functions in storing and supplying the body's calcium as required. Calcium and phosporus in extracellular matrix serves as regulators in remodelling process.

Body estrogen stimulates the receptors located on osteoblasts thus enhancing the bone formation. Parathyroid hormone and vitamin D are the other stimulator and regulators respectively.

1.2.1. Risk Factors

Women in their postmenopausal stage are in the highest risk group of osteoporosis (Figure 1.1). Because in either natural or operational way, menopause significantly decreases the estrogen level in the body, consequently decreasing the rate of bone formation, and leading to bone loss.

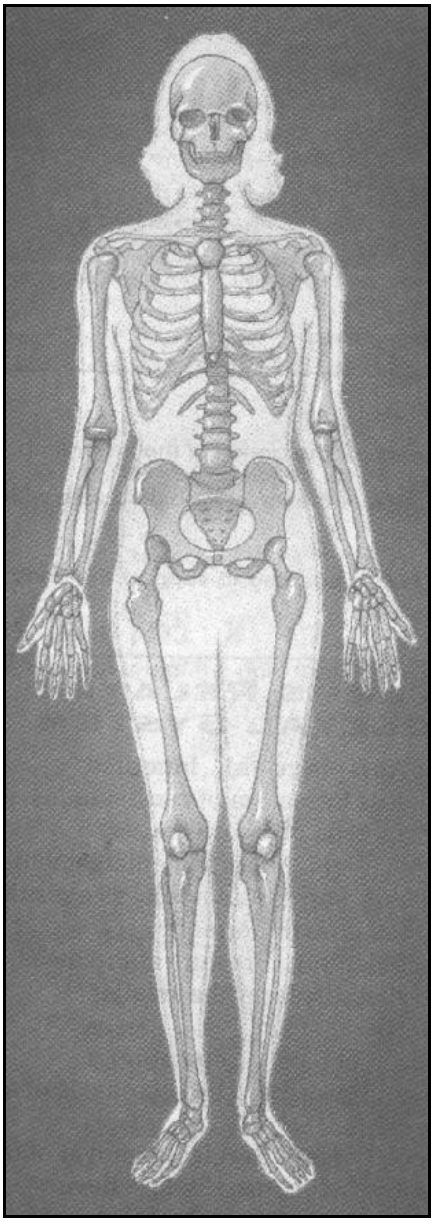


Figure 1.1. Human Skeleton (Shier et. al., 1996)

In the past it is thought that estrogen or hormone replacement therapies (ERT, HRT) can both treat and prevent osteoporosis. After some researches it was found that by decreasing the rate of bone loss, these therapies prevent the illness but there is still no evidence that they can treat it. Additionally, researchers of the Women Health Initiative Study decided to quit their study in 2002 (about 3 years earlier than the finishing date), and recommended alternative treatments, because of the increasing breast cancer risk of women who are taking HRT in the study (WHI, 2002). After that, FDA has changed the labels of HRT drugs from treatment and preventive to preventive usage only (Davidson et. al., 2002).

1.2.2. Personal Medical and Family History

The state of having a fracture history of herself or from her family can increase the risk of osteoporosis. Hormonal illnesses like hyperparathyroidism can lead to problems in bone formation process, and therefore they should be screened for the possibility of osteoporosis.

Women having metabolic (osteomalacia) or neoplastic disorders (myeloma) are also having risks (Grene ,2001)(AMA, 2001). Other risk factors are listed in Table 1.1.

On the other hand there are some cases which can be protective against this illness, like high parity, regular exercise, large body habitus (Johnson et. al., 2000). Total duration of breast feeding is not correlated with bone density. Extended lactation period and multiple pregnancies does not increase the osteoporosis risk. Altough bone mineral density and calcium in body decreases during lactation, the loss normalizes at the end of this period. Plus some studies showed that parity itself is a protective against osteoporosis: bone mineral densities increase proportionally with the number of parity in women upto the age of 69. On the other hand osteoporotic fractures occured more frequently in women over age 70 and in nulliparous women (Jones et. al., 1999). So, in some cases clinican may recommend pregnancy instead of HRT since pregnancy is a high estrogen state.

Age, as expected, is the most important factor in osteoporosis. With aging, many problems arise in the body like natural menopause, poor health dementia,

eyesight problems and they may lead to some accidents resulting in fractures (Grene 2001), (Grossman et. al., 2002).

Table 1.1. Risk Factors of Osteoporosis

MODIFIABLE FACTORS	UNMODIFIABLE FACTORS
Smoking	Personal fracture history
Excessive alcohol consumption	Fracture history in family
Glucocorticoids, anticonvulsants usage	Race (Caucasian,Asian)
Low Ca,Vitamin D diet	Elder age
Estrogen lacking	Dementia
Low body weight (<43 kg)	History of organ transplants
Sedentary lifestyle	Connective tissue disorders
Environmental risks (dark stairs)	Hormonal disorders

There are also some pharmocologic risk factors for bone loss (Sachs, 2001)(Clowes et. al., 2001): Phenytoin sodium (Dilantin) –an anticonvulsant- can lead to osteoporosis in its long term usage.

Glucocorticoids reduce mineralization, mineral apposition rate and osteoblastic activity. High doses can cause loss in trabecular connectivity and fractures may occur in vertebrae and pelvis. These drugs also decrease vitamin D uptake in intestine so indirectly increases the renal calcium secretion (Davidson et. al., 2002).

1.2.3. Physical Examination: Signs and Symptoms

As the disease progresses, some symptoms may come out like; back pain, height loss, neck strain, fractures, midabdominal pain because of ribs resting on iliac crest, forshortened waist that can be recognized with difficulty in finding proper fit clothes etc.(Johnson et. al., 2000). Height loss up to 2 in. can be seen (Cauley et. al., 2001). These physical changes cause the body's center of gravity to

orient to a more forward position (Johnson et. al., 2000). Hence balance problems and consequent accidents resulting with fractures can occur as the disease progress.

1.2.3.1. Who Should Take Diagnostic Test?

The USA National Osteoporosis Foundation constituted some groups needing test: Postmenopausal women below 65 and having one or more risk factors in addition to postmenopausal status, postmenopausal women having fractures, women above age of 65, women taking osteoporosis treatment, women taking HRT for a long time, women having other risk factors (having fracture history, low weight, smoking, taking causative drugs) are recommended for screening.

There are various kinds of scales being used in screening. One of them is the ”simple calculated osteoporosis risk estimation”, (SCORE), and it is described in USA National Osteoporosis Foundation guidelines. These scales consider the risk factors and then assign a risk score accordingly to the patients. In order to have a proper diagnose, bone mineral density measurements are asked from the people having critical scores.

1.2.4. Bone Density Measurements

Bone density tests are standard tests being used in the diagnosis of osteoporosis (Table 1.2). Measurement can be done either at central or peripheral bone sites. Dual energy X-ray absorptiometry (DEXA) and quantitative computed microtomography (QCT) measurements are done from central site. DEXA is the gold standard in bone density measurements. It is mainly applicable to hip and spine (Davidson et. al., 2002)(Cadarette et. al., 2001). QCT directly measures the 3D bone density that is known as the most sensitive method as it can distinguish different tissues of bone. However due to its expensiveness, uneffectiveness in serial usage and high radiation exposure property, it is not frequently used. Other measurement methods are peripheral dual energy absorptiometry, ultrasonometry, single energy X-ray absorptiometry and single photon absorptiometry. Among all measurement methods, single energy X-ray absorptiometry and single photon absorptiometry use single energy source in detection, and hence can not distinguish bone and tissue.

Therefore they are only applicable to wrist, heel and to some huge number of screening situations. As they are not accurate, they only give a general idea of risk and they are generally replaced with DEXA. Ultrasound is the newest technique that is not widely used but highly inspired by the researches. Being the oldest technique, radiography is no longer used for this purpose.

BMD testing compares the patient's BMD measurement with young man's BMD value. The reason of doing this comparison with 20 year old man is that at this age human reaches its peak bone density. The standard deviation (SD) of the patient's BMD result from the mean is computed. If patient's BMD deviates towards below the mean value, (ie. SD is negative) then this number is called as T score.

Table 1.2. Various Screening Methods

	ADVANTAGES	DISADVANTAGES
Dual energy X -ray absorptiometry (DEXA)	Low amount of radiation exposure, fast, effective in serial applications, inexpensive, regularly updated with new softwares	Osteoarthrisis may cause an overestimated result, should be corrected by an expertised operator
Quantitative computarized tomography (QCT)	Most sensitive, can make 3d analysis, many application sites	Large amount of radiation exposure, uneffective in serial applications, expensive
Heel ultrasound (HUS)	No radiation exposure , cheapest, fast, porTable, usable in office environment	Limited application sites, limited accuracy

Table 1.2 (continued)

x- ray	cheap, effective in serial screening applications	oldest technique, limited application areas, radiation exposure,can give a general idea if there is a huge amount of bone loss like 40-50%
physical examination	no radiation exposure, fast, can directly suuply ideas about the risk factors	does not give a quantity value to make comparison, not accurate and may lead to wrong diagnosis if the physician is not expertised.
Chemical evaluation of serum and bone markers	reveals the metabolic sources of osteoporosis if there is any	High degree of biologic variability

In calculating Z score, unlikely from T score, comparison is made with the mean of a human belonging to same age and gender group (ex.gender:female,age:47). However Z score is not used for diagnosis since the mean score reduces with aging. T score is used by clinicians for diagnose. T score critization is done by World Health Organization, WHO (Table1.3).

If T score deviates from the healty young man's BMD by a magnitude of 1 or less than 1, it is accepted as normal. If SD is between 1 and 2.5, this bone has low bone mass namely osteopenia. If it deviates from the mean by a magnitude larger than 2.5, then this bone can be diagnosed as osteoporotic bone. Actually in many cases, osteopenia is the level before the diagnostic osteoporosis. Hence this condition should also be followed in future.

Table 1.3. WHO Evaluation for BMD Screening

SITUATION	T SCORE	STANDARD DEVIATION (SD)
Normal	T > -1	Less than 1
Osteopenia (low bone mass)	-2.5 < T < -1	Between 1 and 2.5
Osteoporosis	T < -2.5	More than 2.5
Serious osteoporosis	{T< -2.5 } + {having 1 or more fractures}	More than 2.5 + having one or more fractures

1.2.5. Nonpharmocologic Treatment

Changing the lifestyle is the first step for the prevention of osteoporosis. Walking programs, low intensity aerobics, gardening and jogging strengths the bones and muscles also improving balance, coordination, reaction times, gait, and flexibility in old patients (NOF, 1998). More intense work out (running, gymnastics), however, stimulates osteogenic formations (North American Menopause Society Management, 2002).

Precautions should be taken to minimize the fracture risks. Home safety should be increased in order to prevent home accidents, since intestinal calcium reabsorption is reduced to 50 % after the age of 65.

Renal enzymatic activities also decreased which affects calcium level in the body, nutrition should be balanced with application of calcium and vitamin D rich diets. Calcium can be obtained from food sources like yogurt, milk, cheese, fish, ice cream, and green vegetables. There are also non diary calcium sources (soybean, orange, brokoli etc.) for women who do not consume diary products.

1.2.6. Pharmocologic Interventions

Treatments approved by FDA involve hormone replacement therapies, vitamin/mineral supplementations, selective estrogen receptor modulators, bisphosphonates and calcitonin.

1.2.6.1. Calcium Treatment

Researches indicate that calcium reinforcement decreases the bone loss and fracture rate in postmenopausal women (Skolnick et. al., 1993). The most common side effect is gastrointestinal disturbances, hypercalcemia and hypercalciuria. Calcium intake should be carefully controlled by women having hypoparathyroidism.

Women having gastrointestinal side effects of calcium like flatus, bloating or constipation may use another source of calcium. Microcrystalline hydroxyapatite is the best absorbed calcium source. It is the only complete bone supplement and it increases bone mass. Other types do not involve all of the minerals required for a healty bone.

1.2.6.2. Estrogen/ Hormone Replacement Therapy (ERT/ HRT)

ERT and HRT are the first accepted therapies both in the treatment and prevention of osteoporosis. Cauley's 6 year study on women above age 65 has demonstrated that ERT/ HRT reduces the relative risk factor by an amount of 0.39 in wrist, and 0.66 in non vertebral fractures respectively compared to the group having no replacement therapy. Moreover, users started the replacement therapy 2 years before the menopause and continued 5 years after the menopause had decreased in wrist and hip fractures risk by 0.29 units, and non vertebral fractures by 0.50 units. Additionally, women who started the same treatment 5 years after the menopause had no significant difference. But researches indicate that even they prevent bone loss, they do not have a satisfactory performance in treatment leg of this illness. Moreover, in 2002 Women Health Initiative stopped their long term study 3 years before its planned finishing time, eventhough the fracture risk of

subjects had dropped by one third with respect to the placebo group. Reason for that was the high and various amounts of adverse cases like 286 heart disease, 290 breast cancer, 212 stroke, and 101 pulmonary embolism out of 16680 women (WHI, 2002). Thus the members of the study recommended not to use HRT in long term and warned the providers not to prescribe these drug combinations as for both prevention and treatment of osteoporosis.

1.2.6.3. Bisphosphonates

These drugs increase hip and spine bone density, reduces bone loss and fracture risks in spine and hip by preventing osteoclast activity on bone surface thus, interfere with the resorption process. (Davidson et. al., 2002) (Johnson et. al., 2000) (Schnitzer et. al., 2000)

Especially, after the latest results on HRT treatments women inclined to use bisphosphonates. Examples of bisphophonate drugs can be given as alendronate (Fosamax®), risedronate (Actonel®), and etidronate (Didronel®).

Calcium, aluminum, magnesium by having anti-acids properties improve the effectiveness of bisphosphonates if taken 2 hours before them. The administration of bisphosphonates has some specifications as: It has to be taken 60 minutes before the meals with an amount of 190 grams of water. Also keeping the stand up position for 30 minutes after the administration is a must. These drugs are contraindicated with hypocalcemia, esophageal abnormalities and renal insufficients. Other side effects of bisphosphonates are abdominal/musculoskeletal pain, nausea, heartburn, esophagus irritation (Schnitzer et. al., 2000) (Lobo et. al., 2000).

The results of the multiple studies indicate that even bisphosphonates seems to be the best alternative in osteoporosis treatment, HRT‘s preventive effect for hip/vertebral fractures is more pronounced than the effect of bisphosphonates. Long term adverse effects of bisphosphonates have not known yet. They cost twice as more as estrogen therapies do (Michele, 2003).

1.2.6.4. Calcitonin

Calcitonin blocks the osteoclasts hence interfering with the bone resorption process (Schnitzer et. al., 2000) (Lobo et. al., 2000). Studies on calcitonin indicate that the number of bone turnover markers and bone density increases or becomes stationary after calcitonin usage. Especially it is known to increase the lumbar spine density at significantly a significant level. However, the reduction in vertebral or nonvertebral fracture risks are not found to be significant.

Calcitonin, being a hormone, is not recommended for breast feeding women. It can be given to patients as nasal inhalation form, or subcutaneous/intramuscular injections. Inhalation is generally preferred. Sufficient calcium and vitamin D should be taken with this medication.

Some researches imply that its injectable form has an analgesic effect so it can be prefered for women having painful fractures. However, it does not seem to be the best solution for osteoporosis treatment so far.

1.2.6.5. Selective Estrogen Receptor Modulators (SERMs)

These drugs are the newest alternative for the treatment of osteoporosis. They include raloxifen (Evista®) and tamoxifen (Nolvadex®, Tamofen®) as the most common examples. Tamoxifen was in use for the treatment of breast cancer. When its positive effect on bone density was recognized it was decided to be used also for osteoporosis treatment. SERMs behave as agonist and antagonist of estrogen according to estrogen receptor specification at different tissues. For instance, while raloxifen stimulates bone formation, it blocks the effect of estrogen in breast and uterus. Hence, it may be a good therapy option for women having a breast/uterus cancer risk.

SERMs may also be an alternative for women having gastrointestinal symptoms and not tolerating bisphosphonate treatments.

In a study comparing the raloxifen (Evista®) and alendronate (Fosamax®) effects (combined and seperate usage) with placebo group for 12 months, it was observed that all medication groups showed significantly higher bone density relative to their baseline values. Fosamax was more effective than Evista with 2.7 %

increase compared to 1.7 % respectively, in femoral neck bone density. The combination group had the greatest density increment with 3.7 % (Johnell et. al., 2002). Yet no correlation information between fracture rates and these density values were found.

On the other hand it has some side effects like leg cramps, venous thromboembolies and induced vasomotor symptoms.

Drug companies categorize the premenopausal stage as a contraindication for SERMs, so women who are recommended to use this class should also use an effective way of birth control.

SERMs can act as an antagonist against warfarin (an oral anticoagulant). So women taking both SERM and warfarin should be under close control. Raloxifen can also antagonize diazepam, diazoxide, and lidocaine (Schnitzer et. al., 2000) (Lobo et. al., 2000). Raloxifen can not be used together with HRT because their individual thromboemolytic event risk can be superposed with their simultaneous consumption (Schnitzer et. al., 2000) (Lobo et. al., 2000).

1.2.6.6. Vitamin D

Vitamin D has a role in intestinal absorption of calcium. It is found inactively in our skin. It becomes active under sunlight. 10-15 min for three times a week sunlight exposure is usually enough for vitamin D activation. It can be derived from foods either. These food sources are eggs, oil, fish, liver salmon and milk (Insel et. al., 2002)

1.2.6.7. Sodium Floride

Flouride simulates osteoblast formation thus helps bone growth. However, using flouride for this purpose has been not approved by FDA yet. Excessive dose may cause irregular bone formation and this leads to fractures .It is simultaneously used with calcium.

To sum up, flouride causes an increase in lumbar BMD, but does not reduce vertebral fracture risk. It may cause gastrointestinal adverse effects (Haguenauner et. al., 2002)

1.2.7. Future Trends in Treatment

Recombinant human parathyroid hormone seems to be the first anabolic drug improving the bone mineral density and bone built markers.

Zoledronic acid (Zometa®) which belongs to the bisphophonates group is the latest FDA approved drug in osteoporosis management in February 2002. It is actually used for the treatment of bone metastases in cancer. Studies with Zoledronic acid administration for 4 times /year vs.1 time/ year groups showed an increase in the spine bone mineral density 5.1 % to 4.3 %, and femoral neck density 3.5 % to 3.1 % respectively. Hence it has been shown that there was no significant difference in fracture rates between 4 time/year and 1 time/year groups. So its administration is decided to be done for 15 minutes for one time in a year by intravenous injection. This drug would be an alternative option for patients who can not tolarate oral bisphosphonate applications (Reid et.al., 2002).

According to a recent research, glycoprotein called *osteoprotegerin* was shown to be superior in effects. It is from tumor necrosis factor receptor family and binds to the enzymes responsible for osteoclast differentiation. Hence it prevents osteoclastic activity and bone resorption. Results also indicate that osteoprotegerin increases if 17 β- estradiol is present in the environment (Reid et.al., 2002).

Vitamin K, being a fat soluble vitamin, primarily functions in the liver, where it is necessary for the formation of several proteins required for blood clotting, including prothrombin (Shier et. al., 1999). Therefore, its defficiency causes prolonged blood clotting time. Vitamin K has different forms: One of these forms is vitamin K1, known also as phylloquinone, found in plants. Second form is vitamin K2, called as menatetrenone, produced from vitamin K1 by a friendly bacteria habiting in human intestine, Escherichia Coli (Shier et. al., 1999).

Tissues vary in their vitamin K needs: For some purposes like blood clotting, vitamin K1 works well; but plenty of research results indicate that vitamin K2 (VitK2) has unique effects on bone health which are not satisfied by the former. Areas where more VitK2 is consumed in the diet have lower fracture rates. It was found to inhibit the resorption of bone caused by the local cellular messenger prostaglandin E2 (PGE2). VitK2 is able to reduce the differentiation into osteoclasts (cells involved in the teardown of bone tissue) from the progenitor cell

types**.** VitK2, increases the programmed cell death (apoptosis) of existing osteoclasts. VitK2 strengthens the bone-building legions of the osteoblasts (cells involved in the manufacture of new bone), by increasing both their numbers and their activity. In the last decade, various clinical trials have been performed using VitK2 and all these studies have found that supplements protect bone health (http://www.vitaspace.com/vitamin_k2.htm).

In one trial, women who took an ultra-high dose VitK2 supplement for 24 weeks increased their bone mineral density by 2.2 %, while placebo (dummy pill) group lost 7.31 % of their bone density. Menatetrenone not only slows, or even reverses loss of bone mass, it also significantly reduces the risk of a fracture. (Orimo et. al., 1998).

In another trial, VitK2 was used in a direct comparison against the bisphosphonate drug etidronate (Didrocal®). VitK2 preserved bone mass, and also slashed fracture risk by two thirds over two years (Iwamoto et. al., 2001)

In a third trial, osteoporotic women taking VitK2 supplements sustained nearly no bone loss over two years, while decreasing fracture risk by 64 % as compared with non-supplementing women (http://www.vitaspace.com/vitamin_k2.htm).

The ability of bones to withstand fractures is not just determined by the *quantity* of bone (as measured by Bone Mineral Density (BMD)), but also by the *quality* of bone – bone "microarchitecture," including especially "trabecular connectivity." Increased quality decreases the rate of fracture. Evidence suggests, and that Menatetrenone's most important effects are on bone *quality,* not bone *quantity* (http://www.vitaspace.com/vitamin_k2.htm).

Clinical trials have found that VitK2 (Menatetrenone) provides as much protection against fracture as drugs that have much more powerful effects on BMD. Clearly, it's bone-protective effects extend to aspects of bone health beyond the BMD numbers (http://www.vitaspace.com/vitamin_k2.htm).

VitK2 provides powerful protection against the loss of trabecular connectivity in laboratory animal models of menopausal osteoporosis. Supplements of this vitamin increase bone quality in young, healthy animals. To get these effects in clinical trials and experimental studies, a specific Menatetrenone supplementation is required. Whereas very little VitK2 exists in the diet, even in the

richest food sources. While the body's friendly bacteria produce some VitK2, little or none of them is absorbed (http://www.vitaspace.com/vitamin_k2.htm).

The body's ability to convert VitK1 to VitK2 is limited, flattening out at levels far below what's used in clinical trials. This ability is further reduced with aging (http://www.vitaspace.com/vitamin_k2.htm).

VitK2's health benefits extend well beyond the skeletal system. Emerging science is now documenting the role of vitamin K – *specifically of Menatetrenone*– in protecting our cardiovascular health, and the health of that all-important organ, the brain (Allison, 2001)

Kameda et. al., (1996) has shown that in contrast to VitK1, VitK2 inhibited osteoclastic bone resorption by unfractioned bone cells and isolated osteoclasts. They first demonstrated that VitK2 induced osteoclast apoptosis, but VK1 did not. Moreover, cyclotemixide inhibited VitK2 induced osteoclast apoptosis. To conclude, VitK2 inhibits osteoclastic bone resorption by targetting osteoclasts to undergo apoptosis, which leads to cell death.

