STRUCTURAL, MECHANICAL AND VISCOELASTIC CHANGES IN OVARIECTOMIZED RAT UPON DRUG TREATMENT AND VIBRATION THERAPY

YANG, XIAO

(B.Sc.(Hons.), Wuhan University of Technology)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOENGINEERING
NATIONAL UNIVERSITY OF SINGAPORE
2012
DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Yang Xiao

14 August 2012
ACKNOWLEDGMENTS

This work was supported by the Academic Research Funding from the Ministry of Education (MoE), Singapore, and I am also very grateful to NUS Department of Bioengineering Scholarship which financially supported me during my four years of doctoral studies in Singapore.

The work would not have been possible without continuous guidance and support from my qualify exam and thesis committee members. I would like to gratefully and sincerely thank to Dr. Taeyong Lee, Prof. James Goh, and Assoc Prof. Toh SL. I would also like to thank the valuable help from coworkers: Chan Yong Hoow, Abhishek Vishwanath Rammohan S, Saara Afzal, Ally Chan, Chen Wenming, especially Padmalosini Muthukumaran and Chen Xiuli who had accompanied me through every hardship. I want to thank Prof. John A Kanis who provided me an opportunity to participate in the development of FRAX® WHO Fracture Risk Assessment Tool.

I would like to acknowledge the support from my parents. They always have faith in me. I would like to express my special thanks to my husband Ismail Ma. Without his understanding and encouragement it would not have been possible for me to finish this work. Alhamdulillah, all praise and thanks be to Allah for endowing me with health, patience, and knowledge to complete this work.
### TABLE OF CONTENTS

Acknowledgments ............................................................................................................. i  
Table of contents .............................................................................................................. ii  
Summary ............................................................................................................................... iv  
List of Tables ....................................................................................................................... vi  
List of Figures ..................................................................................................................... viii  
List of Abbreviations and Symbols ................................................................................... xi

**Chapter 1: Introduction** ........................................................................................................ 1  
1.1 Osteoporosis and skeletal fragility ................................................................................. 2  
1.2 Literature review of bone strength determinants ......................................................... 4  
  1.2.1 Bone mineral density and fracture prediction ....................................................... 5  
  1.2.2 Viscoelasticity of bone ............................................................................................ 6  
1.3 Objectives and hypotheses ........................................................................................... 9  
1.4 Overall design ............................................................................................................... 11

**Chapter 2: Prevention and treatments of osteoporosis** .................................................. 16  
2.1 Medical treatments ....................................................................................................... 17  
  2.1.1 Antiresorptive agents ............................................................................................... 18  
  2.1.2 Anabolic agent ........................................................................................................ 21  
  2.1.3 Combination treatment ......................................................................................... 22  
2.2 Biophysical interventions .............................................................................................. 25  
  2.2.1 Exercise ................................................................................................................ 25  
  2.2.2 Whole body vibration ............................................................................................. 26  
2.3 Ovariectomized rat model ............................................................................................. 30

**Chapter 3: Ibandronate does not block the anabolic effects of PTH** ................................. 32  
3.1 Introduction .................................................................................................................. 33  
3.2 Methods ....................................................................................................................... 34  
3.3 Results .......................................................................................................................... 40  
3.4 Discussion .................................................................................................................... 52  
3.5 Conclusions .................................................................................................................. 61
Chapter 4: Alterations of bone viscosity and geometry upon drug treatments ........62

4.1 Introduction ..........................................................................................................63
4.2 Methods ..................................................................................................................63
4.3 Results ....................................................................................................................69
4.4 Discussion ...............................................................................................................85
4.5 Conclusions ..........................................................................................................91

Chapter 5: Positive correlations between loss tangent and ultimate strength ........93

5.1 Introduction ..........................................................................................................94
5.2 Methods ................................................................................................................94
5.3 Results .................................................................................................................100
5.4 Discussion ............................................................................................................107
5.5 Conclusions ..........................................................................................................111

Chapter 6: Long-term whole body vibration impairs osteoporotic bone quality ...112

6.1 Introduction ..........................................................................................................113
6.2 Methods ...............................................................................................................113
6.3 Results ...............................................................................................................118
6.4 Discussion ............................................................................................................123
6.5 Conclusions ..........................................................................................................126

Chapter 7: Conclusions and future directions ..........................................................128

7.1 Conclusion ............................................................................................................129
7.2 Future work ...........................................................................................................134

Bibliography ...........................................................................................................136

Appendix ................................................................................................................149
SUMMARY

Besides Bone Mineral Density (BMD), viscoelastic properties like viscosity ($\eta$), elastic modulus ($E$) and loss tangent (tan $\delta$) of bone are also crucial determinants of bone strength. However, these properties were seldom assessed in the prevalent drug or whole-body vibration (WBV) therapy. The first phase of this thesis was to examine whether there is a beneficial effect of PTH and ibandronate combined treatment in the ovariectomized (OVX) rat bone. Traditional BMD measurement together with serum biomarker test, micro-computed tomography ($\mu$CT) and a specially designed mechanical test were adopted. Maximum load, strength–strain indices (SSIy) and bone formation marker of the combination group were significantly higher than both monotherapy groups in tibia metaphysis. Compared to the previous studies which showed impedance from bisphosphonates in combination therapy, our phase 1 results revealed that ibandronate does not block the anabolic effects of PTH.

Viscoelastic response of ovariectomy and treatment was further examined using nanoindentation and dynamic mechanical analyzer (DMA) test. Firstly, in the nanoindentation test conducted on distal femoral metaphysis, ovariectomy induced deterioration in $\eta$ and $E$ was observed. Different drugs had selective effects on bone especially in preserving geometry, $E$ and $\eta$. The concurrent administration of PTH and ibandronate was shown to offer an added advantage in maintaining BMD and cortical bone area by an increased periosteal formation and a decreased endocortical resorption. $\eta$ was prominently restored in combined treatment group. It is in accordance with an observed denser alignment of collagen fibers and hydroxyapatite crystals matrix with fewer pores, which may play an important role in hindering fracture propagation.
Secondly, DMA test on cortical samples from control and drug treated femoral diaphysis were conducted. The BMD of femoral diaphysis cortical bone samples did not show any difference between groups, whereas combination treatment was demonstrated to have a better therapeutic effect in terms of a higher ultimate force in bending test, increased \( E' \) (storage modulus) and \( \tan \delta \). This indicates that these viscoelastic changes accompanying ovariectomy and treatment would be ignored in clinical screening by an unchanged BMD value. It is the first time that positive correlations between \( \tan \delta \) and ultimate strength of the bone were observed during normal daily activity frequency range (0.9-6 Hz).

Finally, BMD, mechanical and viscoelastic properties of WBV treated OVX rat were investigated. There is no substantial effects resulting from 16 weeks of low-magnitude, high-frequency vibration treatment (30 Hz, 0.3 g) on tibia BMD and viscoelasticity were found. In fact, the WBV treated rats have significantly lower lumbar BMD than non-treated group. Maximum load from rat vertebrae compression test was significantly reduced in WBV treated rats. This result indicates that the prevalent vibration therapy may not provide a curative effect in all conditions. Since the optimal parameters were still under research, WBV should not be recommended for long-term usage. Taken together, the results of the present work provide a better understanding of pathogenesis of osteoporosis, and present a novel assessment of treatment efficacy in bone viscoelasticity aspect.
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Descriptions</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Result of t-test sample size calculation</td>
<td>15</td>
</tr>
<tr>
<td>2.1.</td>
<td>Antiresorptive medications (FDA=US Food Drug Administration; MPA=medroxyprogesterone)</td>
<td>18</td>
</tr>
<tr>
<td>2.2.</td>
<td>Literature review on different combination treatments of anabolic and antiresorptive drugs (Ratio=Anabolic:Antiresorptive)</td>
<td>24</td>
</tr>
<tr>
<td>2.3.</td>
<td>Literature review on whole body vibration therapy</td>
<td>29</td>
</tr>
<tr>
<td>3.1.</td>
<td>Mechanical property changes of animal groups measured by 3-point bending test. Group averages are represented as mean ± SD</td>
<td>45</td>
</tr>
<tr>
<td>3.2.</td>
<td>Density changes of animal groups measured by pQCT. Group averages are represented as mean ± SD</td>
<td>48</td>
</tr>
<tr>
<td>3.3.</td>
<td>Serum levels of bone formation (P1NP) and resorption (CTX) markers. Average concentration is expressed as mean ± SD</td>
<td>51</td>
</tr>
<tr>
<td>4.1.</td>
<td>Geometrical parameters changes of different groups measured by pQCT. Group averages are represented as mean ± SD</td>
<td>75</td>
</tr>
<tr>
<td>4.2.</td>
<td>Viscoelastic property changes of different groups measured by nanoindentation. Group averages are represented as mean ± SD</td>
<td>81</td>
</tr>
<tr>
<td>5.1.</td>
<td>Dimensions of rat cortical bone specimens. The span length for all the specimens was 5mm. (mean ± standard deviations)</td>
<td>99</td>
</tr>
</tbody>
</table>
5.2. Parameters by pQCT analysis of mid-shaft femoral cross-sectional diaphysis (mean ± standard deviations) 101