Mawatari et. al., (2000) has studied the effect of VitK2 on 3D trabecular microarchitecture in OVX rats by applying VitK2 just after ovariectomy 88 weeks, and analyzed the results by using DEXA and MCT. They reported that VitK2 improves osteoporotic bone loss by significantly increasing trabecular bone volume, fractal dimension, and connectivity sensitivity. However, despite this apparent protection it still remains unknown whether it is possible to reestablish the trabecular connectivity if therapeutic intervention occurs after their trabecular connectivity has been lost.

1.3. Bone Physiology, Structure and Mechanics

Bone differs from any other tissues with its rigid structure. It has so many functions in the body. It protects organs, constitutes a framework for the body, and gives its shape. It carries, transfers loads, and orients itself to different mechanical situations. Bone has the ability to repair itself. It is also responsible for mineral deposition in the body (Webster, 2000)

Bone is composed of inorganic salts, collagen fibers, noncollagen proteins and minerals. In axial skeleton bone is cancellous, and is cortical in appendicular skeleton.

The main function of cortical bone is mechanical. The function of cancellous is mainly metabolic. For example calcium homeostasis in metabolism is supplied from the red marrow sites of cancellous bone (Jee, 1988)

Axial skeleton includes skull, sternum, ribs, vertebrea and pelvis. On the other hand, appendicular skeleton is composed of shoulder, elbow, knee, ankle, foot, wrist, hand, femur, tibia, fibula, ulna, radius and humerus. Human long bone consists of two ends called epiphysis, and one circular shaft called diaphysis. At metaphysis and diaphysis interface, conic shaped metaphysis exists. Epiphysis and metaphysis transfers coming load to diaphysis by distributing evenly to articulating portions. Due to this reason epiphysis and metaphysis crosssections are larger than that of diaphysis. Bone's circular shaft has thick cortical bone whereas ends have thin cortical bone.

In growth stage there is growth plate between epiphysis and metaphysis. It is made up of hyaline cartilage. During the growth stage growth plate and trabeculae of metaphysis are responsible for elongation. This plate becomes trabeculae at the end of growth and it is added to bone epiphysis. So, at the end of growth there will be no strict boundary between epiphysis and metaphysis (Jee, 1988) (Frost,1995) (Jee, 1999).

Outer surface of the bone is covered with a membrane called periosteum. It has role in growth, bone formation and fracture healing. Inner surface is covered with a thin membrane called endosteum. It has osteoclasts (OC), osteoblasts (OB) and bone lining cells (BLC).

Cortical bone constitutes 80 % of total bone mass. Most of the cortical bone is in the shaft of appendicular skeleton.

Cancellous bone constitutes 20 % of the total bone mass. It has vertical plates and between the plates there are rods (struts) which helps to form a network like architecture. The plate-rod combination is called as trabeculae. Red bone marrow is in epiphysis trabeculae. It is mostly found inside of the bone (Jee, 1988) (Frost,1995) (Jee,1999).

. Cortical vs cancellous bone ratio varies with bone type. This ratio is bigger in appendicular skeleton (ex: long bones), and smaller in axial skeleton (ex: vertebrea). Surface/volume ratio is 20 for cancellous bone, and 2.5 for cortical bone. 33 % of the bone surface is cortical, and this surface is mainly outer surface. Remaining 67 % of the surface is cancellous, and this surface is inner surface. Turnover rate is slow in cortical and fast in cancellous bone (Bronner and Worrell, 1999).

Mammalian cortical and cancellous bone has two types: woven bone and lamellar bone. Woven bone is the bone tissue in the developing embryo. Cancellous woven bone is formed by endochondoral and intramembranous ossification. Woven bone has a matrix of coarse fibers, less organized and lives shorter than lamellar. Woven bone is replaced by lamellar bone in human long bones at the age of 2 or 3. It is made up of unit layers called lamellae. Lamellae contains fine fibers

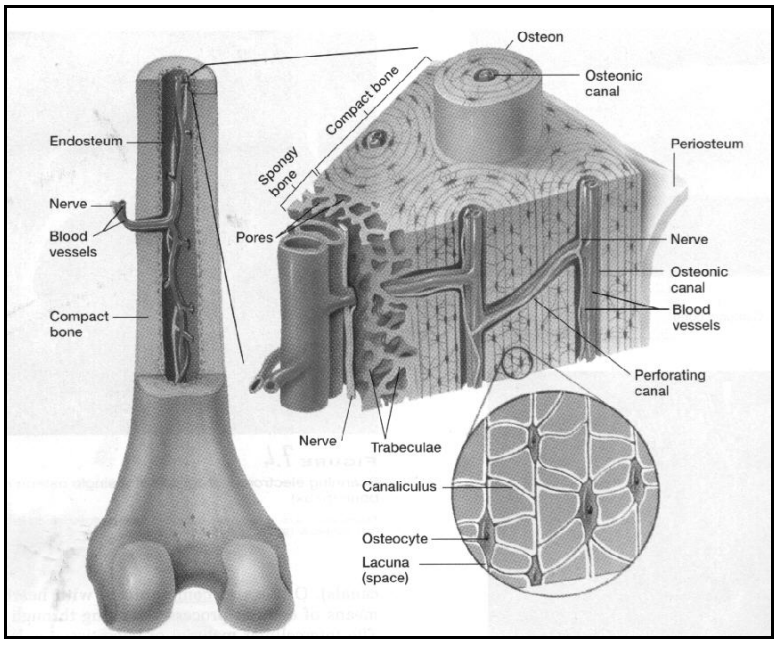


Figure1.2. Bone Structure (Shier D., Butler J., Lewis R.,1996)

Osteon is the cylinder shaped bone cell unit that surrounds an osteonic canal, Haversian system. It is composed of concentric lamellar bones. Cell processes are

achieved by the help of gap junctions (Jee, 1988) (Frost,1995) (Jee,1999) (Ericksen et. al.,1994)(Martin and Burr,1989).

Bone's 65 % is mineral, 35 % is organic matrix and remaining 10 % is proteins. Minerals are rod shape like small crystals. They are mainly impure hydroxiapatite (HA), and foreign substances like polyphosphates and bisphosphonates.

Organic matrix is made up of 90 % collagen. Then cells and water come. Frequently seen proteins are osteocalcin, osteonectin and osteopontin. Protein function in bone cell is still unknown (Gehron and Boskey, 1996).

Blood circulation is necessary for growth, modelling and remodelling. At the ends of long bones, there are periosteal arteries and nutrient arteries. In diaphysis, centrifugally located veins do the circulation. Periosteal arteries feed trabecular bone, and medullary arteries feed cortical bone (cortex). Periosteal and endosteal arteries are connected at bone ends (Figure1.2) (Jee, 1988) (Frost,1995)(Parfitt et. al., 1983).

Vascular system has a role in bone remodelling. They supply various bone cells. Capillaries bring preosteoclasts. Preosteoclasts originate in bone marrow (Jee, 1988).

Endothelial cells also play role in 'coupling' of formation to resorption. Blood flow increases after ovariectomy, and decreases with parathyroid hormone, prostaglandin E2. On the other hand, marrow blood flow decreases with aging(Jee, 1988) (Frost,1995) (Parfitt et. al., 1983).

Cartilage makes interstitial and appositional growth. However bone makes only appositional growth, since bone's mineralized structure has nonexpandible nature. Hence all bone activities occur on surfaces of bone. Bone has two kinds of surfaces: periosteal and endosteal. Endosteal surface is divided into 3 types as cancellous, intracortical and endocortical. Hence, in total there are four diffrent kinds of bone surfaces (Figure 1.3) (Jee, 1988) (Frost,1995)(Parfitt et. al., 1983).

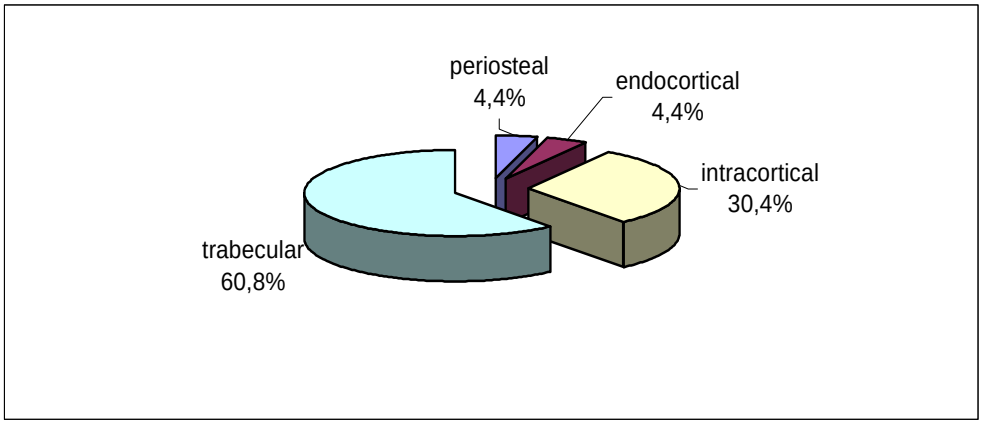


Figure 1.3. Types of Bone Surfaces (Weiss, 1988)

Cancellous surface constitutes 61 % of the total bone surface, since it has a high surface to volume ratio. This ratio for cancellous surface is 20, whereas it is 2.5 for cortical surface. At a particular time, bone can be in one of the three following situations: formation, absorption or resting. Forming surfaces are covered with bone formation cells called osteoblasts. Resorbing surfaces have osteoclasts, and concavities naming as Howship lacunae. Resting surfaces do not have osteoclasts (OC), nor osteoblasts (OB), but have bone lining cells (BLC). Most of the surface is in the stage of resting (Jee, 1988) (Frost,1995)(Parfitt et. al., 1983).

In Table 1.4 surface activity percentages and activity periods for cortical and trabecular bone are summarized.

Table 1.4. Surface Activity Magnitudes and Durations at a Particular Time, (Recker R.R. Ed., Bone Histomorphometry: Techniques and Interpretation, CRC Press, Boca Raton, FL, 1983.

Properties Measured		BONE TYPE	
		cortical	trabecular
Activity % on surfaces	resorption activity (%)	3	6
	formation activity (%)	0,6	1,2
	resting activity (%)	96,6	92,8
Duration of activities	time of resorption (days)	24	21
	time of formation (days)	124	91
	remodelling cycle (days)	148	112
Turnover rate (%/year)		43	26

Bone structural unit in cortical bone is osteon (system). It constitutes 2/3 of the cortical bone volume. Other 1/3 is lamellae and the remnants left from previous remodelling stages. Osteon has a cylindrical structure (Figure 1.4).

A central canal passes longitudinally at the center. In this canal, blood vessels, lymphatic nerves, and connective tissue exists. Osteons are connected to each other with Volkmann's canals. Osteon's wall is made up of 20-30 concentric lamellae. The outermost lamellea of each osteon is called cement line. It is a mineralized matrix made up of collagen fibers.

Bone structural unit in cancellous bone is hemiosteon. Its shape is like a shallow crescent. As in cortical bone, trabecular units are bonded with cement lines (Jee,1998)(Parfitt et. al., 1983).

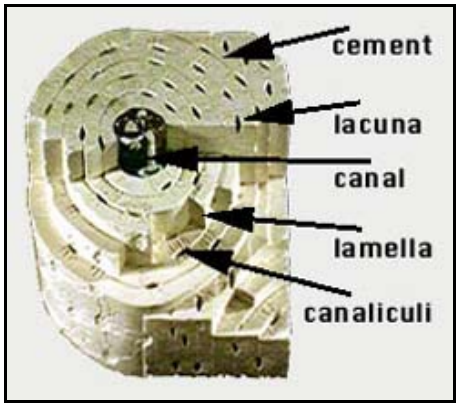


Figure1.4. Parts of Osteon (http://www.trinity.edu/rblyston/bone/intro2.htm)

Osteoclasts (OC): are multinucleated giant cells (Figure 1.5). Their main role is to resorp bone surface by evacuating and creating cavities. These cavities are called as Howship lacunae. OC's are derived from hematopoietic marrow. In resorption, bone mineral dissolves, and collagen and other matrix proteins are digested by proteolytic enzymes. OC has receptors for calcitonin and it responds to PTH, Vit D, and calcitonin. But OC lacks of PTH receptors. So, OB comes into picture and creates sufficient environment for OC resorption(Jee, 1988) (Frost,1995) (Jee,1999) (Ericksen et. al.,1994)(Martin and Burr,1989).

Calcitonin induces osteoplasmic resting, and Vit D_3 induces OC precursors to become mature OC 's. Bisphosphonates, calcitonin, estrogen are used commonly to inhibit the resorption by inhibiting the formation and OC activity. So they

promote OC apoptosis (cell death) (Tomkinson et. al., 1997)(Dawson, 1999)(Fleisch 1997).

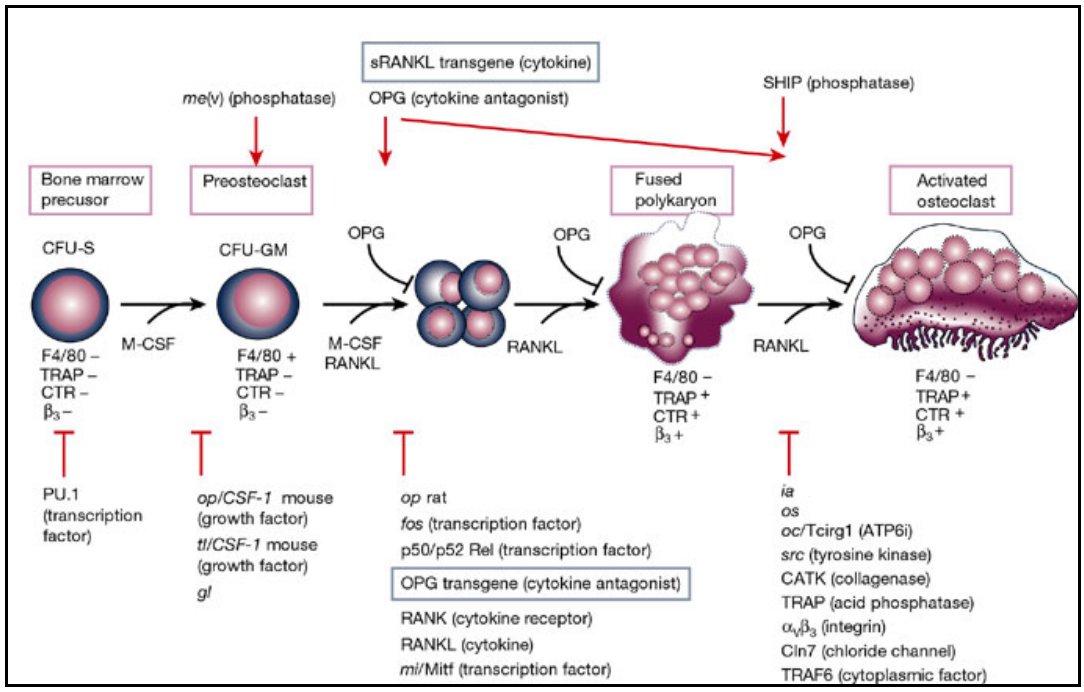


Figure 1.5. Osteoclast Formation (http://www.nature.com/cgitaf/DynaPage.taf?file=/nature/journal/v423/n6937/full/nature01658_fs.html&content_filetype=pdf)

Osteoblasts (OB): Osteoblasts contribute to bone formation by secreting unmineralized bone matrix. They also participate in calcification and bone resorption. The only known bone specific protein is osteocalcin and bone silaprotein. High alkaline phosphatase (ALP) presence, type 1 collagen secretion are the two characteristics of the OB phenotype (ie. existence). OB's secrete mainly type I collagen and bone matrix osteoid. The collagen fibers support mineralization. OB precursors (ie.immature OB's) are located on bone surfaces like periosteum, marrow, stroma, and endosteum. It is not precisely known but strongly believed that pericytes and endothelial cells are the precursors. OB precursors proliferate (= reproduced), differentiate to preosteoblasts then become mature OB's. It is also believed that active OB either becomes BLC in resting period, or becomes an osteocyte, or it is subjected to apoptosis. The bone ALP, osteocalcin markers, and bone collagen degradation can be monitored in blood serum. So they are useful

chemical markers in order to monitore bone formation (Jee, 1988) (Frost,1995) (Jee,1999).

OB synthesize and secrete unmineralized matrix. This matrix is composed of 90 % collagen and 10 % noncolloganeous proteins. Formation occurs in two stages: First stage is matrix formation, and it continues 15 days. Second stage is mineralization, and it happens at osteoid (ie. newly formed bone junction). Mineralization occurs after the matrix formation. The time passed between the end of formation and the start of mineralization is called mineralization lag time. The unmineralized matrix, waiting for mineralization, is called osteoid seam. If mineralization lag time is short, a thin osteoid seam is formed (Ericksen et.al., 1994)(Parfitt et. al., 1983).

Bone is mainly consists of type I collagen and its traces. Collagen fibers give the framework structure and its shape. Mineral HA is inserted in the framework. HA supplies the rigidity of collagen framework. Non collageneous proteins (ex: osteocalcin, bone silaoprotein) are thought to have an important role in bone calcification and HA deposition in bone matrix.

Mineral growth and proliferation are mainly controlled by collagen fibrils and matrix proteins. Bisphosphonates –an antiremodelling agent given to osteoporotic patients- bind HA crystals to surface, and blocks the mineral dissolution. So bone mineral content tends to increase as a result of this situation.

Osteoporosis, also cellular activity, can influence mineral properties. Size and distribution of mineral crystals are also influenced from bone mechanical properties. Although bone mineral density has been correlated to mechanical strength, quantitative computed tomography (QCT) describes the amount of bone in a given volume, not in a given area. QCT gives information about bone's architectural properties, and it allows to derive a bone strength index which is more reliable (Lian et. al., 1999)(Rodan and Rodan, 1995).

Bone lining cells (BLC): When OB's are not in the formation process, it means they are in resting. They cover the resting bone surfaces. These are called resting OB's or bone lining cells (BLC). They occupy the majority of bone surface. They can return to stem cell, preosteoblast, or being subjected to apoptosis. BLC's are capable of forming bone. They may involve homeostatic processes like bone mineral/ mass/ architecture regulation. BLC serves as a barrier between osteocyte

and canallicular system. They are involved in osteoclastic bone resorption by digesting surface osteoid (Jee, 1999) .

Remodelling: is the continuous process of bone destruction and formation. Remodelling supplies biomechanically and metabolically competent bone material. Bone quality changes with time due to several reasons and it needs repairing itself. The products of remodelling includes cement line, secondary osteons (s), hemiosteons (trabeculae) and interstitial lamellae.

Human after age of 2 or 3, primary woven bone is replaced with secondary bone. Secondary bone is continuously destroyed and rebuilt until death. Adult bone turnover period for cortical bones is 20 and for cancellous 1-4 years. This cyclic situation helps to regulate Ca^{+2} homeostasis and repair structural damage (Jee 1988)(Jee 1999)(Frost,1995).

Advantages of remodelling is that remodelling removes dead, damaged, overmineralized bone tissue, and helps microarchitecture to adapt local stresses. Remodelling of trabeculae removes and perforates trabeculae. Remodelling of cortical bone increases porosity and decreases cortical width, hence it reduces strength.

Bone remodelling unit (BMU) does bone turnover and removal and replacement of osteon in cortical and hemiosteon in cancellous bone. BMU cycle includes six stages: Resting, activation, resorption, reversal, formation, and mineralization.

Resting: in human 90 % of cancellous and 95 % of cortical bone is in resting stage when the left percentage is doing reconstruction. In resting, bone surfaces are covered by BLC which may turn OC and OB presursors (Miller et.al.,1992).

Activation: Starting of resorption activity on resting surface is called activation. It is believed to occur because of biomechanical stimulation this cycle requires OC recruitment to bone surface.

Capillary growth is needed for this action. At that time BLC are believed to start digesting endosteal membrane and mineralized bone surface becomes ready for precursor OC cell exposure (Rodan and Rodan, 1995).

Resorption: OC's attach to bone surface and begin erosion. They form Howship lacunae in cancellous bone, and creates resorption cones in cortical bone. Cone direction is parallel to long axis of bone. This stage lasts 1-3 weeks.

Reversal: Lasts 1-2 weeks between resorption and formation. Howship lacunae in cancellous and cutting cones in cortical bone still exist, but there are also mononuclear cells coming from an unknown origin. Coupling of formation and resorption occurs at this stage .

Formation and mineralization: Formation has 2 levels; matrix synthesis and extracellular mineralization. OB are begin to deposit unmineralized matrix called osteoid seam. 70 % of this seam is finished in 5-10 days. Mineralization is completed about 3-6 months in both cortical and trabecular bone (Jee 1988)(Jee 1999)(Frost,1995)(Parfitt, 1983)(Ericksen, 1994)(Martin and Burr, 1989).

BMU of adult skeleton resorps 20 parts of bone but replaces 19 part. Thus creating a negative balance situation. This is called bone remodelling dependent bone loss. Mechanical usage depresses new remodelling units. So remodelling increases with mechanical disuse. Cortical and trabecular bone loss rate increases, trabecular becomes osteopenic, and marrow cavity enlarges (Jee 1988)(Martin and Burr, 1989).

Bone turnover: Is the intensity of remodelling. For ilium and ribs, its frequency depends on surface/volume ratio. At other sites, difference in mechanical loading determines the turnover frequency (Martin and Burr, 1989)(Parfitt, 1983).

Rate of remodelling is dominated by mechanical usage and modulated by PTH, growth hormone 1.25 OH_2, Vit D, and microdamage.

Remodelling can be either in conservation and disuse mode. In conservation remodelling, resorption equals to formation. In disuse remodelling, bone making is less than bone resorption so the result is a net bone loss next to marrow. Latter one is the result of disuse and aging. In this mode, treshold of strain is not exceeded by mechanical performance. This results with osteopenia, thinner cortices and large marrow cawities. If treshold is exceeded, remodelling is damped back to its normal level (Frost, 1995)(Jee, 1988).

Osteoporosis has two types: In true osteoporosis spontaneous bone fragility with fracture occurs without exceeding normal physical activity ranges. This mainly occurs in spine.

Second type, physiological osteopenia reduces bone strength and mass. Fracture does not occur without falls. Fractures affect extremities more than spine (Frost, 1998).

FDA approves some antiresorptive-antiremodelling drugs such as calcitonin bisphosphonates, SERM for prevention and treatment. FDA criterion is to reduce fracture rate, which is a clinical benefit. Some efforts about developing drugs that stimulates bone formation also exist (Dawson ,1999).