5.3. Mechanical properties (mean ± standard deviations) 103

5.4. Spearman rank-order correlations between viscoelasticity, density, bone area and macro mechanical properties 106

6.1. BMD and BMC changes of different groups measured by pQCT. Group averages are represented as mean ± SD 120
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figures</th>
<th>Descriptions</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Overall design of phase 1</td>
<td>12</td>
</tr>
<tr>
<td>1.2.</td>
<td>Bone usage in phase 1.</td>
<td>13</td>
</tr>
<tr>
<td>1.3.</td>
<td>Overall design of phase 2</td>
<td>14</td>
</tr>
<tr>
<td>1.4.</td>
<td>Bone usage in phase 2</td>
<td>14</td>
</tr>
<tr>
<td>2.1.</td>
<td>Incision site and exposure of ovary for ovariectomy surgery in rats</td>
<td>31</td>
</tr>
<tr>
<td>3.1.</td>
<td>Contouring method used to delineate the trabecular bone region. Left: x-ray image with ROI (red) in the center; Right: binary image with black region as the selected ROI</td>
<td>35</td>
</tr>
<tr>
<td>3.2.</td>
<td>A schematic diagram to show the calculation of stability index SSI</td>
<td>37</td>
</tr>
<tr>
<td>3.3.</td>
<td>The tibia specimen in the testing position. (A) μCT rendered image of rat tibia. (B) Details of the three-point bending test consisting of the aluminum base and the roller stamp</td>
<td>39</td>
</tr>
<tr>
<td>3.4.</td>
<td>Visualization of bone loss in a transaction of the tibial metaphysis of an rat from each group at the time of surgery, and two, six, ten and twelve weeks following surgery</td>
<td>42</td>
</tr>
<tr>
<td>3.5.</td>
<td>Changes in trabecular morphology measured by μCT. Top row: Bone Volume fraction (BV/TV), Structure Model Index (SMI) and Trabecular Porosity (Tb.Po). Bottom row: Trabecular Thickness (Tb.Th), Trabecular Separation (Tb.Sp) and Trabecular Number (Tb.N)</td>
<td>43</td>
</tr>
<tr>
<td>3.6.</td>
<td>Positive linear correlation between maximum load ($F_{\text{max}}$) and strength-strain index to the y axis (SSIy)</td>
<td>49</td>
</tr>
<tr>
<td>4.1.</td>
<td>Left: A typical indentation load-displacement curve with holding period indicated by arrow; Right: Non-linear regression curve fitting of displacement-time data in calculation of $\eta$ ($R^2 &gt; 0.99$).</td>
<td>67</td>
</tr>
<tr>
<td>4.2.</td>
<td>μCT rendered image of rat tibia showing the volume of interest (VOI) (left); Visualization of bone loss in a transverse section of the femur metaphysis of a rat from each group at week 12 (right), showing prominent trabecular bone loss in OVX group and</td>
<td>69</td>
</tr>
</tbody>
</table>
positive effects of drugs in negating the bone loss

4.3. Five adjacent pQCT slices from the femur metaphysis of each group at week 12. There was evidence of great trabecular bone loss in the OVX group specimens showing a larger bone marrow area with less dense (red/yellow) trabecular mesh as opposed to highly dense (cyan/white) trabecular bone mesh of SHM specimens. Compared to OVX, trabecular bone was better preserved in PTH, IBN and COM specimens along the longitudinal axis.

4.4. Changes in morphology and volumetric bone mineral density measured by (A) μCT: bone surface to volume ratio (BS/BV), bone volume ratio (BV/TV), structural model index (SMI), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th); and (B) pQCT: mean BMD, trabecular density (Tb.BMD) and cortical density (Ct.BMD).

4.5. SEM surface images of the cortical bone of femur metaphysis from different groups of rat, taken at 10,000X magnification.

4.6. SEM surface images of the trabecular bone of femur metaphysis from different groups of rat, taken at 10,000X magnification.

4.7. Power-law regression analysis of the relationship between viscosity (η) and cortical elastic modulus (Ec) for five different groups at week 12.

5.1. A standard femur diaphysis three point bending test configuration.

5.2. Sinusoidal stress σ applied to linear viscoelastic material, resulting in strain ε and phase lag δ.

5.3. DMA sample preparation procedure. (A) Anatomical position of the sample: femur diaphysis; (B) Specimen was cut along longitudinal axis and posterior site of the femur; (C) Actual size of a typical sample; (D) Testing position on the DMA clamp.

5.4. Visualization of bone geometry in a transverse section of the femur diaphysis of a rat from each group yielded by pQCT.

5.5. The mean tanδ of rat femur diaphysis cortical bone for different groups; error bars represent the standard deviation.
5.6. The mean storage modulus ($E'$) of rat femur diaphysis cortical bone for different groups; error bars represent the standard deviation

6.1. Rat on the platform: 20 min/day, 0.3g, 30 Hz WBV was administered to the VIB group

6.2. In vivo pQCT scans were performed under anesthesia at week 4, 8, 12 and 16

6.3. Vertebra sample placed on the compression test device

6.4. Femur sample placed on the DMA device with 3 point bending configuration

6.5. Percentage change in body weight. Mean value with standard deviation error bar were presented for each group. *: $p < 0.05$ from OVX. †: $p < 0.01$ from OVX

6.6. Visualization of bone loss in (A) longitudinal in vivo PQCT image of tibial metaphysis at week 4, 8, 12 and 16; (B) ex vivo PQCT image of tibial metaphysis at end point week 20; (C) ex vivo PQCT image of lumbar vertebral L3 from each group

6.7. Maximum load, stiffness, typical load-displacement curve and energy to failure in compression test of L3 vertebrae. Mean value with standard deviation error bar were presented for each group. *$p<0.05$ versus OVX rats

6.8. Storage modulus ($E'$) and tan delta measured at 1 Hz by dynamic mechanical analysis (DMA). Mean value with standard deviation error bar were presented for each group. *$p<0.05$ versus OVX rats
**LIST OF ABBREVIATIONS AND SYMBOLS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXA</td>
<td>Dual energy X-ray absorptiometry</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>Tb.BMD</td>
<td>Trabecular bone mineral density</td>
</tr>
<tr>
<td>Ct.BMD</td>
<td>Cortical bone mineral density</td>
</tr>
<tr>
<td>Ct.Ar</td>
<td>Cortical bone area</td>
</tr>
<tr>
<td>Ct.Th</td>
<td>Cortical bone thickness</td>
</tr>
<tr>
<td>Ct.Po</td>
<td>Cortical porosity</td>
</tr>
<tr>
<td>Ps.Pm</td>
<td>Periosteal perimeter</td>
</tr>
<tr>
<td>Ec.Pm</td>
<td>Endocortical perimeter</td>
</tr>
<tr>
<td>CSMI</td>
<td>Cross-sectional moment of inertia</td>
</tr>
<tr>
<td>SS1y</td>
<td>Y axis Strength-Strain Indices</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>BS/BV</td>
<td>Bone surface to volume ratio</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone volume fraction</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>Trabecular thickness</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>Trabecular separation</td>
</tr>
<tr>
<td>Tb.N</td>
<td>Trabecular number</td>
</tr>
<tr>
<td>SMI</td>
<td>Structural model index</td>
</tr>
<tr>
<td>Tb.Po</td>
<td>Trabecular porosity</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of interest</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>$E$</td>
<td>Elastic modulus</td>
</tr>
<tr>
<td>$E_t$</td>
<td>Trabecular elastic modulus</td>
</tr>
<tr>
<td>$E_c$</td>
<td>Cortical elastic modulus</td>
</tr>
<tr>
<td>$H$</td>
<td>Hardness</td>
</tr>
<tr>
<td>$H_t$</td>
<td>Trabecular hardness</td>
</tr>
<tr>
<td>$H_c$</td>
<td>Cortical hardness</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>DMA</td>
<td>Dynamic mechanical analyzer</td>
</tr>
<tr>
<td>$\tan \delta$</td>
<td>Loss tangent</td>
</tr>
<tr>
<td>$E'$</td>
<td>Storage modulus</td>
</tr>
<tr>
<td>$E''$</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>WBV</td>
<td>Whole-body vibration</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>RS</td>
<td>Raman spectroscopy</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 Osteoporosis and skeletal fragility

Bone is a complex, hierarchically organized organ system whose composition and structure are closely related to, and in many ways controlled by, the functional demands made upon it (Morgan et al., 2008). Besides providing mechanical strength and protection to inner soft organs, bone is also the primary site for hematopoiesis and mineral storage. Osteoporosis is a worldwide public health problem. It is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures. Normal bone turnover involves a balance between the process of bone resorption and bone formation in which osteoclast resorb bone and osteoblasts secrete new organic matrix into the resorption cavity (Manolagas et al., 2000). In postmenopausal women, the rate of bone turnover increases dramatically and can remain elevated for up to 40 years, leading to continuous, progressive bone loss (Garnero et al., 1996). The basis for the increased bone turnover is thought to be due in part to a shortening of lifespan of osteoblasts and a prolongation of the lifespan of osteoclasts (Manolagas et al., 2000). Several interacting factors, such as clinical, medical, behavioral, nutritional and genetic variables, contribute together to the risk of osteoporotic fracture (Cooper et al., 1992). Fractures induced by osteoporosis cause a great burden to society due to increased mortality and disability and the resultant expenses of caring. In 1990, there were 1.7 million hip fractures alone worldwide; with changes in population demographics, this figure is expected to rise to 6 million by 2050, as reported by World Health Organization (Kanis et al., 1994).

The pathogenesis of osteoporosis is mainly due to the depletion of estrogen caused by post-menopause or ageing effects. The most prevalent clinical tool for osteoporotic
fracture prediction and treatment efficacy assessment is the bone mineral density (BMD) measurement by dual-energy x-ray absorptiometry (DXA) or quantitative computed tomography (QCT). While DXA measures areal bone density (g/cm²), QCT determines volumetric density (g/cm³). Previous large prospective studies have shown that a reduction in BMD causes increase in fracture risk (Hui et al., 1988). In the subsequent studies, BMD at various skeletal sites were assessed. Among different sites, femoral neck BMD is shown to be the most representative predictor for osteoporotic fracture (Melton et al., 1993, Cummings et al., 1994, Marshall et al., 1996). However, after years of clinical studies, some researchers believe that BMD measurement alone does not provide sufficient evaluation of the trabecular architecture (Hildebrand et al., 1999), actual stiffness or the ability to withstand an applied force on bones (Stürmer et al., 2006). Furthermore, it is becoming generally accepted that high BMD does not always relate to low fracture risk in elders (Wilkin and Devendra, 2001, Cefalu, 2004, Kumasaka et al., 2005) and drug-treated patients (Watt et al., 2000).