1.4. Animal Models

FDA recommends the use of animals experiencing osteopenia after OVX. FDA requires data from rat or human primate which satisfy these conditions. Rat studies must finish in 12 months duration. This time equals to 4 year of human treatment. Study must include histological evaluation, bone density measurement, biochemical turnover marker, biomechanical testing for bone strength (Kimmel, 1996) (Thompson et. al., 1995).

1.4.1. Rat as an Animal Model in Osteoporosis

Ovx rat model has the gold standard among the animal models of osteoporosis. Ovx rat model simulates most important clinical features of estrogen defficient human skeleton. Similarities exist between human and rat osteoporosis: First of all, the development of osteopenia, and a rapid phase of bone loss occurs during early stages of estrogen deficiency. Secondly, increased turnover is related with rapid phase of bone loss induced by estrogen deficiency just like in humans. Thirdly, estrogen treatment prevents osteopenia by depressing bone turnover. Lastly, bisphosphanate compounds depress turnover in rats as in humans.

On the other hand, model has also some limitations such as, processes of remodelling in growing rat is not valid in adult skeleton. Rat model is poor in order to study intracortical effect since modelling is low in rats. Ovx rat does not develop fragility fractures. The limitation due to growing rat can be minimized by using 10 month old female rat (Kimmel,1996).

1.5. Scope of the Study

In this study, different types of bioactive agents were used to investigate their single and combined effects on biomechanical properties of osteoporotic rat bone. Among them, the best known agent estrogen was chosen as the standard. This hormone, being naturally present in the body, is the most commonly chosen drug for osteoporosis, especially for patients undergoing ovariectomy procedures. Together with menopause, it is known that bone tissue degeneration and many other symptoms like hot flushes, heart diseases, or some psychological problems start. For most of these problems, estrogen stays as the gold agent in treatment. However, despites these benefits it is also known as a high risk drug for some other estrogen receptor carying tisuues like breasts and uterus. High amounts or long term uses of estrogen is known to be associated with hyperplasia or cancer development in these tissues which restricts its clinical uses. Therefore, in this study, it was aimed to add a drug having similar structure to estrogen. Such that, it will work as agonist (same way) in the bone tissues, but antagonist (opposite way) in the others to estrogen therapy. Raloxifen was chosen for this purpose, since it has the ability to act on estrogen receptors on bone as estrogen agonist, while those on other tissues like breast and uterus as estrogen antagonist. So, the study was designed to see the combined effects of these two agents on different tissues (bone and uterus) with ovariectomized rat model. This combination is thought to bring a new approach to osteoporosis therapies, since it is not applied currently in clinics, despite both agents being prescribed as seperate and with some other drug combinations.

As the second new approach to osteoporosis treatment, VitK2 was chosen to be used alone, or in combination with raloxifen in same animal model of osteoporosis. Although VitK2 is well documented on its protective effect on bone structure and decreased incidence of bone fractures owing to its specific role on bone metabolism (carboxylation of bone Gla protein, BGP) Its rebuilding or repairing effect on bone tissue is not completely established. So, this unclear part of VitK2-bone relation was aimed to be clarified with the present project by using this vitamin on rats. Besides this property, VitK2 is also proved to have protective effects on cardiovascular health, which is also a serious problem in postmenopausal women and gets severe with use of certain drugs like raloxifen. Considering these

opposite properties of the two agents, they were decided to be applied together as one of the experiment group to see their combined effect on bone.

As a very widespread problem, there is not an established effective treatment for osteoporosis yet. Hence, many clinical trials are carried out with use of bone density measurements and fracture probabilities as the evaluation methods. However, the risk of fracture is obviously an environmentally modifiable parameter which may occur in a person with a higher bone density. For these reasons, it is thought that comparison of bone density information with biomechanical tests, on osteoporotic bones are necessary preclinical information before any such trials on osteoporosis. By mechanical testing, bone overall behaviour upto fracture, and comparison of current methods used in the calculation of material properties (i.e.strain gauge technology vs beam theory) are aimed to be studied.

CHAPTER 2

MATERIALS AND METHODS

2.3. Materials

17 β-estradiol and Methylcellulose (M 0512) were purchased from Sigma Chemical Co. (St. Louis, USA).

Raloxifen was kindly given as a gift from Gülhane Military Academy of Medicine (Ankara, Turkey).

Vitamin K2 was a generous gift of Eisai Co., Ltd. (Tokyo, Japan)

Female, 2 months old, virgin Wistar rats were obtained from Dr. Refik Saydam Central Institude of Hygiene (Ankara, Turkey). Thomson et. al. stated that magnitude of response to OVX is greater in young rats than in old rats.

Benzyl alcohol was obtained from Fluka.

Ketalar® (ketamin, 50mg/ml) was obtained from Eczacıbaşı Inc. (İstanbul, Turkey) and Rompun® (2 % xylasine) was obtained from Bayer Turk Chemical Inc. (İstanbul, Turkey).

2.4. Methods

2.4.1. Animal Care and Ethics

All the studies on animals were performed in accordance with ethical guide lines for animal care, and all work was reviewed and approved by the ethical committee of Ankara University, Department of Veterinary Medicine. Rats were weighted on the first day premedication,and postmedication stages of the study. Rats were housed randomly in wire cages as 3-4 in one cage in the Animal Care Quarters of

Engineering Sciences Department, METU, where they were maintained at constant temperature (20-22°C), and humidity conditions (30 % - 50%) at 12 hour day/night cycles. They were allowed to recover from transport and handling. Picture indicating animals and laboratory environment is shown in Figure 2.1.

No special diet was used during the experiments. Rats were fed with standard rat chaw and tap water *ad libitum*. After acclimation for one month, they were divided into 7 groups randomly (Table 2.1.)

Figure 2.1. Animals and Laboratory Environment

2.2.2. Ovariectomy Operation Procedure

Ovariectomy operations were performed by Vet. Dr. Hasan Bilgili from Ankara University Department of Veterinary Medicine.

15 mg/kg Xylazine (Alfazine) was injected to subjects as premedication, following this stage 10 mg/kg Ketamin (Ketamidor) was administered for general anestesia.

The operation site was limited to arcus costalar region in cranial (up, above), and inguinal region in caudal (down, below). Then identified region was shaved and cleaned by antiseptic solutions (Baticon).(Figure 2.2.a) Later, subjects were laid down to the operation table, and surfaces except the operation site were covered with sterilized fabric. 2 cm skin incision (cut) was made along linea alba line. (Figure 2.2.b)

Table 2.1. **Experiment and Control Groups**

GROUP NAME (N)*	LABEL	MEDİCATİON (ML/DAY)	TREATMENT PERİOD (MONTHS)	ROUTE OF APPLICATİON
Sham (8)	(S)	_	_	_
Control (6)	(C)	_	_	_
Ovx+Estrogen (5)	(E)	3	3	Sc
Ovx+Raloxifen (7)	(R)	3	3	Oral
Ovx+Vit K (8)	(K)	5	2	(K) Sc
OVX+Estrogen+ Raloxifen (8)	(E+R)	3(E)+3(R)	(E) 3 (R) 3	(R) Oral (E) Sc
OVX+Raloxifen+ Vit K (8)	(R+K)	3(R)+5(K)	(R) 3 (K) 2	(R) Oral (K) Sc

*n shows the number of subjects in each group

Following this step m.obliques externus abdominis, m.obliques internus abdominis (=muscles covering abdominal cavity) and periton (=membrane covering abdominal cavity) were opened, and then omentum was deviated and ovarium were seen (Figure 2.2.c). After this, ovarium were ligatured with 3/0 polyglactin 910 (Vicryl) suture from mesovarium in its proximal and from cornu uterus in its distal (Figure 2.2.d). Following ligaturation, bilateral ovariectomi was carried out (Figure 2.2.e). Bleeding was taken under control. M.obliques externus abdominis and m.obliques internus abdominis were sutured with 3/0 polyglactin 910 (Vicryl).

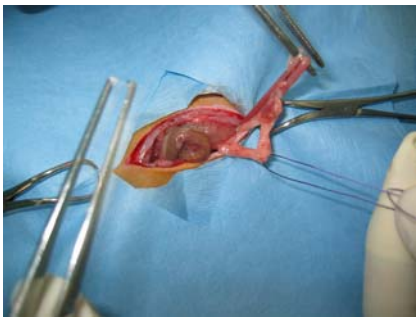
Following this step, the connective tissue under the skin was stiched with 4/0 Chrome catgut (absorbable), and as a last step, outermost skin was sutured with nonabsorbable monoflaman nylon sutures (Figure 2.2.f).

After the operation, abdominal region was disinfected with blue Terramicin spray and a soft bandage was also applied. 5mg/kg Alfoxil 250 mg therapy was applied to subjects for one week following operation. They were monitored and looked after carefully in postoperation period. About 3 months after the surgery drug treatments has started. Bilateral ovariectomy was applied to all groups except Sham operated group.

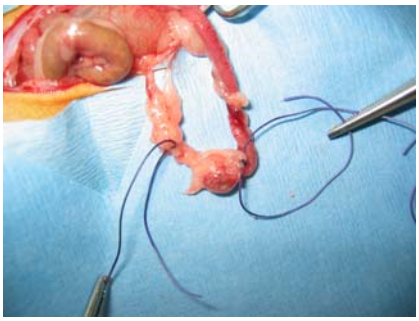
This group was anesthesized and same procedure was applied without removal of the ovaries.

(a) shawing

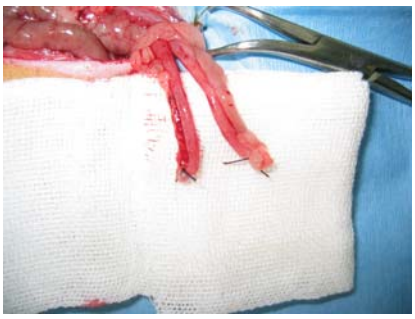
(b) 2 cm skin insicion



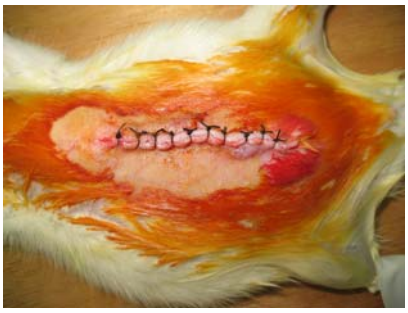
(c) omentum was deviated



(d) ovarium were ligatured



(e) bilateral ovariectomy



(f) outermost skin was sutured

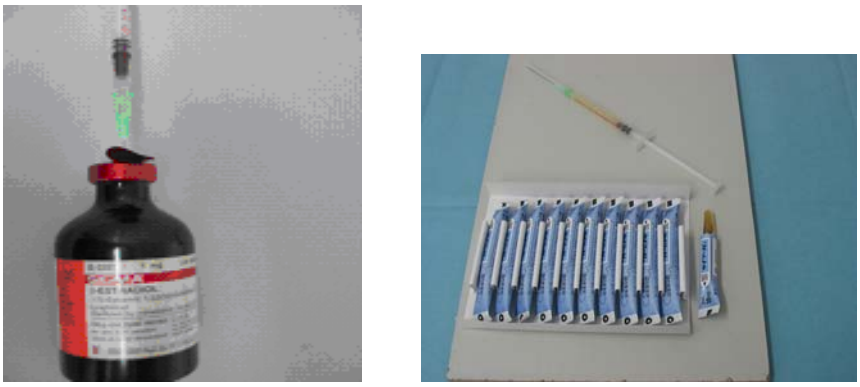
Figure2.2. Stages of Ovariectomy Operation

2.2.3. Preperation and Application of Drug Solutions

Estrogen was prepared by disolving in Benzyl Alcohol (5%) and 95 % Corn Oil solution to obtain a final form of 6.25 μg in 0.3 ml solution (one dose). (Preparations were performed in GATA). One dose of drug was administered subcutaneously to rats daily in E and E+R groups for 5 times a week and total treatment lasted for 12 weeks.

Vitamin K2 was supplied by the Japanese medicine company in its own solution as ready to use. Daily subcutaneous injection was done to K and R+K groups with a dosage of 0.5 ml/rat (30 mg/kg rat). This was repeated for 5 times a week and total treatment lasted for 8 weeks (Figure 2.3).

Raloxifen oral solutions were prepared with Microcristal cellulose, Cabosil, Mg.Steorat and are administered daily by using a simple gavage, at a dose of 0.3 ml/rat. Raloxifen was orally administered to R, and R+K, and E+R groups for 5 times a week, and total treatments lasted for 12 weeks (Figure 2.4).



(a) (b)

Figure 2.3. Subcutaneously Injected Estrogen (a) and Vitamin K2 (b)

(a) (b)

Figure 2.4. (a) Raloxifen and Gavage (b) Oral Administration of Raloxifen

2.2.4. Tissue Collection and Processing

At the end of medication period, rats were sacrificied with Lystenon® administration intraventicularly with a dosage of 2 ml/rat.

During sacrification intracardial blood was taken and collected in tubes. Then, they were centrifuged for 10 minutes at 5000 RPM (revolution per minute). Supernatant part involving blood plasma was removed and kept in 4ºC until use in biochemical analysis.

Uterus and right tibia of each rat were taken for histological and histomorphometrical investigations.

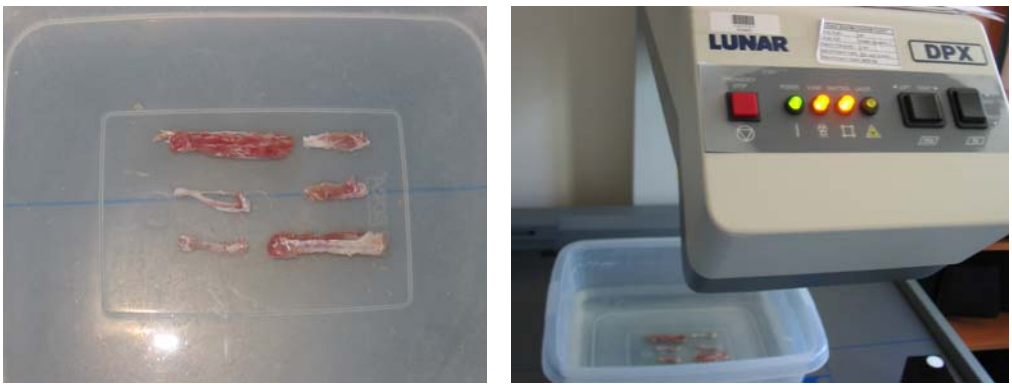
Left tibia, both sides of femora, and lumbar vertebrae (Figure 3.4 a) were removed, placed in separate tubes as wrapped in saline –soaked gauze sponge in order to keep the water content and stored at -20º C until DEXA measurements and biomechanical testing.

2.2.5. Bone Mineral Density Measurements Using Dual Energy X-Ray Absorptiometry

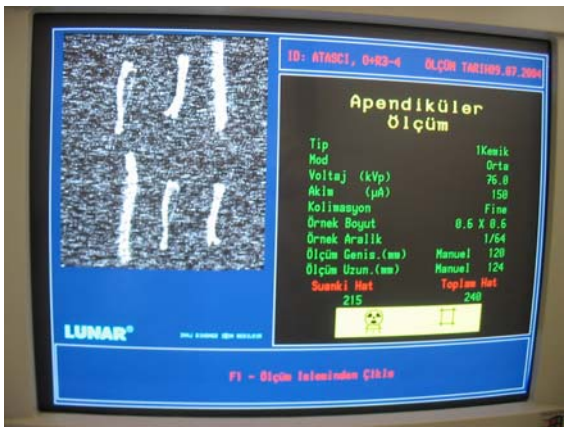
Immediately before the biomechanical testing, in order to get the bone mineral densities and bone mineral contents, right tibia/femora and lumbar bones were scanned with Lunar-DPX-IQ (Madison, Wisconsin, USA) DEXA device

involving small animal software (Medical Center of Middle East Technical University). The appendicular acquisition program was used. Mode of evaluation was detailed. The setting of the instrument was 76.0 KV and 150 μA. Collimation was fine. Distilled water was used to simulate the soft tissue presence while scanning the bones with densitometer (Figure 2.5).

After scanning whole bones six regions of interest (ROI) were selected from proximal, distal and midspan locations for tibia and femora in order to see the partial loosening in bone trabecular and cortical sites.



(a) (b)



(c)

Figure 2.5. (a) Specimens Ready for BMD Screening (b) BMD Equipment (c) Related Software

2.2.6. Histological Evaluations

After the removal of uterus, samples they were fixed in Bouien's fixative (saturated formaldehyde: picric acid:acetic acid, 15:5:1, v/v) for 6 hours. The specimens were dehydrated gradually by incubating for 1 h in each of 70, 80, 96 % and finally absolute alcohol. The samples were then incubated in methyl benzoate for 24 h in order to soften the materials. The samples were immersed first into benzene/paraplast (1:1) mixture, and, then into pure Paraplast® for 6 h in a vacuum oven and embedded in paraplast. The blocks were cut in sections 5-6 μm in thickness with a rotary microtomer (Leica RM 2025). The sections were stained with Crossmon Modified Triple stain for light microscopic examinations.

2.2.7. Biomechanical Testing

2.2.7.1. Three Point Bending Test

Before starting to the tests, soft tissues including periosteum were completely removed from the bones and crossectional properties (a/p width, m/l width, length, thickness) were measured with a Mitutoyo brand micrometer.

Measurements of bending strains on femurs under three point bending test were done by using strain gauges that were attached to the bone surfaces, and resulting data was collected by a strain indicator device (Vishay Instruments', USA). Right femur and tibia measurements, however, were done with conventional three point bending setup that yields load-displacement data by using a Lloyd LS 500 Universal Testing Machine (Figure 2.6c)

Femora and tibia were put on the test set up as its anterior face being in tension and posterior face being in compression.

During both tests same conditions were used. Deflection rate was decided as 2 mm/min for both tibia and femora (w,w/o strain gauge). Roller supports were designed as 3 mm in diameter. The supports are custom made in OSTİM (Figure 2.6.d). Span length was decided as 15 mm for both femora and tibia as specified span length to thickness ratio. 500 N load cell was used in during the tests (0.5 %

accuracy). The data acquisition rate was 1 data per second for strain indicator, and 2 data per second for Lloyd Universal Testing Machine.

2.2.7.2. Surface Preparation and Application of Strain Gauge

First of all bone surface, where the gauge is going to be applied, was smoothened and cleaned from dirt and grease Figure 2.6.(a)(b). The gauge was grapped and a proper orientation was decided.Then the gauge was transfered via cellophane tape.

Adhesive is applied properly than gauge was allowed to dry (Figure 2.6.e). Then welding of cables is done on to gauge. Gauge was connected to the strain indicator via these cables. During strain gauge tests, Vishay Strain Indicator and Lloyd Universal Testing Machine were operated simultaneously in order to get the load value and its corresponding strain value at the same time.

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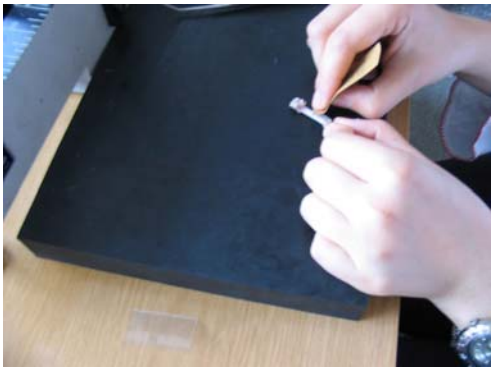
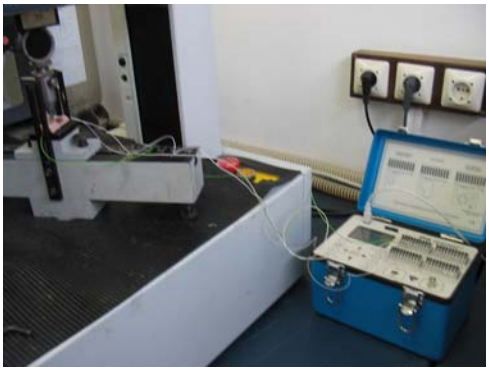


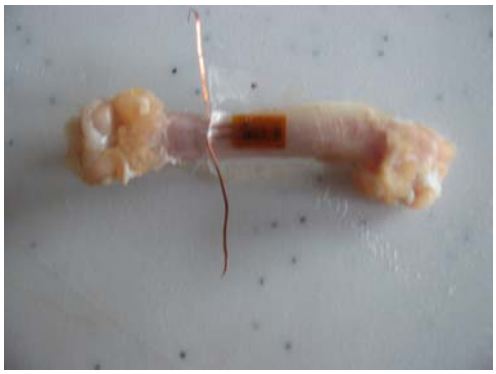
(a) smoothening the surface

(b) cleaning the surface



(c) Lloyd Universal Standart Testing Machine and Vishay Strain Indicator

(d) Lloyd Three Point Bending Set Up and Custom Made Roller Supports



(e) Strain Gauge Applied on Testing Bone

Figure 2.6. Strain Gauge Application

2.2.8. Methods Used for Mechanical Analysis

2.2.8.1. Mechanical Properties and Behaviour

Stress strain diagram is the characteristic of a material, and gives important information about material performance in testing.

Stress strain digram of typical structural steel in tension (Figure 2.7) is used to introduce the properties which are also tried to be determined in this study on bone specimens.

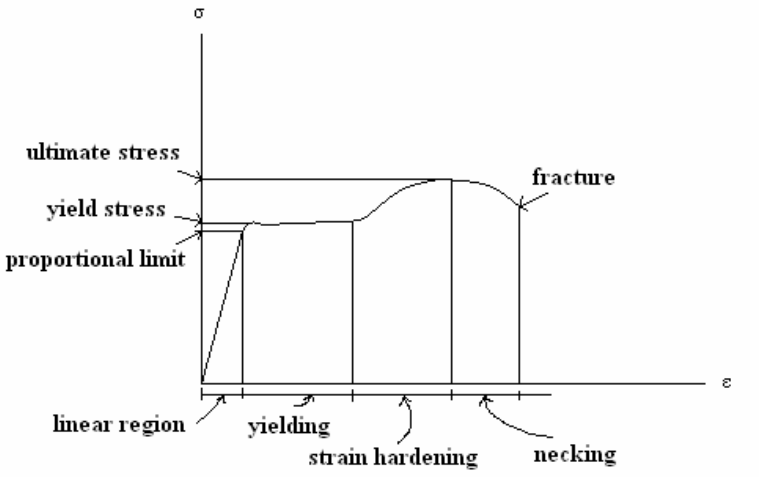


Figure 2.7. Stress Strain Digram of Typical Structural Steel in Tension (Gere and Timoshenko,1990)

Proportional limit : is the last point where stress and strain varies proportionally with each other. If two variables are proprtional with eachother, their ratio should be equal to a constant. Hence proprtional relationship is also linear (Gere and Timoshenko,1990). Proportional load limit is the magnitude of load at this specified point.