Besides BMD, numerous other critical determinants of bone quality have been investigated with the development of techniques, for instance, the bone structural analysis. The current generation of DXA and QCT is now able to perform hip structural analysis which is widely used together with BMD in clinical prediction of osteoporotic fracture. Another invasive technique, micro-computed tomography (μCT), has provided an opportunity to define three-dimensional microstructural aspects of bone and in conjunction with newer methods of measuring bone strength (Turner et al., 2000). However, due to the high dose exposure of radiation to acquire micro-level structure, μCT is mainly used in research purpose. In addition, several serum and urine biochemical
markers are available which provide an index of the overall rate of bone remodeling/time point. These biomarkers are collagen degradation products from remodeling process, i.e., C-terminal cross-linked telopeptide of type I collagen (CTX) and N-terminal pro-peptide of type I collagen (P1NP). Although biomarker is a useful non-invasive tool to study the dynamic bone remodeling for both experimental and research purpose, the validity of this measurement is subject to the dietary and nutritional changes in individual. Recently, bone viscoelasticity, a new determinant which correlates with material load-bearing capacity, has raised a growing concern (Ammann et al., 2007, Brennan et al., 2009). The viscoelasticity of bone arises from the void collapse, densification of cancellous bone and natural viscoelastic response of collagen as a polymer. Certain viscoelastic properties of compact bone (i.e. loss tangent) have been demonstrated as an effective tool to test human bone (Yamashita et al., 2001). Furthermore, Les (2005) reported that long-term ovariectomy (induced osteoporosis) decreases ovine compact bone viscoelasticity. These previous studies showed deterioration in viscoelasticity of bone may possibly associate with the development of osteoporosis. However, there is still a lack of characterization of various treatment effects on osteoporotic bone viscoelasticity. For a better understanding of pathogenesis and drug efficacy in osteoporosis, timely investigations into bone viscoelasticity is necessary. This chapter will provide a brief overview of the determinants of bone strength in general, and more attention will be given to emerging techniques for viscoelasticity measurement and applications.

1.2 Literature review of bone strength determinants

Strength of bone is dependent on two main individual physical/structural factors: quantity and quality (Chesnut et al., 2001). Bone quantity consists of density and size, which
together form bone mass. Bone quality consists of structural and material properties. In this chapter, two distinctive surrogates of quantity and quality -- BMD and viscoelasticity, will be reviewed and discussed respectively.

1.2.1 Bone mineral density and fracture prediction

BMD measurement by dual energy X-ray absorptiometry (DXA) is widely used to diagnose osteoporosis and evaluate treatment effect (Kanis et al., 1997). The World Health Organization (WHO) in 1994 defines osteoporosis as a BMD value that is 2.5 standard deviations (SDs) or more below the mean of a young adult of the same sex as the subject (T-score) (Kanis, 1994). The question of how well does measurement of BMD predict occurrence of osteoporotic fracture has been under debate for decades. Early precursors in the field proposed a rule that the lower the BMD, the greater the fracture risk (Kanis, 1994, Marshall et al., 1996). This finding was based on a meta-analysis of existing clinical data of non-treated patients. This substantial work was further extended by two research groups, in which the data from antiresorptive drug treated patients were taken into account. According to Wasnich and Miller, risk reduction was estimated to be 54% for an 8% BMD increase (Wasnich and Miller, 2000). A more recent study reported a risk reduction of 3% for a 1% BMD increase (Cummings et al., 2002). Thus, the earlier study determined that changes in BMD appeared to explain most of the observed risk reduction, whereas the more recent study determined that it explained only a fraction of the observed risk reduction. This discrepancy may partially reveal the non-linear and non-proportional relationship between BMD increase and fracture risk reduction. There seems to be (1) a markedly reduced fracture risk even with small increase in BMD; (2) a fracture risk reduction that may occur immediately (i.e. within the
1st year of treatment) and not increase despite subsequent BMD build; and (3) no dosage-related fracture risk reduction despite dosage-related increases in BMD. These meta-analyses suggest that other factors may partially account for the some of the variability between increase in BMD and the observed risk reduction with treatments.

In order to explain the loose correlation of fracture risk and BMD increase, Michelotti and Clark’s research group has successfully characterized the femoral neck length and hip fracture risk (Michelotti and Clark, 1999). Their results have recognized the increased fracture risk associated with decreased BMD, and more importantly, associated with both smaller bone diameter and decreased cortical thickness. More recently, a research group has established the use of biochemical markers and paved the way for measuring bone turnover in osteoporosis (Delmas et al., 2000). Furthermore, recent studies of drug therapies for osteoporosis have focused on the relationship between bone turnover and fracture risk prediction. These promising studies suggest that besides BMD, there are other unexplored surrogates of bone strength prediction, especially in the field of bone quality. Viscoelasticity, which will be discussed in the next subsection, is one of them.