Modulus of elasticity: If a matreial behaves elastically and shows a linear relationship between stress and strain, this material is called linearly elastic. Modulus of elasticity is the slope of the straight line in the linear region. This linear relationship can also be expressed as $\sigma=E*\varepsilon$, which is also known as Hooke's Law.

Yield stress: at this point proportionality between stress and strain no longer exists. From this point, large deformation occurs without a noticable increase in force.

Strain hardening: after the large deformations in yielding, material goes under strain hardening meaning that its atomic and crystalline structure starts to change. It results with increased resistance.

Ultimate strength: load eventually reaches to its maximum value. The stress occured at that point is the ultimate stress, and load required for this stress is the maximum load. It reflects the general integrity of the organic and inorganic components and their orientations in bone structure (Cowin)(2000).

Fracture: continuing the test causes a reduction in load, and fracture occurs.

2.2.8.2. Strain Energy Concept

The strain energy concept is of fundamental importance in applied mechanics, and principles of strain energy is widely used when determining the structure response to loading conditions.

Strain energy is defined as the *energy absorbed* by the loading specimen during the loading process. It is denoted by U. It is equal to the work done by the load. It is equal to the area below the load-displacement curve. (Figure 2.8)

$$U = W = \int_0^{\delta} P_1 \, d\delta_1$$

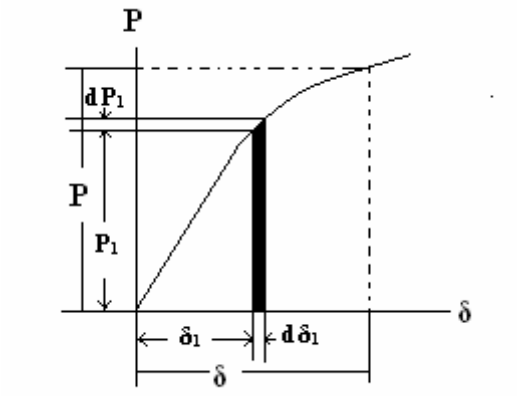


Figure 2.8. In a Prismatic Bar Subjected to Statically Applied Tensile Load, (Gere and Timoshenko,1990)

Strain energy U stored in beam is equal to work done, and is calculated similarly in previous case as (Figure 2.9):

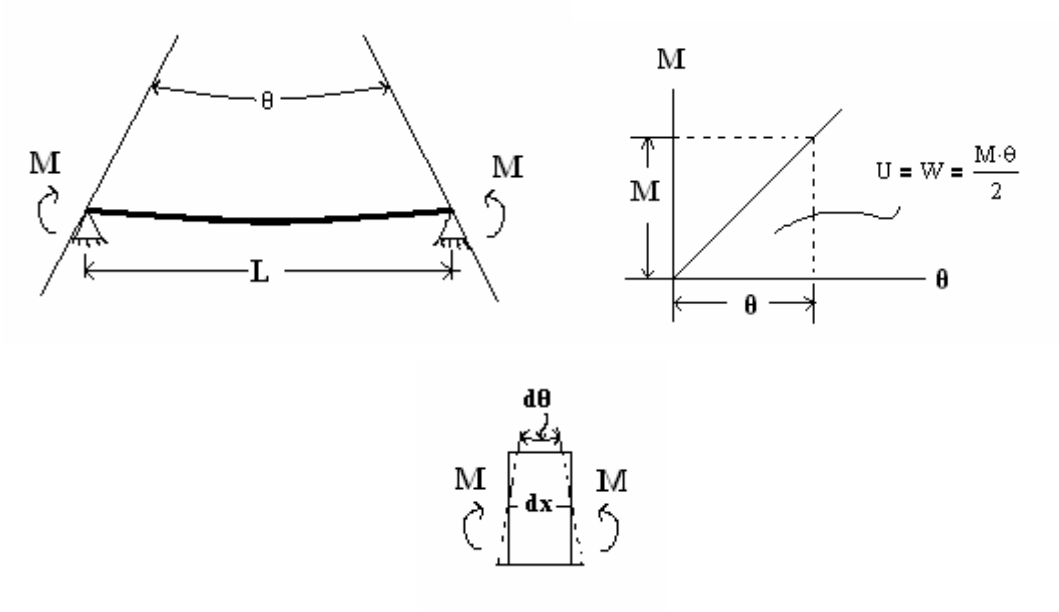


Figure 2.9. Strain Energy for Beams, Provided that Beam Obeys Hooke's Law, (Gere and Timoshenko,1990)

$$U = W = \frac{M \cdot \theta}{2} \quad , \qquad \theta = \frac{M \cdot L}{E \cdot I} \quad , \qquad U = \frac{M^2 \cdot L}{2 \cdot E \cdot I}$$

It can be realized that above formula is valid for constant moment case. For a condition of moment with varying x (like in three point bending case existing in this study) equation becomes

$$U = \int \frac{M(x)^2}{2 \cdot E \cdot I} dx$$

For three point bending, which is also a varying moment case, the proper equation is derived by the researcher as below:

Curvature is the second derivative of deflection (basic differential equation of the deflection curve of a beam)

$$d\theta = \frac{d^2}{dx^2} v \cdot dx = \frac{M \cdot dx}{E \cdot I}$$

So the strain energy can be rewritten as:

$$U = \int \frac{E \cdot I}{2} \cdot \left(\frac{d^2}{dx^2} \nu \right)^2 dx$$

deflection with a function of x is:

$$\nu = \frac{P \cdot x}{48 \cdot E \cdot I} \cdot \left(3 \cdot L^2 - 4 \cdot x^2 \right) \qquad 0 < x < \frac{L}{2}$$

by putting these values into the strain energy equation :

$$\frac{d^2}{dx^2} \nu = \frac{P^2 \cdot x^3}{24 \cdot E \cdot I}$$

result comes out as:

$$U = 2 \cdot \int_0^{\frac{L}{2}} \frac{P^2 \cdot x^3}{24 \cdot E \cdot I} dx = \frac{P^2 \cdot L^3}{96 \cdot E \cdot I}$$

According to Gere and Timoshenko (1990), the strain energy of a simply supported beam under concentrated load on midspan can be calculated as:

$$U = \frac{P \cdot \delta}{2} = \frac{24 \cdot E \cdot I \cdot \delta^2}{L^3}$$

If δ is written in terms of load from the deflection formula,

$$\delta = \frac{P \cdot L^3}{48 \cdot E \cdot I}$$

It can be easily seen that this result equals to the result derived by the researcher:

$$U = \frac{P \cdot \delta}{2} = \frac{P}{2} \cdot \frac{P \cdot L^3}{48 \cdot E \cdot I} = \frac{P^2 \cdot L^3}{96 \cdot E \cdot I}$$

or

$$U = \frac{P \cdot \delta}{2} = \frac{24 \cdot E \cdot I \cdot \delta^2}{L^3} = \frac{24 \cdot E \cdot I}{L^3} \cdot \left(\frac{P \cdot L^3}{48 \cdot E \cdot I} \right)^2 = \frac{24 \cdot E \cdot I}{L^3} \cdot \frac{P^2 \cdot L^6}{48^2 \cdot E^2 \cdot I^2} = \frac{P^2 \cdot L^3}{96 \cdot E \cdot I}$$

From this result, it can be concluded that strain energy for a simply supported beam under concentrated load P, can be calculated from area under the load deflection curve, or from the derived formula for any load level P if the necessary material properties exist.

In this study strain energy is computed for two levels of P (energy absorbed in elastic region and energy absorbed until fracture) in femur, and one level (energy absorbed until fracture) for tibia.

In addition to strain energy in bending, strain energy of shear will be stored in beam elements. However beams having greater lengths than depths (ie. L/d>6), strain energy due to shear is relatively small, so can be disregarded (Gere and Timoshenko,1990). A similar result was also found for deflection (due to bending versus due to shear), and confirmed with literature as can be seen in APPENDIX.

2.2.8.3 Linear Beam Theory

Linear beam theory is used as a model for bone bending tests.

When a beam is loaded, its straight longitudinal axis is deformed into a curve This curve is called as deflection curve.

Finding deflections at some specific points along the axis is important. Especially in building design, deflections are always checked whether or not they are under the maximum permissible values. Large deflections bring poor appereance and too much flexibility. In Figure 2.10 deflection of a simply

supported beam having a concentrated load on its midspan can be seen. It also simulates the three point bending.

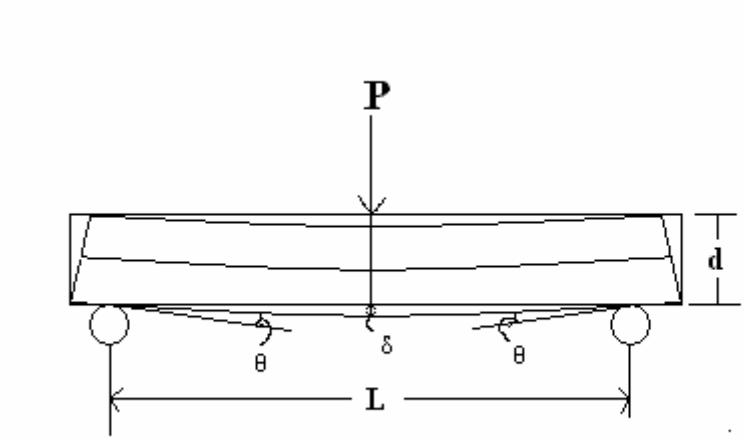


Figure 2.10. Deflection of Simply Supported Beam Having Concentrated Load On its Midspan

The maximum deflection of a simply supported beam having a concentrated load on its midspan can be calculated the following expression (Gere and Timoshenko,1990):

$$\delta = \frac{P \cdot L^3}{48 \cdot E \cdot I}$$

where:

δ refers to deflection in mm,

P refers to applied load in N,

E refers to modulus of elasticity in MPa,

and I refers to crossectional moment of inertia of the beam in mm^4.

This expression can not be derived without making the following assumptions:

Loads only act in the plane of bending

Material of the beam is linearly elastic and follows Hooke's Law of curvature, that is

$$\kappa = \frac{1}{\rho} = \frac{-M}{E \cdot I}$$

where:

κ is the curvature

ρ is the radius of curvature

Material is homogenous

Beam area (A) and modulus of elasticity (E) are constant

The displacements on beam are infinitesimally small ($\Delta x \approx 0$)

The beam rotation angle on supports are infinitesimally small ($\Delta \theta \approx 0$)

Plane perpendicular to the longitudinal axis of beam remains plane after the loading

The Poisson's ratio is neglected as a result of this "plane remains plane" assumption

There is a surface along the longitudinal direction that the axis along this surface is neither subjected to tension or compression.This axis is called neutral axis.

Strain measured in transverse crossection changes linearly, and it is proportional to the distance from the neutral axis.

Bone is anisotropic, heterogeneous, having variable crossection. However beam deflection theory in mechanics of materials is applicable to bone mechanics if above assumptions are made.

Bending tests are useful in order to measure the intrinsic and extrinsic mechanical properties of long bones.

Intrinsic properties are the material properties that depend on materials geometrical dimensions, but extrinsic ones are independent from geometry.

Bending causes compressive stress on the load applied surface and tensile stresses on the opposite surface. Since bone is weaker in tension than compression failure generally occurs in tension side.

Bending can be applied on three points or four points of the specimen. Three point bending is more practical since there is one load application point. In three point bending maximum load, bending moment and deflection ocuurs at midspan. Hence midspan is the most critical location in three point bending. However it has the disadvantage of high shear stress near the load application point. In four point bending, two point loads are applied. The span between the application of these two loads is free from shear stresses. So deflection due to bending can be studied more

safely. On the other hand these two loads need to be applied simultaneously and equally.

In order to achieve this, the application surface must be regular. However this is not the case in bone. Even with smoothening the surface, it is very difficult to achive perfectly symmetric loading. So three point bending test is more preferable than the four point one.

In this study Modulus of Elasticity of femur bone is calculated with two different methods.

In the deflection method, E is calculated from the derived equation given above. In this equation P and δ data are obtained from experiment, L is decided before testing, and I is calculated from the crossectional dimensions measured before the testing. From this method strain can be calculated indirectly (Turner and Burr 1993).

In the second method, Modulus of Elasticity is calculated with calculated stress and direct strain measurements obtained from strain gauges.

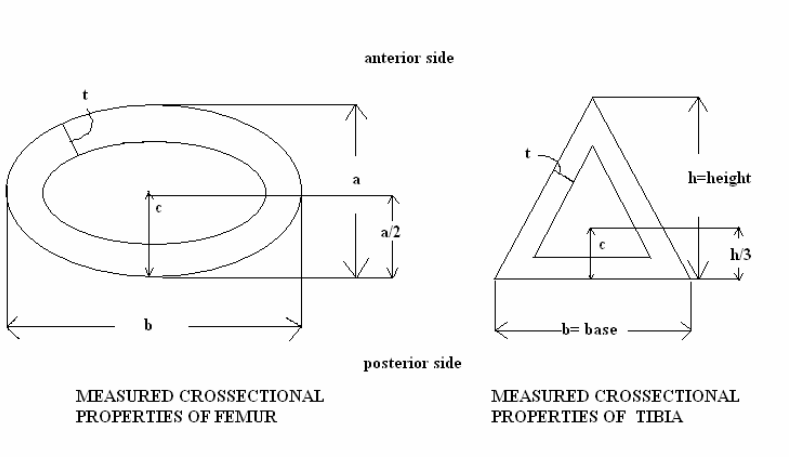


Figure 2.11. Measured Crossectional Dimensions

2.2.8.3.1 Calculating the Modulus Of Elasticity by Deflection Method

$$E = \frac{P \cdot L^3}{48 \cdot \delta \cdot I}$$

$$I = \frac{\pi}{64} \cdot \left[a \cdot b^3 - (a - 2 \cdot t) \cdot (b - 2 \cdot t) \right]$$

where :

P is the load at midspan

δ is the deflection at midspan

a is anterior posterior diameter

b is medialateral diameter

t is the thickness of the femur diaphysis (Figure 2.11)

2.2.8.3.2 Calculating the Modulus Of Elasticity with Direct Strain Gauge Data

$$M_{max} = M_{midshaft} = \frac{P \cdot L}{4}$$

$$\sigma = \frac{M \cdot c}{I}$$

$$E = \frac{\sigma}{\varepsilon}$$

where:

M is the bending moment at the midspan

P is the load at midspan

σ is the stress due to bending moment

c is the distance from the neutral axis to the outermost surface

The modulus of elasticity calculation for tibia is similar to the case in femur, except crossectional moment of inertia (I). I for tibia can be calculated as:

$$I = \frac{1}{36} \cdot \left[b \cdot h^3 - (b - 2 \cdot t) \cdot (h - 2 \cdot t)^3 \right]$$

where:

b is the base

h is the height

t is the thickness of triangular crossection

ε is the strain data obtained from experiment.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Three Point Bending (TPB) Test Results of Femur Bones

Data obtained from Lloyd Universal Testing Instrument were used to determine the mechanical properties of femur undergoing three point bending test. By using data obtained, load vs deflection curves were drawn as shown in Figure 3.1.

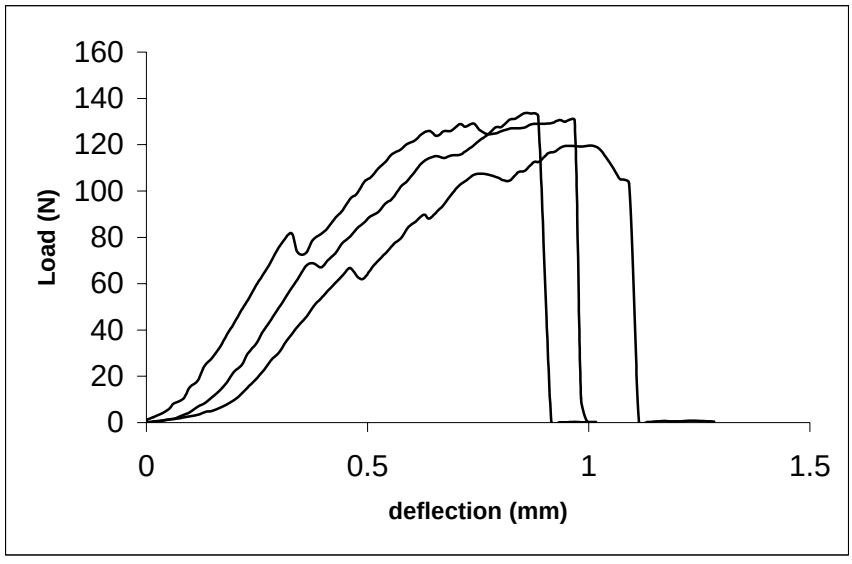


Figure 3.1. Typical Load vs Deflection Curves of Femora (three specimens from group R+K)

3.1.1. Comparison of Proportional Load Limit for All Study Groups Using Femur Three Point Bending Results

In Figure 3.2, Proportional load limit (PL) values of seven groups are shown. As can be seen in this Figure, E+R group has the maximum PL , the ovariectomized and untreated control group C has the minimum PL as expected. R group (those treated with raloxifen only) has the second highest PL value among all treatment groups and this value is also very close to that of sham operated group S. Comparing group R with group E, indicated that R results in a better PL value than single E, but their combination (E+R) is much more effective than the administration of these two drugs individually. Group K that was treated with VitK2 only, had the lowest PL value among the treatment groups. The combination group R+K performes much better than K but worse than R. Thus, it may be concluded that raloxifen involving groups have the three highest PL values among the treatment groups. Above results also indicate that the drug additivity in E+R combination is not encountered in R+K combination in femur bones. Statistically group mean values are not different from each other. Tukey's Multiple comparison test for the groups also resulted with $p>0.05$.

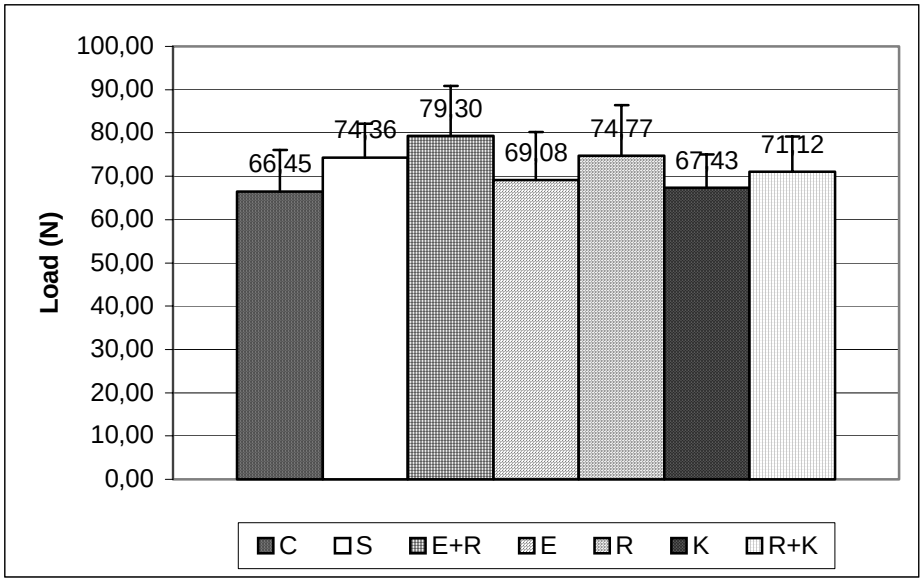


Figure 3.2. Comparison of Proportional Limits of All Groups' Femora Under Three Point Bending Test {(E+R)>S>R>(R+K)>E>K>C}

In the statistical analysis, one-way analysis of variance (ONE WAY ANOVA) and, Tukey's Multiple Comparison Test is used for the post-test evaluations.

3.1.2. Comparison of Yield Load for All Study Groups Using Femur Three Point Bending Results

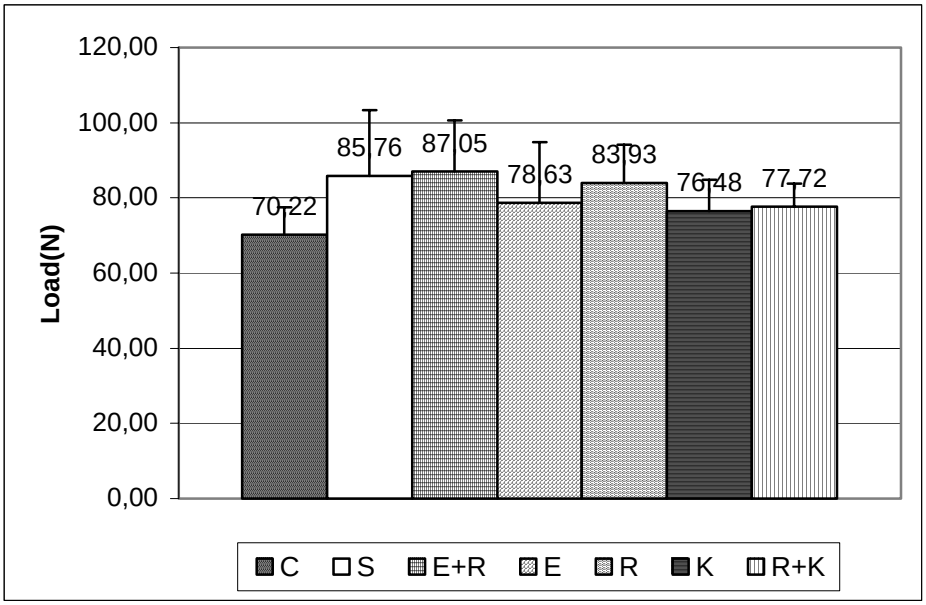


Figure 3.3. Comparison of Yield Load Results of All Groups' Femora Under Three Point Bending Test {(E+R)>S>R>E>(R+K)>K>C}

As seen in Figures 3.2 and 3.3, yield load (YL) of all groups behave similar to PL. E+R has the highest YL value possibly due to drug additivity. As in PL case, Group R is higher than group E. Unlike in PL, YL of E slightly exceeds R+K combination. Group K, has the lowest YL, but its value is much closer to treatment groups than C-the untreated control. These PL and YL analysis indicate Vit K2 has positive effect on femur to some extent. However, as in the previous case, all the differences were statistically insignificant ($p>0.05$).

3.1.8. Comparison of Yield Strength for All Study Groups Using Femur Three Point Bending Results

As shown in Figure 3.4, sham operated group S has the maximum yield strength YS. So it can be said that this group has the strongest bone among all. Group C, which is the ovariectomized untreated control group has the lowest value as expected and the difference between these two groups is statistically significant with $p<0.05$. Other significant differences were obtained between groups S vs R+K, and groups S vs K ($p<0.05$). K has the smallest yield strength value within treatments. Group E has the best yield strength value of treatment groups. While group R is in the second place, the combination E+R follows the individual administrations of E and R. However, the differences between E, R, E+R are not significant ($p>0.05$). The differences between the group mean values, however, are statistically significant ($p<0.01$).

Thus, it can be concluded from these results that VitK2 supplementation is not as efficient as estrogen or raloxifen on osteoporotic bone, but still providing some degree of protection or healing that is not encountered in the untreated group.

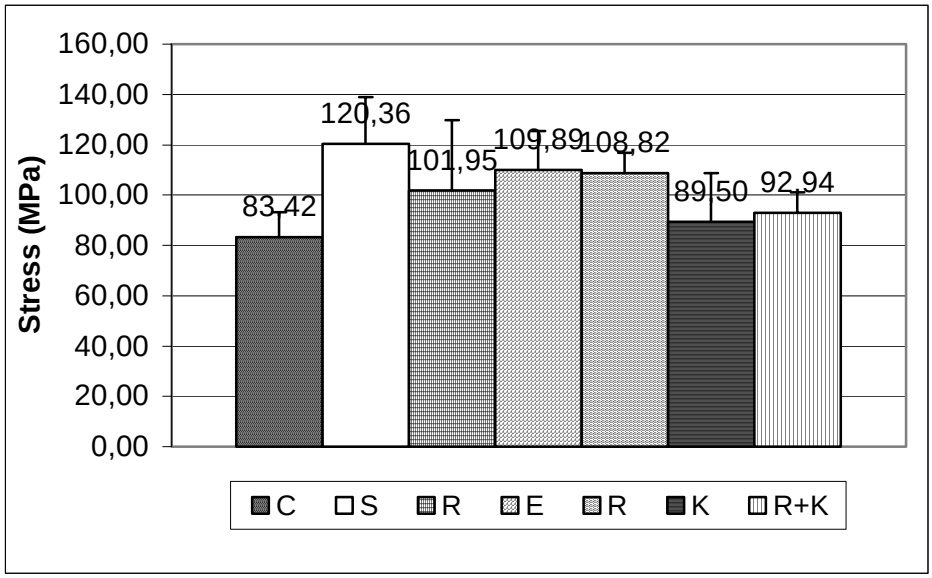


Figure 3.4. Comparison of Yield Stress of All Groups' Femora Under Three Point Bending Test {S>E>R>(E+R)>(R+K)>K>C}

3.1.9. Comparison of Modulus of Elasticity for All Study Groups Using Femur Three Point Bending Results

According to the results of Modulus of Elasticity (E) for all groups (Figure 3.5) the highest modulus belongs to S and the lowest belongs to C. Among the treatment groups, R has the highest modulus of elasticity, E has the second, and R+K has the third highest modulus. But these differences are again statistically insignificant. ($p > 0.05$)

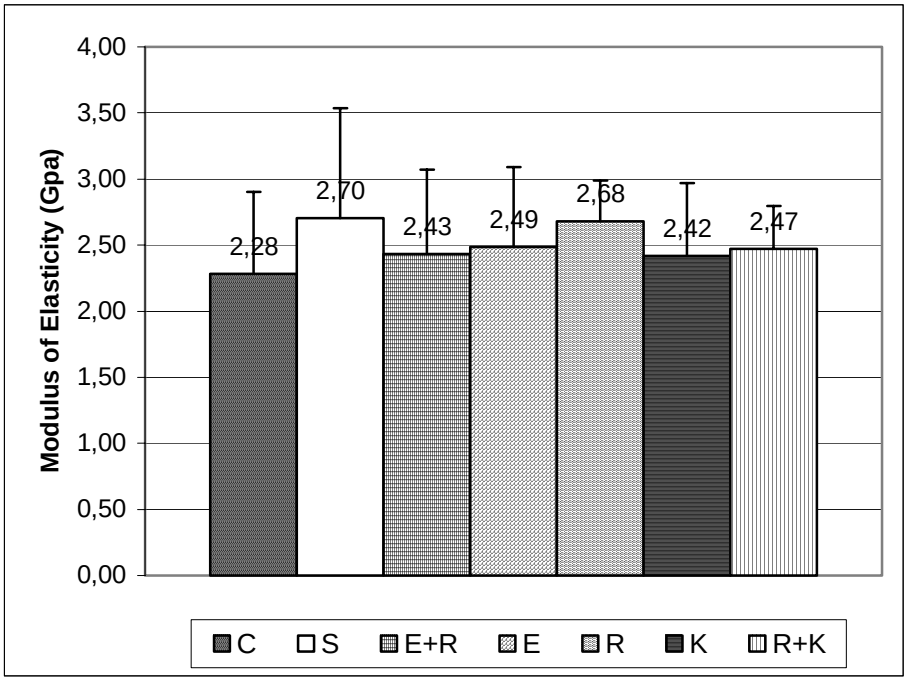


Figure 3.5. Comparison of Modulus of Elasticity of All Groups' Femora Under Three Point Bending Test {S>R>E>(R+K)>(E+R)>K>C}

According to the Modulus of Elasticity results, drug performance order has changed most probably as a result of involvment of deflection parameter in the modulus calculations. In addition to this, any wrong measurement of a specimen's geometrical property (diameters, thickness etc.) is multiplied with an order of 3 in

numerator and degree of 4 in denominator I. (Theory for the equation, $E=P*L^3/(48*\delta*I)$, was given in the Materials and Methods Chapter).

So strength values may be a more reliable parameter than modulus of elasticity especially for such small specimens.

3.1.10. Comparison of Maximum Load for All Study Groups Using Femur Three Point Bending Results

The combination group E+R has the highest maximum load (ML), with the other combination R+K being the second among all groups (Figure 3.6).

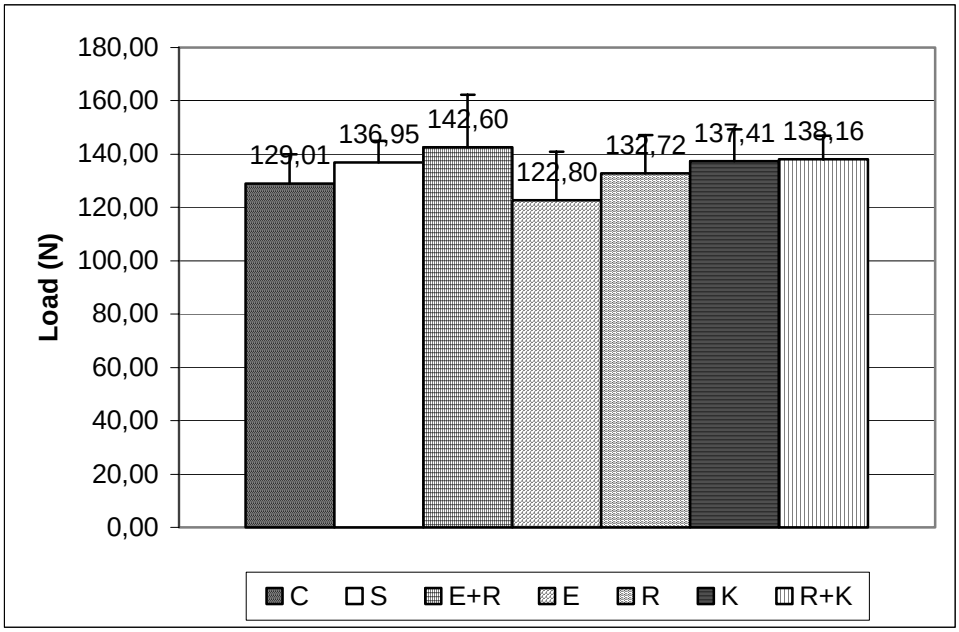


Figure 3.6. Comparison of Maximum Load of All Groups' Femora Under Three Point Bending Test {(E+R)>K>(R+K)>S>R>E>C}

Interestingly, in ultimate load analysis, group K comes into picture by being as effective as other approved ostoporosis drugs. It gives the third highest ultimate load results of all groups with its single administration. It is followed by Sham operated group, S. Ultimate load results of E treatment group was found to have the lowest value when administered alone, thereby, indicating the importance of combination with R.

Raloxifen combinations are again the highest values and raloxifen interaction with VitK2 also works at the ultimate stage of loading.

Hence it can be concluded that the risk of fracture is the least in raloxifen treated combination groups and K treated groups. From this conclusion it can be suggested that raloxifen is more beneficial in skeletal metabolism when used together with these agents. VitK2 seems to provide a specific effect on femur as load approaches to its maximum capacity in bending.

3.1.11. Comparison of Energy Absorped In Elastic Region for All Study Groups Using Femur Three Point Bending Results

As shown in Figure 3.7, E+R and R have the first and second highest energy absorption in their elastic range, respectively. Group S is the third and R+K is the fourth in this ordering. VitK2 treatment groups have the lowest energy absorption before yielding.

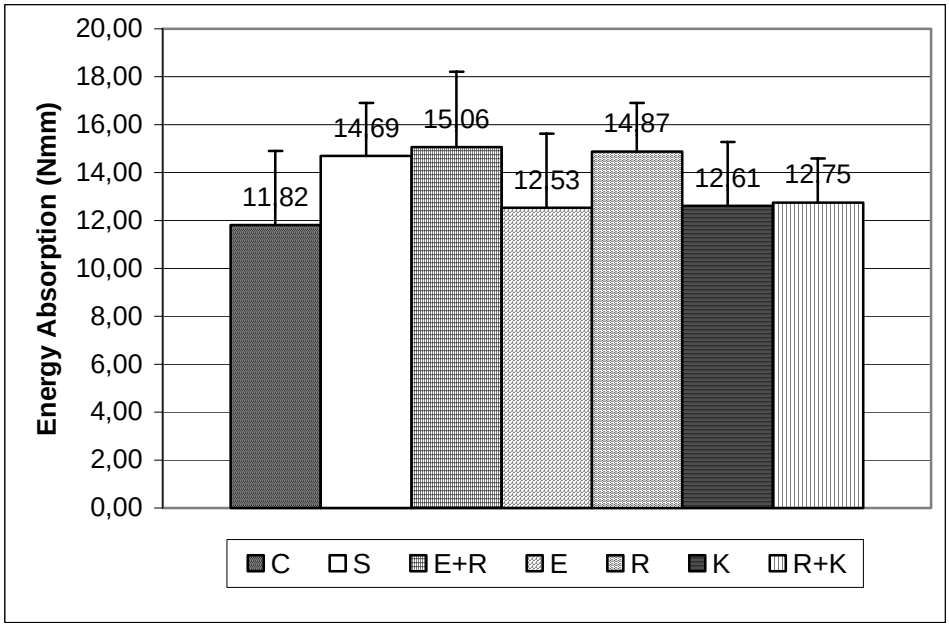


Figure 3.7. Comparison of Energy Absorption In Elastic Region of All Groups' Femora Under Three Point Bending Test {(E+R)>R>S>(R+K)>K>E>C} ($p=0.1943 > 0.05$)

Group E+R absorbed the maximum energy upto elastic limit. While group R has second and R+K results in third highest value in total energy absorption. Single

raloxifen application absorbs less energy than the combination group. Raloxifen and its combinations perform better than E and even greater than S. This proves that raloxifen has not only preventive but also treatment effect.

The same result was concluded with another study carried out on osteoporotic bone by (Michele et al., 2003).

3.1.12. Comparison of Energy Absorption Until Fracture for All Study Groups Using Femur Three Point Bending Results

As shown in Figure 3.8 energy absorption until fracture results show that E+R has the best energy absorbed among treatment groups while E and R are slightly less than the combination. R+K also has high energy absorption capacity. So combinations are also effective in energy absorption until fracture.

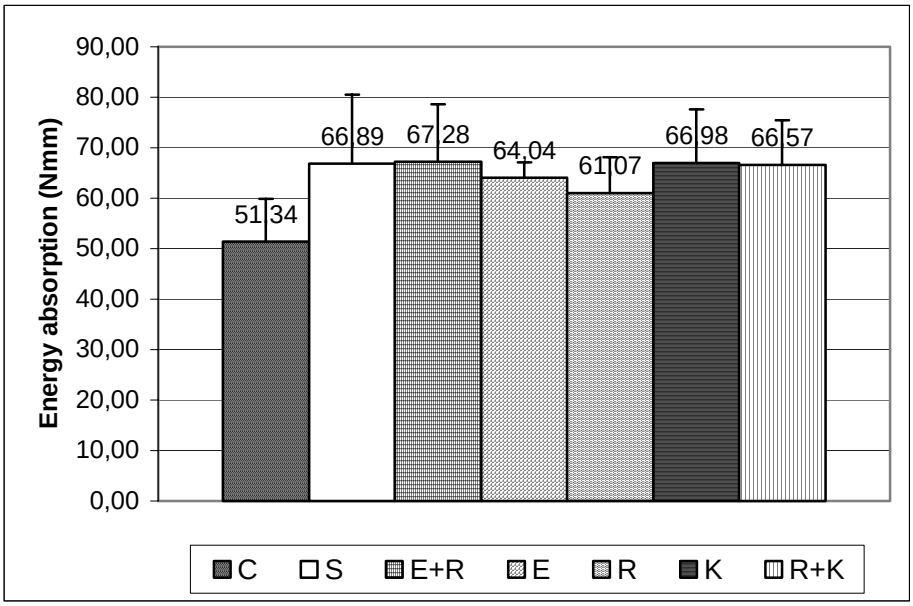


Figure 3.8. Comparison of Energy Absorption Until Fracture of All Groups' Femora Under Three Point Bending Test{(E+R)>K>S>(R+K)>E>R>C}

Raloxifen was pointed as being especially effective in reducing the fracture risk of trabecular structure of bone, like vertebral bone. But femur diaphysis –where the three point bending test is mainly acted on- is the cortical bone.

Considering results of mechanical test data it can be suggested that VitK2 has a good performance in overall bending of femur diaphysis and it is effective in cortical bone in femur. A similar conclusion was also made by Iwamoto et al, (2003) from BMD and histological results.

All treatment groups and sham group S are close to each other ($p>0.05$), and they are all better than C. It is important to notify that, since there is no load transfer from trabecular to cortical region, in three point bending of femur, trabecular bone resorption can not be measured with the above mechanical behaviours of femur. Hence, with TPB tests it is not expected to evaluate the osteoporosis in trabecular bone.

3.1.8. Comparison of All Mechanical Properties of Femur Bones Measured with Three Point Bending Test

Table (3.1) summarizes the three point bending behaviour of femur diaphysis for all study groups.

From the numerical results of mechanical properties obtained by femur three-point bending tests following evaluations can be made:

Raloxifen and estrogen are effective individually in osteoporosis prevention because, in all mechanical properties calculated their values are greater than ovariectomized-untreated control group C. Their individual performances are moderate in low stress levels and slightly less than moderate in high stress levels. Raloxifen and estrogen combination has not only preventive but also treatment effect, since 5 out of 7 analysis, the values of this combination are larger than the unoperated and untreated baseline group, S. This combination is even greater than the uses of these agents alone in 5 out of 7 cases. This combination performs well both in low stress levels (YL, YS) and high stress levels (ML).

Therefore, it can be concluded that the combination is effective in cortical bone matrix in all stress levels. So, it may be a good approach to use them in combination form in treatments and in preventive therapies.

Table 3.1. Overall Mechanical Testing Results of Femur

FEMUR TPB	PL (N)	YL (N)	YS (MPA)	E (GPA)	ML (N)	ENERGY-ELASTIC (NMM)	ENERGY-FRCT. (NMM)
C	66.45	70.22	83.42	2.28	129.01	11.82	51.34
S	74.36	85.76	120.36	2.70	136.95	14.69	66.89
E+R	79.30	87.05	101.95	2.43	142.60	15.06	67.28
E	69.08	78.63	109.89	2.49	122.80	12.53	64.04
R	74.77	83.93	108.82	2.68	132.72	14.87	61.07
K	67.43	76.48	89.50	2.42	137.41	12.61	66.98
R+K	71.12	77.72	92.94	2.47	138.16	12.75	66.57

Owing to known protective property against osteoporosis, the response of raloxifen, estrogen and their combinations to mechanical stress would be significantly larger if the treatments had started just after the ovariectomy. Demster et al. (1995) has shown that osteoclast surface is maximum at the 35th day postoperatively. Moreover, bone formation rate is increased (in order to compensate highly increased the octeoclast activity) and reaches to its maximum at the 30 th day postoperatively.

Vitamin K effect is little in low stress levels, but its performance is much better than individual performances of raloxifen and estrogen in high stress levels. Also by considering the previous study results (Kafantari et al., 2000), it can be suggested that VitK2 functions in the orientation of collagen fibers in cortical bone that makes bone stronger under high bending stress levels. Kafantari et al, proved in their studies that estrogen deficiency due to ovariectomy causes bone collagen

decline. Martin and Boardman also showed (1993) that collagen fiber orientation is a strong parameter determining the bending stiffness of bovine bone. It is also thought that both trabecular and cortical bone are affected from the collagen orientation property of the bone. Binkley et al. (2002) found out that intermolecular crosslinks are responsible for the mechanical strength of collagen and Kafantari et al. (2000) states that any reduction in fibril crosslinking causes rapid loss of fibril stabilization and, consequently decreased mechanical properties of bone.

Vit K2 effect on bone quantity can be investigated more precisely by using pQCT since it is a more sensitive method than the other quantity measuring devices. It is also important to notice that most studies suggest VitK2 as being more effective in bone quality rather than bone quantity (http://www.vitaspace.com).

VitK2 combination with raloxifen also performs better than individual performances of K and R. In ML it exceeds the others, so can be also considered as effective in high stress levels. However, when compared with E+R combination, R+K performance falls behind of it.

3.3. Calculating the Modulus of Elasticity with Direct Strain Measurements

3.3.1. Comparison of Modulus of Elasticity for All Study Groups Using Femur Three Point Bending Results

In the previous modulus of elasticity calculations, load-deflection data collected from Lloyd Universal Testing Instrument were used in flexural deflection equation. As explained in Materials and Methods there is no strain variable in this equation. So, in this approach strain value is neither calculated nor measured.

In the second test method, strain data was simultaneously collected via a strain indicator, while the same load-deflection data was being collected with Llyod Instrument. By this way strain values could be directly measured for each specimen.

At the end of this dual process, load-strain data for each specimen were obtained. After calculating the stresses and dividing these values to corresponding strains, modulus of elasticity values were calculated (Figure 3.9).

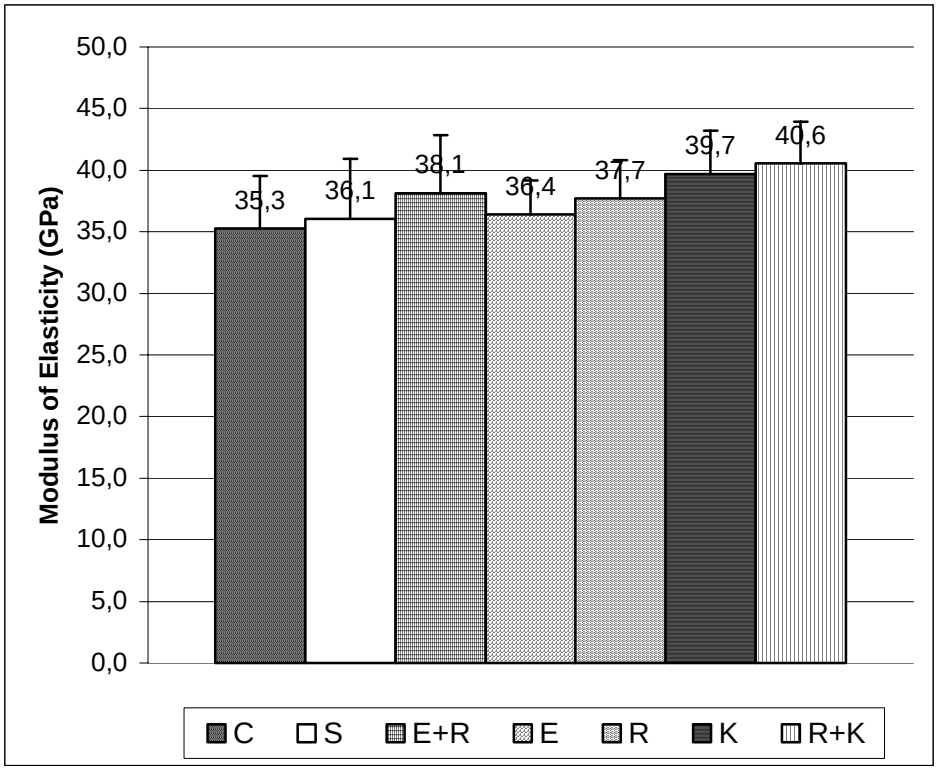


Figure 3.9. Comparison of Young's Modulus of Elasticity for All Groups' Femora Under Three Point Bending Test (calculation with directly collected strain data from strain indicator){(R+K) > K>(E+R)> R >E > S >C }(p=0,5438>0.05)

Comparing the modulus of elasticity for all groups shows that p value is greater than 0.05 for mean values and multiple comparison of groups are not significantly different from each other (as in the case of previous calculation).

However, the mean values of these two approaches were different from each other significantly. In fact some studies in literature also found significantly different results between direct strain approach and beam-deflection approach (Table 3.2).

Turner (1993) observed the difference in moduli of elasticitiy obtained from gauge application in one study and deflection formula used in another study on rat tibia. This work was thought to compare the elastic moduli of two approaches in one study.

Table 3.2. Comparison of Modulus of Elasticity Calculations within Themselves and with Literature

BONE TYPE	FEMUR			TIBIA		
	This Study		Erickson et al.*** (2002)	This Study	Turner et al. (1991)	Akhter et al.*** (1992)
	Ed*	Es*	Es	Ed*	Ed	Es
	(GPa)	(GPa)	(GPa)	(GPa)	(GPa)	(GPa)
Mean	2,70	36,1	26,0	5,53	7,6	29,4
sd	0,55	4,8	7,3	1,40	- **	- **

Ed: Modulus of elasticity calculated by deflection formula in beam theory

Es: Modulus of elasticity calculated with direct strain data obtained from strain indicator

*All of these values belong to sham operated control group, S

** No standard deviation was mentioned for that study

*** Strain gauge application was done in vivo

It was also aimed to see the ultimate behaviour of rat femur in bending and obtain the ultimate strain values via strain gauges. In studies done with other species (ex. sheep), obtaining the ultimate strain via diectly bonded strain gauge would not be seen, since the gauge reached to its ultimate strain before the specimen. So some other techniques were developed like strain clips (Whan et al,.2003). However in this study no such problem occured in strain gauge applications, meaning that strain gauge performed well till the ultimate strength of the rat femur. Ultimate strain of rat femur, obtained via strain gauge in this study could not be compared with other works, since such a trial was not encountered in literature. So this study seems to be one of the few strain gauge studies on rat bone in literature. Other few strain gauge studies have been done on femur in vivo, and for small strain activities, like exercising wheel (Keller, 1982)(Spendler,1989)(Erickson et al., 2002)(Akhter et al., 1992). This work is intentionally done in vitro in order to see the difference between deflection approach and direct strain approach more clearly.