1.2.2 Viscoelasticity of bone

As a biological material, the bone exhibits a complex hierarchical structure with micro-components dynamically undergoing remodeling, which results in complicated mechanical properties (Akkus et al., 2004). The viscoelasticity of bone arises from the void collapse, densification of cancellous bone and the natural viscoelastic response of collagen as a polymer (Garner et al., 2000). Collagen fibrils are strengthened by mineral deposits, and vice versa. It is not yet clear whether viscoelastic parameters are a dominant player in prediction of fracture risk. However, viscoelastic properties are known to be
significant predictors of the impact strength of polymers which share similarities with bone collagen (Nielsen and Landel, 1994). The viscoelastic properties of bone have been shown to correlate with the ultimate strength and toughness of the tissue (Yeni et al., 2004). If changes in the viscoelastic properties of bone material accompany osteoporosis, then fracture risk could be increase dramatically without altering BMD.

Currently, there are two common experimental methods to determine bone viscoelasticity. The first method, which is traditionally used in large animal models, is a determination of bone tangent delta (tan δ), storage modulus ($E'$) and loss modulus ($E''$) by Dynamic Mechanical Analyzer (DMA). During the experimental process, a sinusoidal stress is applied and the strain in the material is measured. Frequency of the applied stress and temperature is usually varied. Smith and Keiper, had, for the first time, measured the dynamic mechanical properties of bone and reported a frequency-dependent property (Smith and Keiper, 1965). Later on, Lakes et al. described the viscoelastic response of bone under tensional loading as function of temperature (Lakes and Katz, 1979). These novel studies suggest that the viscoelastic behavior of bone may have a pronounced effect on its fracture behavior under dynamic loading. Since different parameters and conditions were adopted in early investigations, fair comparisons of the results between different research groups seemed problematic. Thus, a standardization of the use of DMA to assess the viscoelasticity of bone is conducted by Yamashita in 2001. Effects of stress levels, temperature, size etc. were tested in this study. Les et al. (2004) have successfully extended the previous work to further characterize effects of architecture, mineralization and remodeling of the bone on viscoelastic properties of bone. In 2005, Les and his research group applied their findings to osteoporotic bone and observed that long-term
ovariectomy decreases ovine compact bone viscoelasticity. Furthermore, chronic kidney disease was recently found to decrease compact bone tanδ without alteration in BMD (Iwasaki et al. 2011). Despite the encouraging results from large animal models (i.e. bovine, ovine), viscoelasticity of small animals (i.e. rodents) is not yet unveiled, partially due to the size limits. Studying viscoelasticity in small animal offers an advantage of faster experimental outcome due to the shorter life cycle, and less ethical issue involved.

A newer method, which has gained more attention nowadays, is to investigate viscosity ($\eta$), elastic modulus ($E$) and hardness ($H$) by Nanoindentation. This method has been applied in recent years to measure $E$ and contact hardness $H$ of bone matrix at high resolution of load and displacement (Oliver and Pharr, 1992). Brennan et al. (2009) has reported selective modifications of $E$ and $H$ by different treatment in osteoporotic rat model. In one recent study, the precursory Voigt model was successfully used to describe the creep behavior of bone matrix during the holding period of indentation (Kim et al., 2010). Viscosity $\eta$ of young and old bone was calculated during creep behavior. The results indicated that prolonged creep deformation (lower $\eta$) can play an important role in the long-term degradation of mechanical stability of osteoporotic bone. However, $\eta$ of osteoporotic bone, in comparison with healthy/treated bone, has not been rigorously investigated in previous studies.

As discussed above, DMA and Nanoindentation have their merits and flaws. While DMA is prevalently used to characterize whole bone tan δ in macro size specimens, Nanoindentation is employed in mapping hierarchical nano-scale viscosity and elasticity. Nevertheless, the ultimate objective of these two different techniques is to further develop their potential application in the field of osteoporosis and its treatment.
1.3 Objectives and hypotheses

As reviewed in section 1.2, bone viscoelasticity has revealed its potential correlation with fracture. Comparing to conventional bone strength predictor BMD, however, the application of bone viscoelasticity is yet to be thoroughly studied. Research gaps for the current study of bone viscoelasticity and the response of different osteoporotic therapies are summarized below:

- Anabolic and antiresorptive mono treatments have been proven effective in treatment of osteoporosis. However, whether the concurrent use of these two different treatments would produce an additive effect by reducing fracture risk is still controversial. In addition, none have used viscoelastic parameters to investigate the existence of additive effect in concurrent treatment.

- The detailed mechanism of Whole Body Vibration (WBV) therapy, as a newly emerged non-pharmaceutical mean to treat osteoporosis, has yet to be explored and understood. None have used viscoelastic parameters to investigate the existence of beneficial effect from WBV therapy.

- It is not yet clear whether bone viscoelasticity plays an important role in the prediction of osteoporotic fracture risk, as compared to BMDs.

The main aim of this study is to explore the drug treatment and vibration therapy induced changes occurred in osteoporotic bone strength. Bone strength here, is characterized by both conventional quantity parameters – BMDs, ultimate load, etc., and also by material quality means – bone viscoelasticity, μCT indices etc.