This difference between the two approaches can be explained with the reasons listed on the following page:

Great amount of deformation occurs where the loading unit is in contact with bone surface (Turner and Burr,1993). This causes the header to sense a deflection larger than the actual one on loading site. This situation leads to overestimation of bone deflection in tension side. It can be seen more clearly by comparing the elastic limit strains calculated by strain formula given by Turner and Burr (1993), and measured with strain gauge, they are 34800 με (sd.8000) vs 2540 με (sd.460) respectively.

It is argued that if loading span L is very short, shear stresses will cause 10 % additional deflection (Akhter et al., 1992). It is also added that for bone specimens the ratio of length to width would be 20:1 to guarantee insignificant shear deflection (Levenston, 1995). In this study set up and span dimensions were decided according the recommendatitons made in literature for proper three point bending test (Turner and Burr, 2000). Then, ratio of shear deflection to bending deflection was calculated analytically, and it was seen that the contribution of shear deflection did not exceed 2.9 % (APPENDIX) which is in agreement with the result given by C.K.Wang, 1983. Hence the shear contribution was neglected in calculations.

Study conducted by Erickson et al.(2002) differs from this study as far as the procedure and the condition (ie. in vivo versus in vitro). The difference in elastic moduli might be the result of this fact. In literature no in vitro strain gauge study on rat femur subjected to three point bending was encountered.

Direction and shifted location of gauge, gauge reinforcement behaviour on bone specimen, electrical and magnetic sound errors, noise due to welding, vibration etc. (Dally and Riley, 1991) may be effective in results. However careful handling and obeying the gauge instructions, these factors can be overcome.

Sources of errors might include the assumptions made during the application of linear bending theory, like constant bone crosssection throughout the bone, plane perpendicular to longitudinal direction remains plane after the loading (hence Poisson's effect is neglected), strain varies linearly and proportional to the distance from the neutral axis, Hooke's Law is valid throughout the whole section. (Cordey and Gautier, 1999) (Gere and Timoshenko, 1990). However, heterogeneous, anisotropic, and viscoelastic characteristics of bone tissue may be effective.

At the end of this discussion, it was realized that results obtained from experimental data are much more reliable than theoretical models if they are carefully performed.

It may be thought that the results are reliable, despite mentioned sources of errors, since all experiments in this study were done by the same researcher under similar conditions.

3.3. Three Point Bending Test Results of Tibia Bone

3.3.1. Comparison of Ultimate Strength for All Study Groups Using Tibia Three Point Bending Test Results

As demonstrated in the Figure 3.10, tibia specimens do not have a yield point in bending. The load deflection curve rise upto a maximum load level and then falls suddenly at break providing the ultimate strength value. Thus, indicating that femur and tibia have anatomically different bone structures.

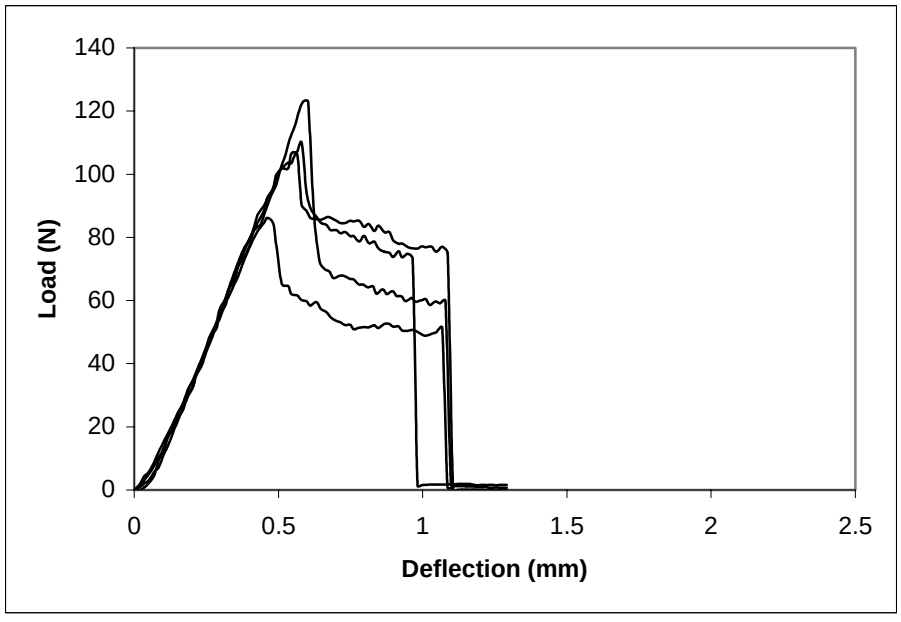


Figure 3.10. The Typical Brittle Failure Behaviour of Tibia for Group K.

This figure also shows the more brittle behaviour of tibia compared to femur. So, instead of proportional limit and yield calculations, the values of the

maximum loading point were computed. Since they reach to maximum load level with a linear proportionality, where the deflection formula valid for elastic range is applicable.

Maximum stress, the ultimate value of stress that a specimen can carry under bending, for all groups, has shown in Figure 3.11. E+R has the largest maximum strength among all treatment and control groups.

The second maximum strength group is the other combination, R+K. The order continues with R and E as being the third and fourth values respectively. Considering all results up to this point, it can be said that raloxifen is both effective in combination groups or in its single supplement in tibia under bending. Estrogen is also effective but its effect is relatively lower than raloxifen. VitK2 locates just between the control and the baseline group implying that it is the least effective single supplementation among these three drugs, yet effective compared to untreated controls.

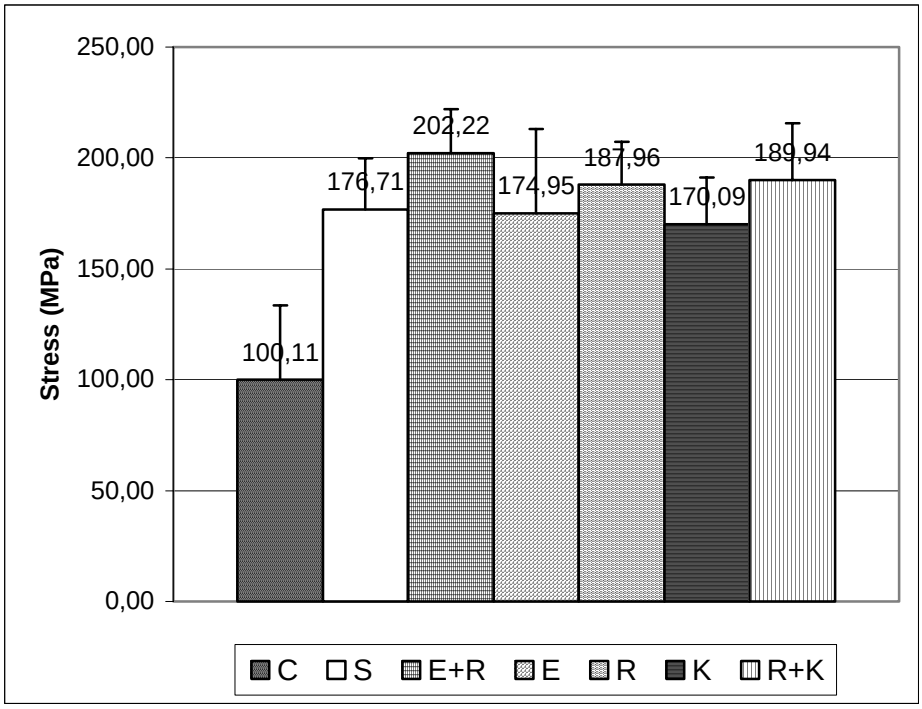


Figure 3.11. Comparison of Ultimate Strength of All Groups' Tibia Under Three Point Bending Test {(E+R)>(R+K)>R>E>S>K>C}($p<0.001$ between group C vs other groups)

The additive effect of Vit K2 may be much more significant if the rats were fed with Ca or Vit D deficient diet as Mawatari et al. (2000), and Akiyama et al. (1999), has done in their study with ovariectomized rats, Mawatari investigated the effect of VitK2 with Ca deficient diet. They started treatment just after ovariectomy and continued for 8 weeks as done in our study. They observed significant increase in trabecular architecture.

Iwamoto et. al., (2003) applied VitK2 on young orchidectomized (ORX) rats and they did bone histomorphometry study on tibial shaft which is mainly cortical and tibia proximal which is mainly trabecular. They found out that histologically VitK2 shows its effect more stongly on cancellous sites than cortical sites. They also mentioned that VitK2 affects protectively on the cancellous bone by normalizing the increased eroded surface to bone surface (ES/BS) ratio. Iwamoto also stated that even at high dose of VitK2, the effect on cancellous bone is mainly the suppression of bone resorption (ie. an antiresorptive effect) rather than stimulation of bone formation. This high dose VitK2 effect on cortical bone was explained with its suppression of osteoclastic activity. Supporting this idea, Kameda et al., suggested the possibility of inhibition of osteoclastic bone resorption by VitK2 through targetting osteoclasts to undergo apoptosis, at which cells are lead to death.

Although the differences between treated groups are not statistically different from each other and sham operated group ($p>0.05$), all of the treated groups as well as sham operated group were significantly different than the untreated control ($p<0.001$ multiple comparison of C with other groups). This implies that tibia is highly degenerated by osteoporosis that occurred after the ovariectomy procedure Thompson et al.,(1995) also found high tibial degeneration owing to osteoporosis created by ovariectomy in rats.

3.3.2. Comparison of Modulus of Elasticity for All Study Groups Using Tibia Three Point Bending Test Results

Moduli of elasticity were compared for all groups in Figure 3.12. Combination groups are again the highest values among all groups.

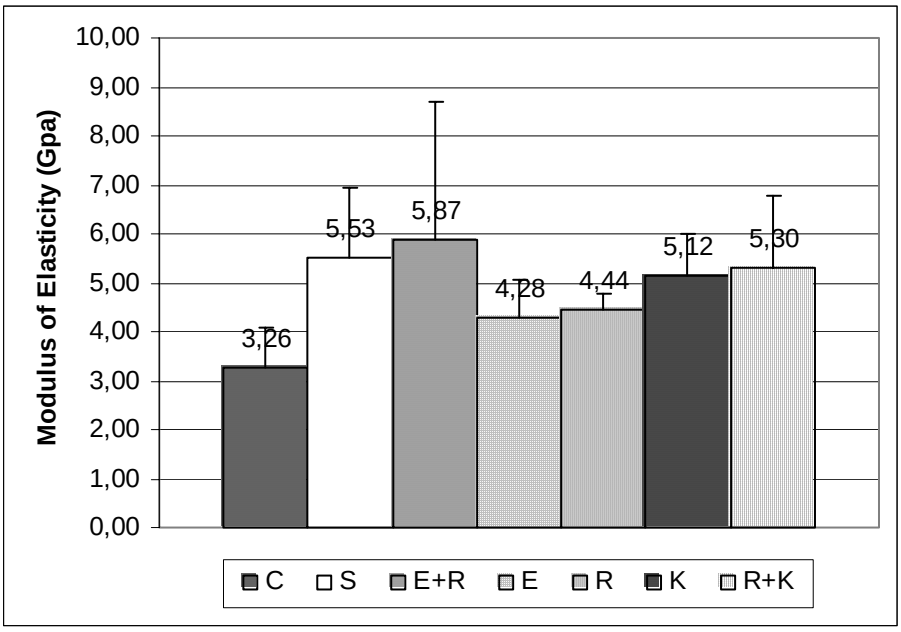


Figure 3.12. Comparison of Modului of Elasticity of All Groups' Tibia Under Three Point Bending Test {(E+R)>S>(R+K)>K>R>E>C}

VitK2 individual supplementation was also found to be the highest among single drug administrations. So, it can be concluded that R is effective in combination groups and VitK2 is effective in both individual and combination supplements ($p = 0.1180 > 0.05$).

3.3.3. Comparison of Energy Absorption Until Fracture for All Study Groups Using Tibia Three Point Bending Test Results

Energy absorption until fracture or the total energy required to fracture the material was compared for all groups in Figure 3.13.

The maximum energy required to fracture was observed for the E+R group, followed by the individual drug treatments.

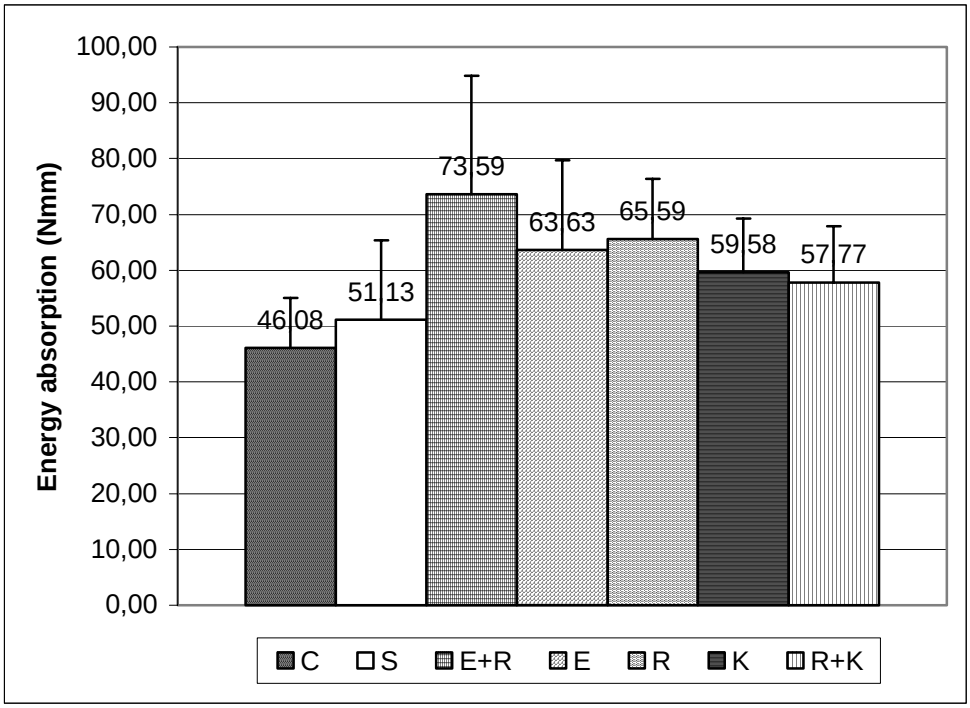


Figure 3.13. Comparison of Energy Absorption Until Fracture of All Groups' Tibia Under Three Point Bending Test {(E+R)>R>E>K>(R+K)>S>C}, ($p = 0.0255 < 0.05$ E+R and other groups

R+K combination, however, requires the least amount of energy to fracture the tibia. But the E+R combination is also statistically different from all groups. By looking at the results of modulus, ultimate strength and energy absorption data, it can be clearly said that E+R drug interaction is highly effective in tibia.

3.3.4. Comparison of All Mechanical Properties of Tibia Bones Measured with Three Point Bending Test Test

Table 3.3 summarizes the three point bending behaviour of tibia for all study groups.

From the tibia mechanical testing results, it is easily observable that ovariectomy extremely weakens tibia. Especially in ultimate strength analysis, mechanical results of ovariectomized group C is significantly less than sham and treatment groups with very small p values (<0.001).

Table 3.3. Three Point Bending Results of Tibia

TIBIA	ULTIMATE STRENGTH (MPA)	MODULUS OF ELASTICITY (GPA)	E-ABS FRACTURE (NMM)
C	**100,11**	3,26	46,08
S	176,71	5,53	51,13
E+R	202,22	5,87	**73,59**
E	174,95	4,28	63,63
R	187,96	4,44	65,59
K	170,09	5,12	59,58
R+K	189,94	5,30	57,77

On the other hand E+R combination seems to be the most efficient treatment for tibial osteoporosis. Because after 3 months of untreated menapousal stage, E+R treated group performes significantly better than unoperated group. It also behaves significantly better than all other treatment groups ($p = 0.0255 < 0.5$).

The other combination group R+K seems to be the second effective therapy for this study. In tibia analysis, fracture was occured without yielding. This means tibia was subjected directly to high stress levels. This property is similar with the R+K's femur performance being high at high stress levels despite low measures in other mechanical properties. So, it can be suggested that this combination is also effective in high stress levels of tibia.

When evaluating the individual performances, R appears in the first place (as in femur case), K is second (unlikely from femur case), and E takes the third. The reason of why VitK2 behaves better than estrogen in individual administrations in tibia may be again explained by its positive effect in high stress situations. It may have an effect on collagen fiber orientation in cortical bone.

Biomechanical testing is the only method of measuring whole bone's response to load, and simulating the effect of load on bone in real conditions. Meulen et al.,(2001) mentioned that looking at only mineral contents or geometrical properties do not give information about structural integrity of bone under mechanical loads and thus might be misleading and whole bone bearing capacity can only be precisely assessed with mechanical testing. So biomechanical results are important in terms of mimicing the response of treated osteoporotic bone under loading. As Ferretti et al.(1995) and Jämsä et al. (1998) stated, crossectional architecture of bone is an important parameter in bending and stiffness. The axial crossectional moment of inertia, is a good geometric parameter indicating the architectural efficiency or abnormality of femoral crossectional structure (Crenshaw et al., 1981)(Gasser et al.,1995).

3.4. Results of Bone Mass Density (BMD) Measurements

As mentioned before bone proximal and distal metaphysial sides have mainly trabecular, diaphysis (mid shaft) has mainly cortical structure. Thompson et. al. (1995) states that FDA Guidelines does not specify skeletal regions for optimal analysis of osteoporosis. However they also mentioned that OVX effect is generally investigated in different parts of the skeleton such as proximal tibia, distal femur and lumbar spine. The effect of osteoporosis is different in trabecular and cancellous parts of the skeleton (Omi et.al., 1995). So measuring mineral density for total bone may hidden the serious loss in some particular sites (ex: trabecular sites).

Due to this fact we devided femur and tibia into 6 parts as tibia proximal (TP), tibia distal (TD), tibia midshaft (TM), femur proximal (FP), femur distal (FD) and femur midshaft (FM) and while measuring their BMDs (Figures 3.13-3.17).

3.4.1. BMD Measurements of Tibia for All Study Groups

In Table 3.4 overall BMD results also exists for above mentioned six regions (TP, TD, TM, FP, FD, FM). It can be stated that group E+R has the maximum BMD values for trabecular proximal and distal of both femur and tibia. Thus, implying that this drug combination is significantly effective in trabecular sites.

This result is paralel to the E+R mechanical performance (Ito et al., 2003). Vit K and its combinations have relatively low BMD values in both femur and tibia. But their mechanical performances were better than control, C in all levels, and even better than some treatment groups in high stress levels. This situation may imply that Vit K2 and its combinations are more effective in bone matrix rather than mineral deposition. Mawatari et al., (2000) in their study obtained a significant increase in trabecular architecture. They also mentioned about an unknown mechanism of effect of Vit K2 on trabecular connectivity if treatment occurs after the loss of trabecular connectivity. Since this situation was satisfied in our case (with start of Vit K2 treatment after the trabecular bone loss -3 months postovariectomy), we might have obtained such results owing to this property of VitK2.

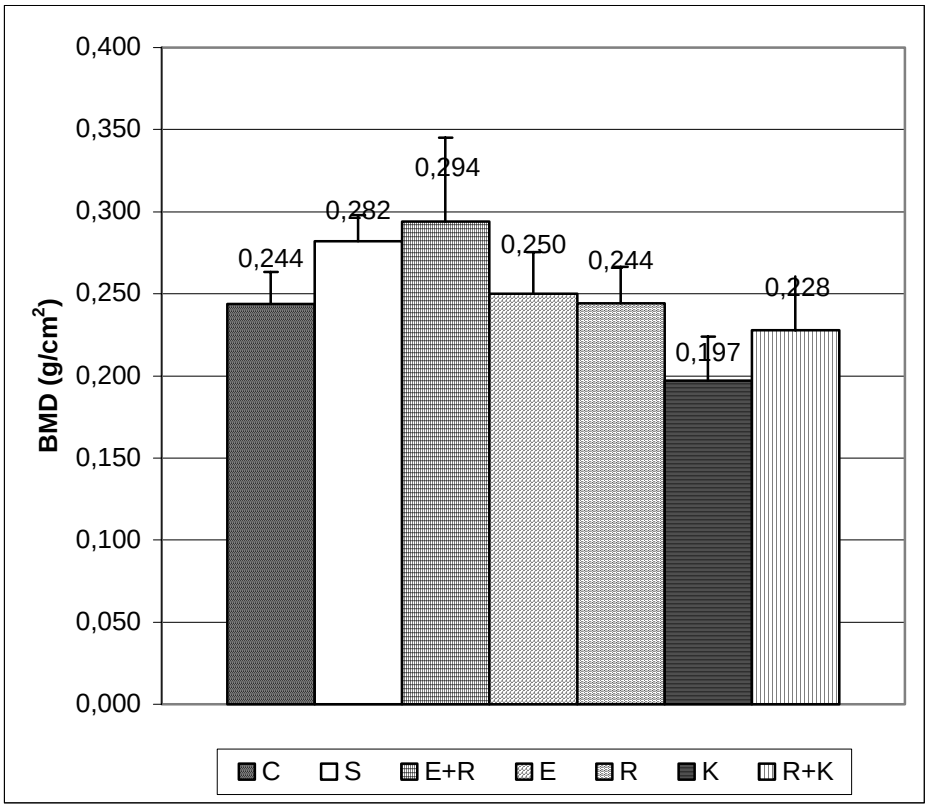


Figure 3.14. Comparison of BMDs of Proximal Tibia for All Groups
Means are different from each other ($p < 0.0001$), S and E+R different from K ($p < 0.001$), E+R different from R+K ($p < 0.001$).

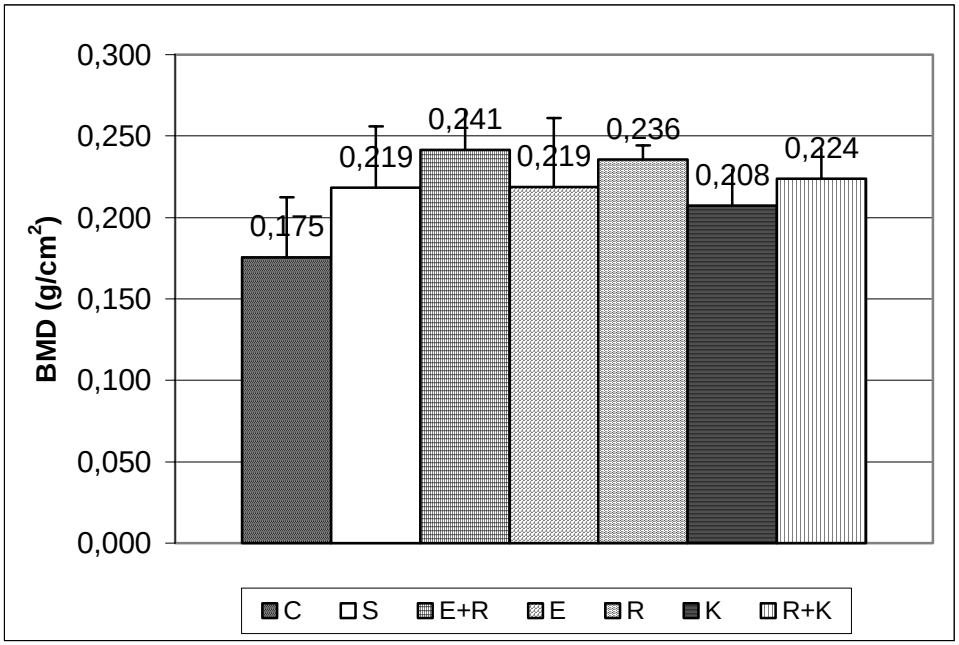


Figure 3.15. Comparison of BMDs of Distal Tibia for All Groups
Means are not different from each other ($p = 0.0605 < 0.05$);
E+R is different from C ($p < 0.05$)

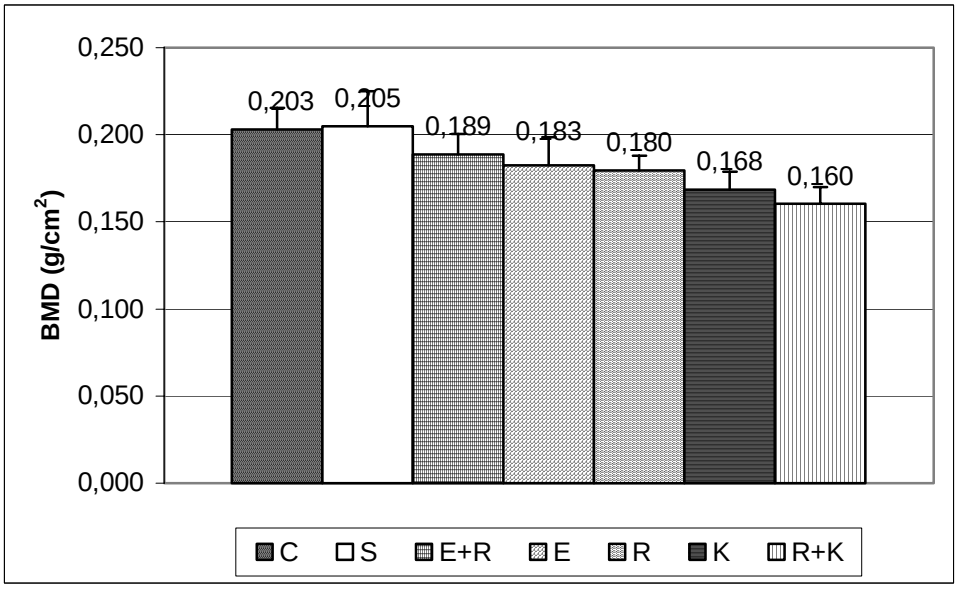


Figure 3.16. Comparison of BMDs of Midshaft Tibia for All Groups
Means are different from each other ($p < 0.0001$); C is different from K and R+K ($p < 0.001$);C is different from R ($p < 0.05$);S is different from R ($p < 0.05$); S is different from K and R+K ($p < 0.001$);E+R is different from R+K ($p < 0.01$)

3.4.2. BMD Measurements of Femur for All Study Groups

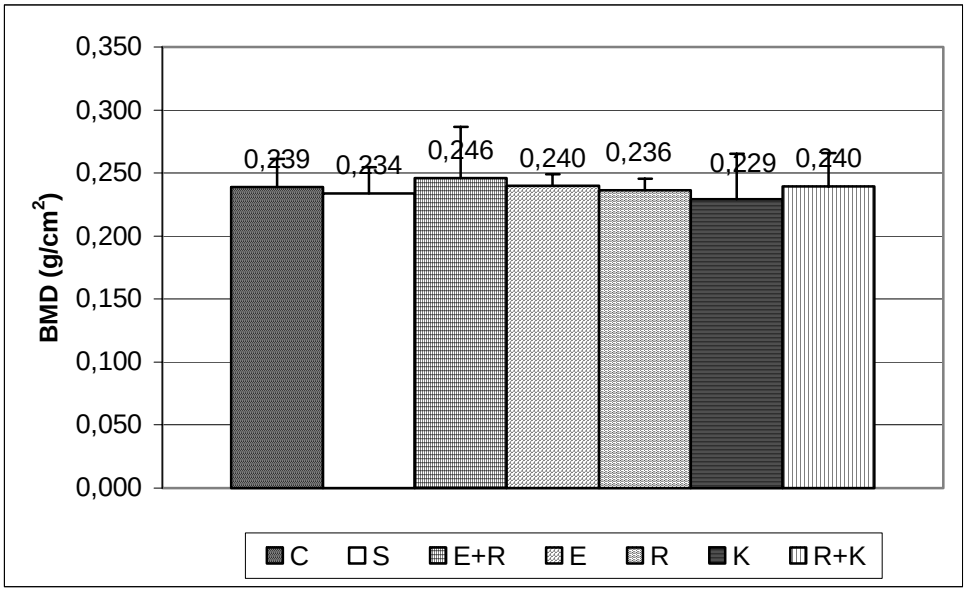


Figure 3.17. Comparison of BMDs of Proximal Femur for All Groups

Means are close to each other ($p = 0.9615 > 0.05$)

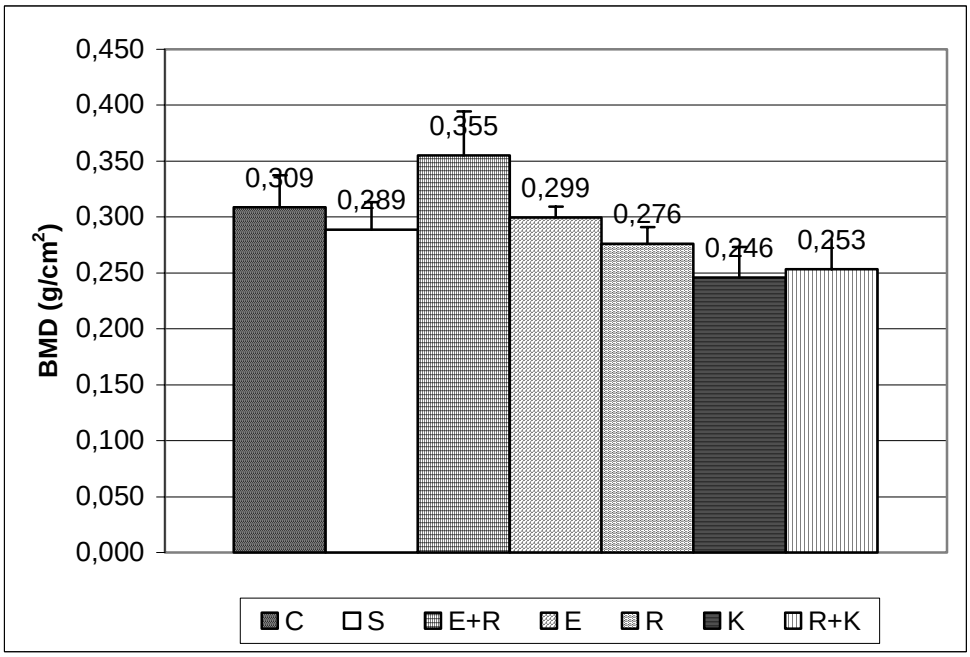


Figure 3.18. Comparison of BMDs of Distal Femur for All Groups

Means are different ($p < 0.0001$);C is different from K and R+K ($p < 0.05$), S is different from E+R ($p < 0.01$);E+R is different from E ($p < 0.05$), and R ($p < 0.001$) and K($p < 0.001$); E is different from K ($p < 0.05$)

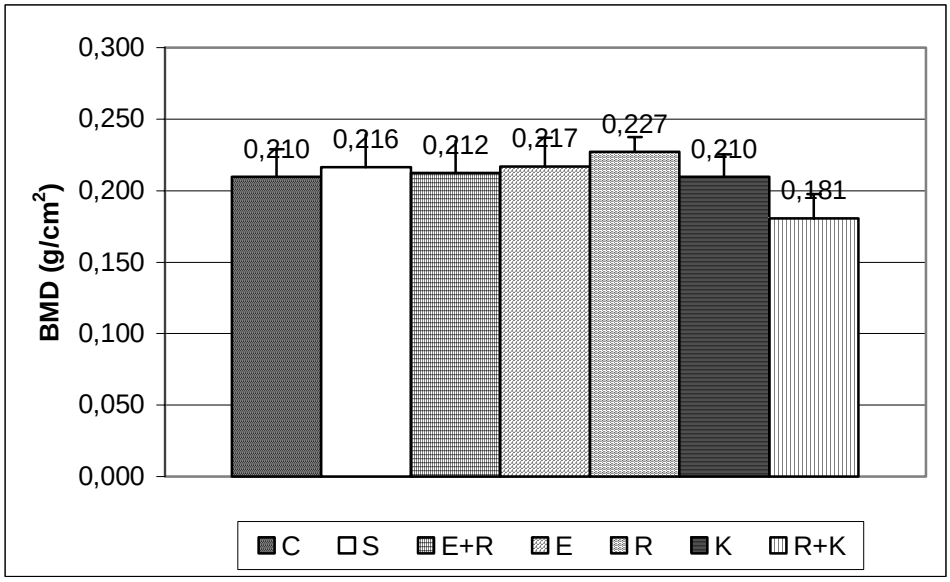


Figure 3.19. Comparison of BMDs of Midshaft Femur for All Groups
Means are different from each other ($p = 0.002 < 0.05$);
R+K is different from S ($p < 0.01$), E+R ($p < 0.05$), E ($p < 0.05$); and R ($p < 0.001$)

Table 3.4. Overall BMD Results of Tibia and Femur

BMD (G/CM^2)	TP	TD	TM	FP	FD	FM
C	0,244	0,175	0,203	0,239	0,309	0,21
S	0,282	0,219	0,205	0,234	0,289	0,216
E+R	0,294	0,241	0,189	0,246	0,355	0,212
E	0,25	0,219	0,183	0,24	0,299	0,217
R	0,244	0,236	0,18	0,236	0,276	0,227
K	0,197	0,208	0,168	0,229	0,246	0,21
R+K	0,228	0,224	0,16	0,24	0,253	0,181

On the other hand, ovx control group (C), unexpectedly, had 3 better BMD results than sham group, S and some treatment groups. A similar result was also demonstrated in a study by Turner et. al.(1993). They stated that bone enlargement exists in ovariectomized rat resulting in increase in BMD values without retaining

the mechanical properties. So it can be said that only BMD results can not be informative enough and even might be misleading in the evaluation of bone mechanical performance as Meulen et. al. has also mentioned in 2001.

Peripheral Quantitative Computed Tomography (pQCT) System has been approved to be an effective tool in evaluating geometric properties of bone in experimental studies (Jamsa et al., 1998)(Ferretti et al., l993). So it can be used to estimate biomechanical parameters of bone for drug analysis, since it is more sensitive than any other quantity determining tools as explained in CHAPTER I.

Eventually from these results we suggest that raloxifen, estrogen and VitK2 is effective in preventive bone loss. But when raloxifene combines with other agents like estrogen and VitK2, it creates different outcomes owing to drug interactions. Thus, these combined therapies (especially raloxifen estrogen combination) are thought to have treatment effect in both bone strength and quantitative structure. A combination effect with raloxifene was also observed by Johnell et al., (2002). They used alendronate – raloxifen combination in their studies and found out that the combination group has superior quantitative parameters than their individual contribution.

3.5. Results of Biochemical Analysis of Blood

Alkaline phosphatase (ALP) activity in blood is seen as an indication of osteoblastic effect so it is used as bone formation marker. Hence its level is relatively more important than Ca, Mg and P levels in the blood of osteoporotic patients. As shown in Table 3.5 alkaline phosphatase is maximum in R group, this can be expected since Martel et.al. (2000) states that raloxifen stimulates estrogen sensitive parameter, ALP, in human endometrial cells. Numerically, ALP in other treatment groups are higher than controls but these differences are not significant. The reason of this insignificance may be the result of the development of a new steady state of remodelling homeostasis as stated by (Thompson et. Al., 1995) or some differential drug interactions at different sites.

Table 3.5. Biochemical Analysis of Blood Samples of All Study Groups

BLOOD	CA (MG/DL)	P (MG/DL)	MG (MG/DL)	ALP (G/L)
C	9,52	6,74	2,14	200,67
S	9,88	7,76	2,36	214,50
E+R	10,53	12,28	3,45	220,14
E	11,40	11,80	3,37	267,25
R	10,63	7,87	2,46	457,86*
K	9,80	7,51	2,39	289,00
R+K	9,58	7,10	2,20	282,67

Statistical comparisons

Ca : groups are not different p=0,0635>0.05

P : groups are different p=0,0036<0.05

Mg : groups are different p<0,0001

Alp :*Groups are different from each other (p<0,0001),

R is different from all other six groups (p<0.01)

All blood values are minimum in control group.

On the other hand Demster et.al. (1995) also emphasized that remodelling of rat metaphysis is difficult to determine, and therefore the osteoblastic effects of drugs cannot be defined easily with biochemical parameters. As also known from studies involving clinical data, blood measurements for osteoporosis determination is still in research level and care must be taken while using them in desicion stage (Moyad et.al., 2003).

3.6. Results of Histological Examinations of Uterus

Histological examinations of uterus of estrogen group has shown edema in the endometrium (area from the lumen to the first muscle region) and enlargement in uterus as correlated with the diameter of this edema. Besides degeneration in connective tissue starting near the epithelial site was highly observed in this group. As a result of movement of this degeneration to inner parts, the normal fiberous structure of the connective tissue has also been lost.

However, neither destruction of epithelium (of endometrium) nor the change in uterus dimensions have occurred in groups not involving estrogen. As a result of degeneration in blood vessel epithelium, blood cells were observed to go out of the vessels in endimetrium, myometrium and perimetrium regions. When the uterus of an estrogen specimen (Figure 3.20) is compared with a raloxifen specimen photography (Figure 3.21) taken under same magnifications, it could be easily concluded that enlargement in uterus dimentions has occurred in the former case. In R alone treatment group, the angiogenesis (increase in blood vessel number was observed, which normally occurs in growing uterus) was observed as well as a healthy epithelial layer with high mitosis event within epithelial cells. The degeneration effect of estrogen was also observed in E+R group at degrees differing (Figure 3.22-23) in each sample most probably owing to the protective role of raloxifen at different rates in different organisms. Histological photographs for these specimens also show the high angiogenesis (which was observed for raloxifen), increase in uterine diameter owing to edema and presence of white blood cells (as observed in estrogen group) all at a level comparably less than the single applications of E and R. There were no differences between OVX control (Figure 3.24) and sham groups (Figure 3.25) except lymphosyte infiltration at some regions in OVX. These two group photos would be more indicative of angiogenesis and connective tissue degeneration events of E and R groups, since they have not occurred in sham and OVX. Histological studies of effect of VitK2 (Figure 3.26) on rat uterus indicated that endometrium structure is in a good condition, but there is an increase in lymphocyte infiltration. Also, weakening in muscles (tunica müskülaris) that forms the miyometrium was observed in this group (Figure 3.27). However, this outcome has not occurred in R+K (Figure 3.28) group suggesting some enhancement with drug combination. The increased angiogenesis of previous raloxifen groups was present in R+K group, too.

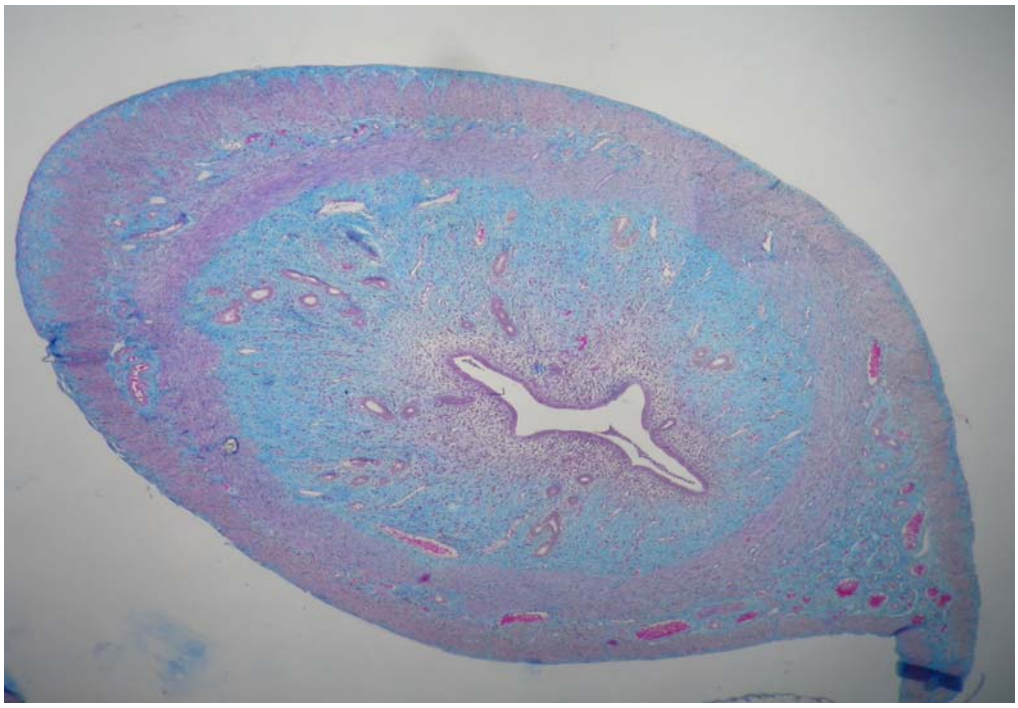


Figure 3.20. Light micrograph of Uterus of OVX-Rat Treated with Estrogen for 12 weeks

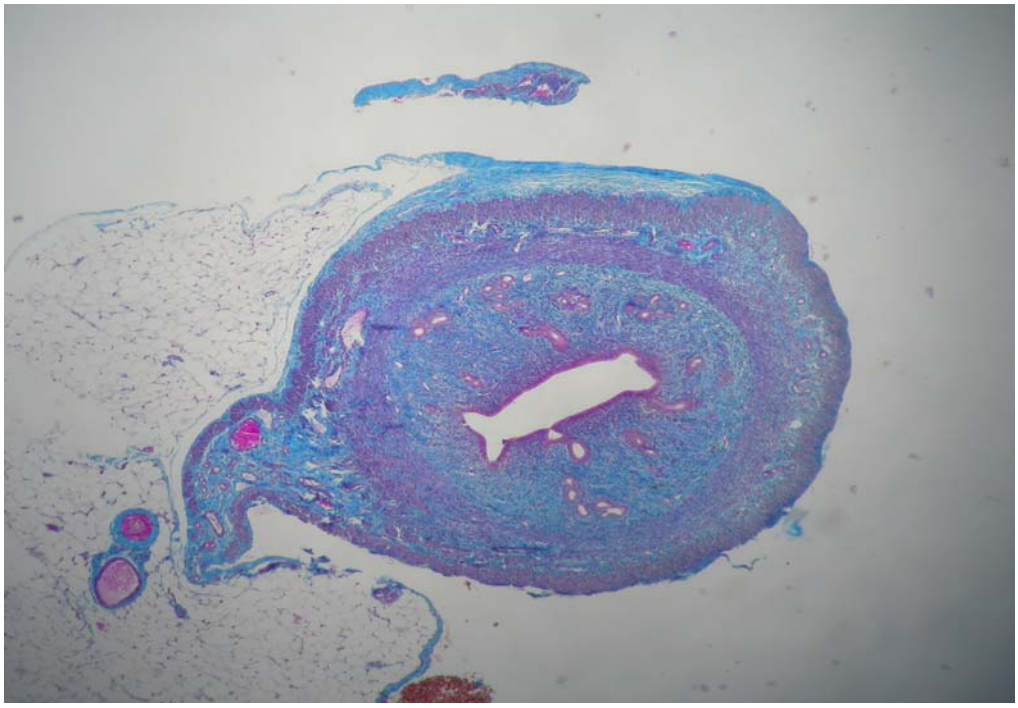


Figure 3.21. Light micrograph of Uterus of OVX-Rat Treated with Raloxifen for 12 weeks

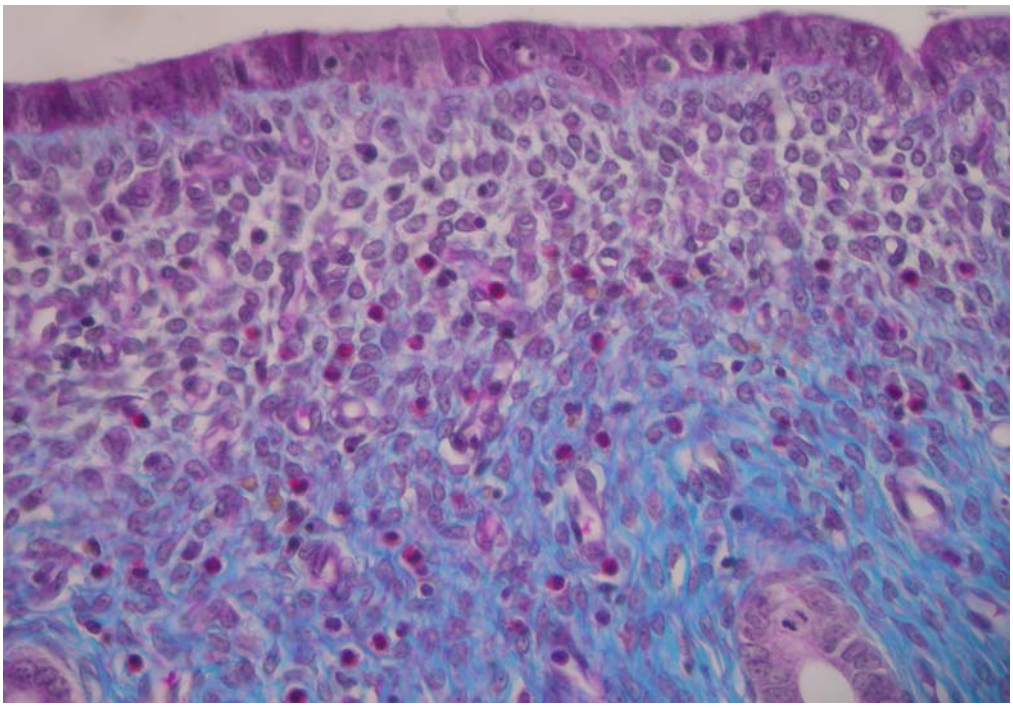


Figure 3.22. Light micrograph of Uterus of OVX-Rat Treated with Estrogen+Raloxifen for 12 weeks