The specific objectives of this study are to:
• Study the density and microarchitectural changes in ovariectomized (OVX, an established method to induce osteoporosis which will be discussed in Chapter 2) rat bone given treatment of parathyroid hormone (anabolic), bisphosphonate (antiresorptive) and combination treatment using BMD and μCT measurements.

• Investigate viscoelastic properties differences (viscosity \( \eta \) and \( \tan\delta \)) in OVX rat bone between vehicle and drug treated groups using nanoindentation and DMA.

• Investigate viscoelastic properties differences (\( \tan\delta \)) in OVX rat bone between vehicle and WBV induced mechanical stimulation groups using DMA.

• Examine the correlations between bone viscoelasticity and traditional bone quality parameters (BMD, ultimate strength, stress, stiffness).

The hypotheses are:

• The combination treatment offers an advantage over mono therapy.

• Nano-viscoelasticity (viscosity \( \eta \)) of osteoporotic bone can be influenced by treatment.

• Macro-viscoelasticity (\( \tan\delta \)) of osteoporotic bone can be influenced by treatment. And it is correlated with ultimate strength of the bone.

• The long-term use of WBV can alleviate the bone loss due to osteoporosis, in terms of bone mineral density and bone strength.

• The rat skeleton which received WBV therapy will response and damp oscillatory stress more efficiently.

The results of the present study may provide a better understanding of pathogenesis of osteoporosis, and will present a novel evaluation of different osteoporotic medication efficacy in bone viscoelasticity aspect. It is understood that there is a difference between
rodent and human bone inherent structure. However, due to ethical issues, tests on larger animal models are not within the scope of this study.

1.4 Overall design

According to the two different types of anti-osteoporotic therapies which will be investigated – medical treatments and whole body vibration, the proposed measurements are divided into the following two phases.

Phase 1: medical treatments (Chapter 3, 4, 5)

Ninety female Sprague Dawley rats (Laboratory Animal Centre, National University of Singapore) aged 10 to 12 weeks were housed at 25°C under a 12:12-hour light-dark cycle and fed with a standard rodent diet (Harland, Model T.2018S) and water ad libitum. The animals were subjected to OVX or sham surgery one week after acclimatization at the animal holding unit. The rats were divided into the following 5 groups: (1) Vehicle-treated sham operated group (SHM); (2) Vehicle-treated ovariectomized group (OVX); (3) hPTH (1-34) treated ovariectomized group (PTH); (4) ibandronate-treated ovariectomized group (IBN) and (5) combined hPTH (1-34) and ibandronate treated ovariectomized group (COM). Human parathyroid hormone (hPTH (1–34), Sigma-Aldrich, Singapore) and ibandronic acid (Roche Diagnostics GmbH, Mannheim, Germany) were diluted in 0.9% saline. Starting from week 4 post-surgery, 10 μg/kg body weight of hPTH (1-34) (Gittens et al., 2004) and/or 7 μg/kg body weight ibandronate (Bauss et al., 2002) or its vehicle (0.9% saline) was administered subcutaneously once a week until week 12 to the respective groups. All animal experiments were conducted in accordance with the approved protocol from the Institutional Animal Care and Use Committee (IACUC) at National University of Singapore.
For destructive studies (i.e., mechanical tests), every two weeks several animals from each group were euthanized by carbon-dioxide asphyxiation as indicated by arrow in Fig. 1.1.

![Diagram](image)

Fig. 1.1. Overall design of phase 1.

After sacrifice of the animals, four bones (right and left tibia, femur) were harvested and distributed for different purposes (Fig. 1.2): Proximal tibia bone and rat serum will be used in Chapter 3 to establish the OVX model with drug treatments; Distal femur bone will be consumed in Chapter 4 to characterize the nano-level viscoelastic change; Diaphyseal femur bone will be adopted Chapter 5 to characterize the macro-level viscoelastic change.
Phase 2: whole body vibration therapy (Chapter 6)

Twelve ex-breeder female Sprague-Dawley (SD) rats of the age 6-8 weeks were subjected to OVX or SHM surgery. The rats were divided into the following 3 groups: (1) sham surgery operated rats group (SHM); (2) ovariectomized rats group without WBV treatment (OVX); (3) WBV treated ovariectomized rats group (VIB), with n=4/group. Starting from 4th week post-surgery, 0.3g, 30 Hz WBV was administered to VIB group, 20 min/day for 16 weeks (Fig. 1.3). At weeks 20, animals were euthanized by carbon-dioxide asphyxiation.
Fig. 1.3. Overall design of phase 2.

Femur, tibia and lumbar vertebral bones were harvested and distributed for different purposes (Fig. 1.4): Proximal tibia and spine bone will be used to establish the OVX model with WBV therapy and diaphyseal femur bone is planned to be used to characterize the macro-level viscoelastic change.
Sample size

t-test analysis was performed by Dr. Chan Yiong Huak (Head of the Biostatistics) to calculate sample size of the study, based on preliminary data of 12 weeks mean BMD of OVX (613 ± 28 mg/cm\(^3\)) and SHM (780 ± 11 mg/cm\(^3\)) groups. The result manifested that a minimal of two animals per each group was enough to give statistical power. Also, we have adopted mixed-model and Bonferroni test. The analysis has taken into account that two bones are from same rat sample, using rat as the random factor.