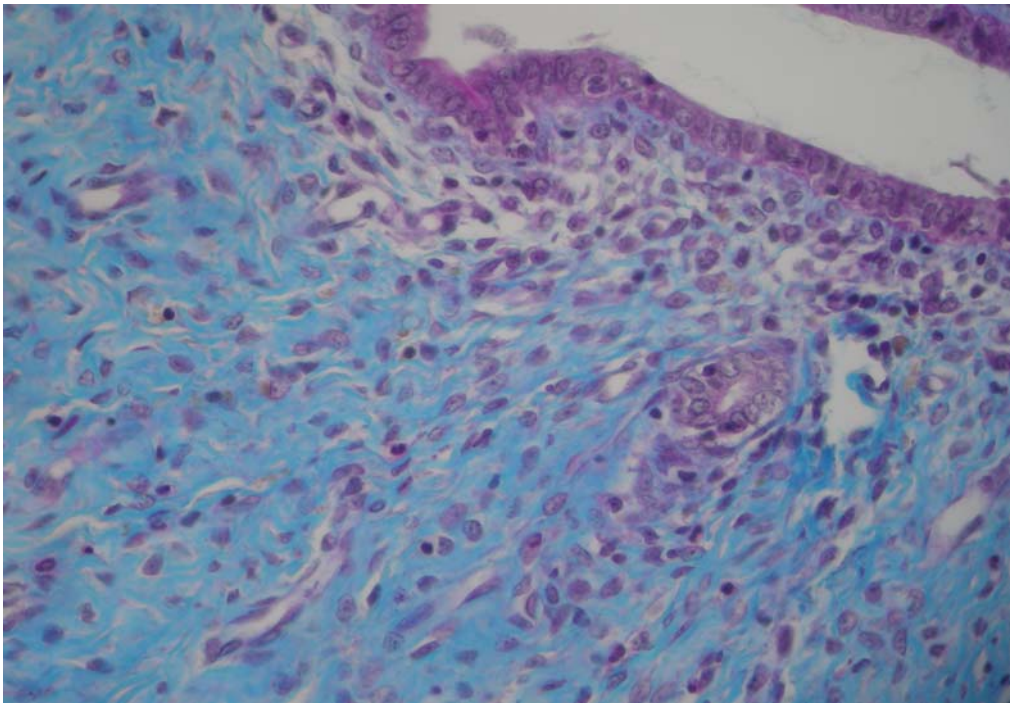


Figure 3.23. Light micrograph of Uterus of OVX-Rat Treated with Estrogen+Raloxifen for 12 weeks

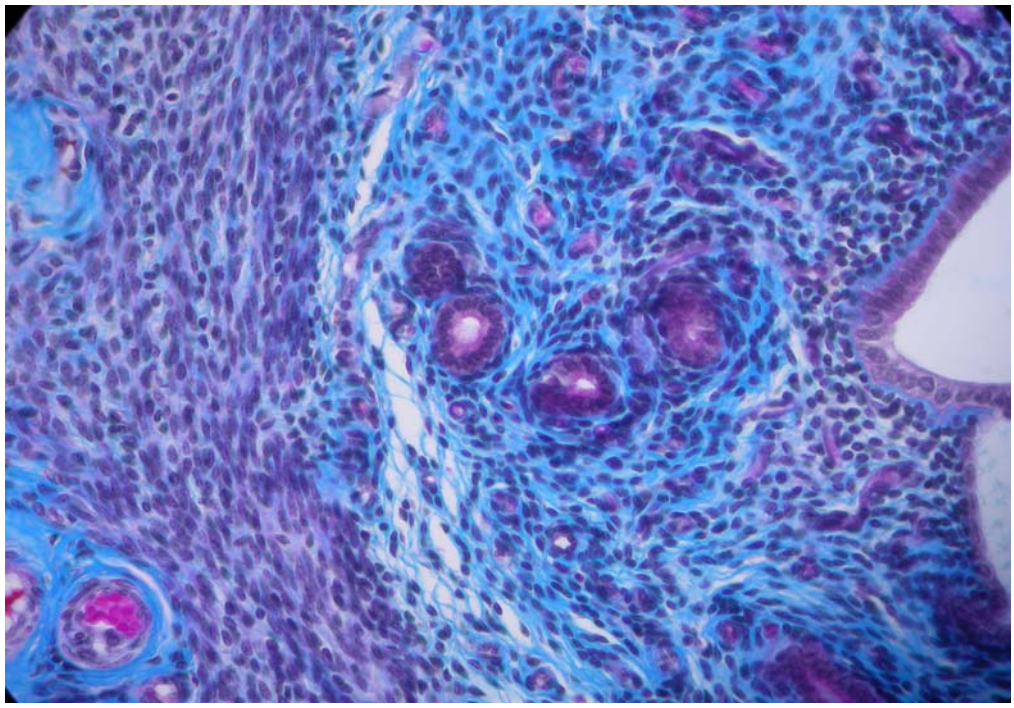


Figure 3.24. Light micrograph of Uterus of Untreated OVX-Rat

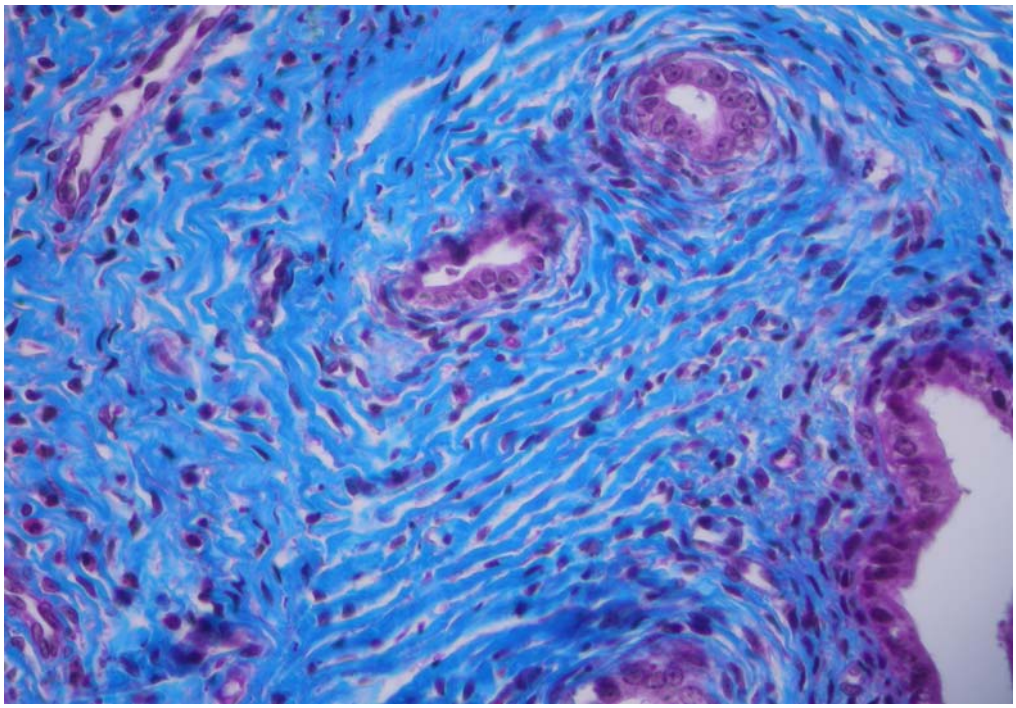


Figure 3.25. Light micrograph of Uterus of Sham Operated-Rat

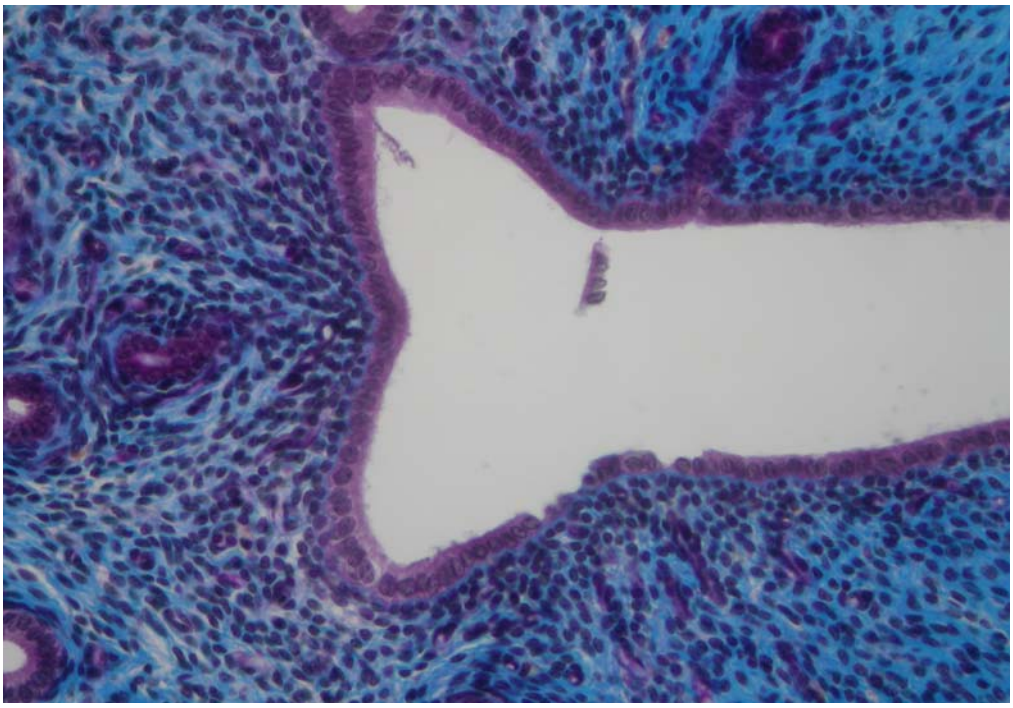


Figure 3.26. Light micrograph of Uterus of OVX-Rat Treated with VitK2 for 8 weeks showing the endometrium structure

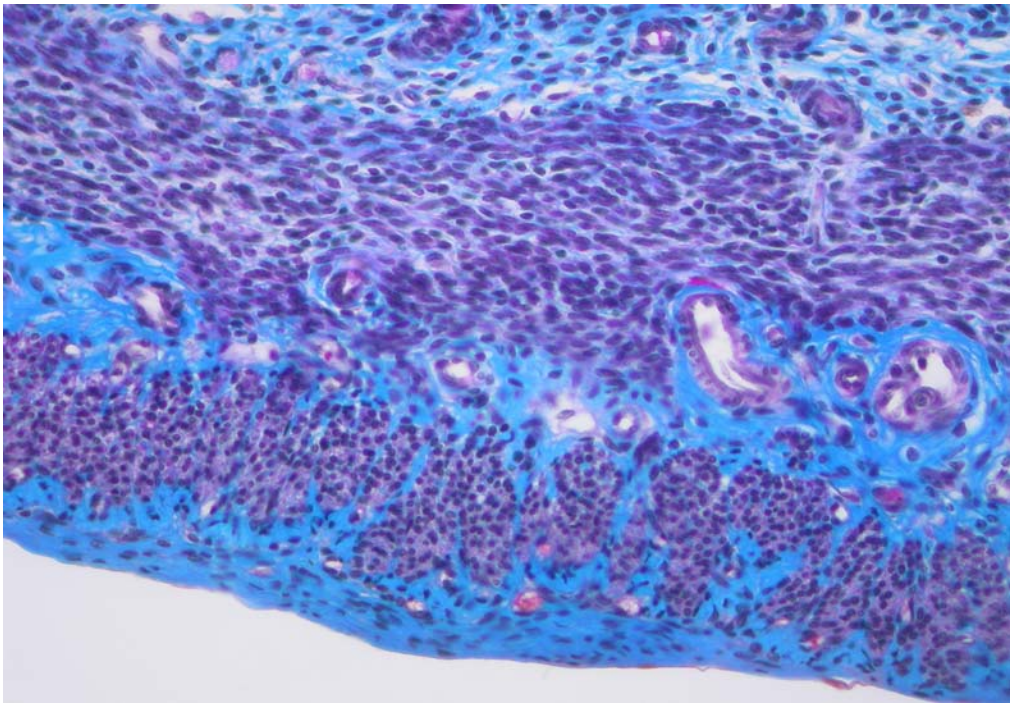


Figure 3.27. Light micrograph of Uterus of OVX-Rat Treated with VitK2 for 8 weeks showing degeneration in myometrium muscle layer

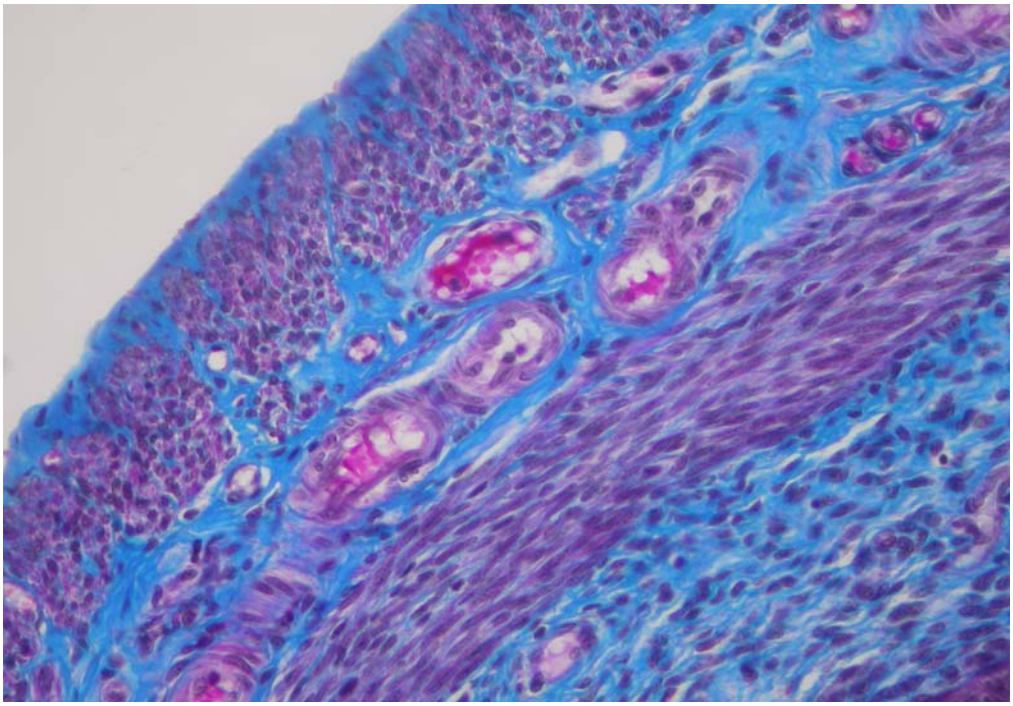


Figure 3.28. Light micrograph of Uterus of OVX-Rat Treated with VitK2 (8 weeks)+Raloxifen for 12 weeks

3.7. Results of Body Weight Changes Upon Ovariectomy and Treatments

It is well known that ovariectomy stimulates weight incrementation (Thompson et.al., 1995)(Jarvinen et. al., 2003). Thompson et al also demonstrated that increased body weight is entirely because of increased body fat. So it can be said that excessive weight gaining in postovariectomy period can be harmful.

The body weights of rats were measured before ovariectomy, 3 months postovariectomy and at the end of the study (3 months after medication). Initial weights were between 140-150 grams for all groups before starting to study. Changes in body weight three months postovariectomy and three months after medications were shown in Table 3.6.

Table 3.6. Body Weights of Rats Three Months Postovariectomy and Three Months After Medications, weights are in grams.

GROUPS	PRE-MEDICATION	POST-MEDICATION
C	275	283,17
S	212,61	220,28
E+R	251,00	247,11
E	238,50	241,13
R	252,28	249,44
K	270,83	278,08
R+K	252,44	248,89

As can be seen in Table 3.6 (ovx control group) C, gained the maximum amount of weight and sham gained the minimum. Group E is the group that gained second minimum amount of weight. However in all R containing treatment groups weight loss was observed upon therapy. Estrogen, raloxifen and their combination might therefore be considered as effective in the reduction of weight increment. Group K gained the maximum weight among treatment groups, while its combination with raloxifen (R+K) loosing weight which might be due to the presence of raloxifen in the treatment. Hence it may be suggested that estrogen, raloxifen and their combination reduces the weight increase adverse effect of ovx and raloxifen is even effective when it is in combined with other agents.

CHAPTER 4

CONCLUSIONS

In this study raloxifen, estrogen and VitK2's preventive and treatment effect on osteoporosis was investigated. It was observed that antiresorptive agents are effective in ovx prevention, but their combination is much better than their separate use in mechanically, histologically and quantitatively. Femur mechanical test results showed that E+R combination is the most effective treatment and all treatments result in numerically (though not statistically significant) higher values of the measured properties for this bone compared to the untreated control group. It is an important outcome because it implies that E+R is effective in every loading condition. Single drug actions are found to be better especially in mechanical properties that involve intrinsic factors of the specimen. VitK2 performs better in some mechanical properties (like E absorption at fracture) but worse in others (like PL, YL etc.) compared to other treatment groups indicating that this vitamin plays a specific role in modifying bone structure thus, rendering bone stronger (effective) under some conditions (especially in high stress). However, similar to estrogen case, its combination with raloxifen performs better than its individual administration. So it can be concluded that raloxifen shows additive effect while it is used with estrogen hormone or VitK2 on osteoporotic rat bone. Although the results of the combinations are not significantly different in all cases, when considered together with the benefits in combined use of these drugs (especially considering the side effects of each single drug) this difference gains much more importance in osteoporosis therapy. With combinations it was aimed to reduce the adverse effects of estrogen on uterus and mammary glands with using raloxifen, this

idea appears to be achieved according to the results of histological evaluations of uterus of all groups showing degeneration in the endometrium and increased uterine weight of estrogen groups, which is not present in raloxifen and prevented in combination groups at different levels, all being better than the E alone case. Biochemical analysis of the blood samples shows numerical increase in bone formation (ALP activity) compared to both controls. The highest ALP activity was observed in R group, which was lower in combinations. Thus, suggesting that a different mechanism of bone repair in osteoporosis has occurred.

Additionally, during the mechanical tests, total mechanical behaviour of rat femur up to fracture was observed by collecting load deflection and strain gauge data simulatenously, which was not encountered in literature before. The shear effect in three point bending was calculated with a simple structural analysis, and it is shown that its insignificant contribution can be neglected, provided that proper depth to span ratio, and experimental set up dimensions were chosen.

When complicated bone structure and high number of parameters effecting it is considered, it is expected to get many unsolved questions in trials of different bioactive agents. In order to better understand the mechanism of an agent on osteoporotic bone, and to get exact interaction of the combined therapies, more controlled experiments involving genetically controlled organisms should be carried out in a dose-response manner. Also, it can be concluded that, a single type of testing method is not reliable in evaluation of osteoporosis, especially in terms of fracture risk.

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APPENDIX

An analysis was made in order to investigate the shear contribution on bone three point bending test, and to compare the result with the one in conventional beam from structural analysis:

The deflection equation derived from the unit load method is the following (Gere and Timoshenko, 1990):

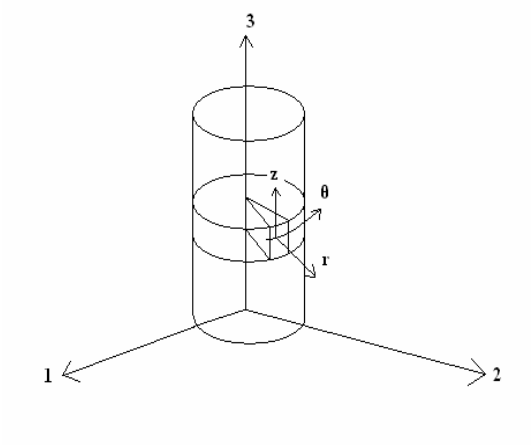
$$\delta = \frac{P \cdot L^3}{48 \cdot E \cdot I} \cdot \left(1 + 12 \cdot \frac{fs \cdot E \cdot I}{G \cdot A \cdot L^2} \right) \qquad (1)$$

where :
P is the load at midspan
Lis the span length
E is the modulus of elasticity
fs is the form factor defined for shear (Gere and Timoshenko, 1990), and it is decided as 2, for this analysis (fs=2 for thin tubes)
G is the shear modulus of elasticity
A is the transverse crossectional area

$$G = \frac{E}{2 \cdot (1 - \nu)} \qquad (2)$$

ν is the Poisson's ratio, and it is decided as the arithmetic mean of the values given for canine bone, (Currey, 2000)
ν_{zr} =0.29, ν_{rz}=0.45, then ν=0.37, and G will be:

$$G = \frac{E}{1.26} \qquad (3)$$



for one femur (from sham group) :

P=74.36 N

L=15 mm

A=22.814 mm2

I= 4.97 mm4

fs=2

For different values of E, deflection due to bending and deflection due to shear is calculated.

Since E varies significantly depending on the calculation method ie. deflection method versus stress strain method (*see* RESULTS for details).

If we put value of fs, equation (1) becomes:

$$\delta = \frac{P \cdot L^3}{48 \cdot E \cdot I} + \frac{P \cdot L}{2 \cdot G \cdot A} \qquad (4)$$

first term is the amount of deflection due to bending, δ_b

second term is the amount of deflection due to shear, δ_s

For E =2.33 Gpa (calculated from deflection method) deflections will be:

$$\delta_b = \frac{74.36 \cdot 15^3}{48 \cdot 2.33 \cdot 1000 \cdot 4.97} = 0.45 \quad \text{in mm.}$$

$$\delta_s = \frac{74.36 \cdot 15 \cdot 1.26}{2 \cdot 2.33 \cdot 1000 \cdot 22.814} = 0.013 \quad \text{in mm.}$$

$$\frac{\delta_s}{\delta_b} = \frac{0.013}{0.45} = 0.029$$

For E =36.1 Gpa (calculated from direct strain method) deflections will be:

$$\delta_b = \frac{74.36 \cdot 15^3}{48 \cdot 36.1 \cdot 1000 \cdot 4.97} = 0.029 \quad \text{in mm.}$$

$$\delta_s = \frac{74.36 15 \cdot 1.26}{2 \cdot 36.1 \cdot 1000 \cdot 22.814} = 8.532 \times 10^{-4} \quad \text{in mm.}$$

$$\frac{\delta_s}{\delta_b} = \frac{8.532 \times 10^{-4}}{0.029} = 0.029$$

Thus from the results above, it can be concluded that for any value of E, shear contribution to beam deflection is negligibly small (less than 3%) for the specimen having above properties.

By this analysis it can be observed that decided dimensions for experimental test set up used in the study obey the recommendations and standards (*see* RESULTS).

It can be also realized that, calculated shear contribution in bone three point bending test by using linear beam theory is quite similar to the contribution in a simple rectangular beam having depth d and span L. T o illustrate this, in Wang's (1983) study about the relative significance of shear deflection to bending moment deflections, it is mentioned that, the ratio of shear deflection, to bending deflection is 0.03 for a beam having a depth to span ratio equals to 1/10.