Table 1.1. t-test sample size calculator shows at least 2 rats were needed to achieve 80% and 90% statistical power.

<table>
<thead>
<tr>
<th>2 Sample T</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mean diff</td>
<td>167</td>
</tr>
<tr>
<td>sd diff</td>
<td>28</td>
</tr>
<tr>
<td>alpha 5%</td>
<td></td>
</tr>
<tr>
<td>sample size</td>
<td>2-sided</td>
</tr>
<tr>
<td>80% power</td>
<td>1.44135</td>
</tr>
<tr>
<td>90% power</td>
<td>1.590903</td>
</tr>
</tbody>
</table>

Thus, the phase 1 study was conducted with two rats per SHM and OVX group, three rats per treatment group in the beginning. However, in the process of experiment we do notice the limitation that the sample size was calculated from preliminary pQCT data, which may not be compliant with other testing methods, i.e., mechanical test. Therefore, later on more rats were added into several critical time-points (week 0, 4, 8, 12) to further confirm the result (Fig. 1.1).
CHAPTER 2

PREVENTION AND TREATMENTS OF OSTEOPOROSIS
2.1 Medical treatments

The patients who have osteoporosis by bone mineral density criteria, with a T-score of -2.5 or below, should begin medical treatment. After years of clinical studies in the treatment of osteoporosis, two distinct classes of drugs, namely the antiresorptive and anabolic agents, proved to be effective and are now available on the market. Antiresorptive medications like estrogens, selective estrogen receptor modulator (raloxifene), bisphosphonates (alendronate, risedronate, and ibandronate) and calcitonins, work by reducing rates of bone resorption. Anabolic agent, teriparatide (parathyroid hormone), works by accelerating bone remodeling which includes both bone resorption and formation.

The currently approved medications include alendronate (Fosamax), risedronate (Actonel), and raloxifene (Evista) for prevention and treatment of osteoporosis; Teriparatide (Forteo) and nasal calcitonins spray (Miacalcin) for treatment only; and estrogens or combinations of hormones (hormone replacement therapy [HRT]) for prevention only.
2.1.1 Antiresorptive agents

Table 2.1 Antiresorptive medications (FDA=US Food Drug Administration; MPA=medroxyprogesterone)

<table>
<thead>
<tr>
<th>Antiresorptive medications</th>
<th>FDA status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bisphosphonates</strong></td>
<td></td>
</tr>
<tr>
<td>Fosamax (Alendronate)</td>
<td>Approved for prevention and treatment</td>
</tr>
<tr>
<td>Actonel (Risedronate)</td>
<td>Approved for prevention and treatment</td>
</tr>
<tr>
<td>Bonviva (Ibandronate)</td>
<td>Approved for daily use</td>
</tr>
<tr>
<td>Zometa (Zolendronate)</td>
<td>In phase 3 trials</td>
</tr>
<tr>
<td><strong>Calcitonins</strong></td>
<td></td>
</tr>
<tr>
<td>Nasal spray</td>
<td>Treatment of osteoporosis</td>
</tr>
<tr>
<td>Oral calcitonin</td>
<td>In phase 3 trials</td>
</tr>
<tr>
<td><strong>Estrogens/hormones</strong></td>
<td></td>
</tr>
<tr>
<td>Premarin (conjugated estrogen)</td>
<td>Prevention if other medications</td>
</tr>
<tr>
<td>Prempro (conjugated estrogen MPA)</td>
<td>contraindicated or not tolerated</td>
</tr>
<tr>
<td><strong>SERMS</strong></td>
<td></td>
</tr>
<tr>
<td>Evista (Raloxifene)</td>
<td>Approved for prevention and treatment</td>
</tr>
<tr>
<td>Lasofoxifene</td>
<td>In phase 3 trials</td>
</tr>
<tr>
<td>Basodoxifene</td>
<td>In phase 3 trials</td>
</tr>
<tr>
<td>Arzoxifene</td>
<td>In phase 3 trials</td>
</tr>
<tr>
<td><strong>Anabolic medications</strong></td>
<td></td>
</tr>
<tr>
<td>Forteo (teriparatide)</td>
<td>Treatment of osteoporosis</td>
</tr>
<tr>
<td>Preos (PTH [1-84])</td>
<td>In phase 3 trials</td>
</tr>
</tbody>
</table>

As shown in Table 2.1, there are mainly five groups of antiresorptive medications:

**Estrogen/SERM**: the estrogen depletion is the main source of osteoporosis which causes the increased survival rate of osteoclast and an elevated resorption. A direct refill of the depleted hormone was thus being considered. Estrogens are proven to be extremely
effective at reducing bone turnover and preserving or increasing bone mass in healthy postmenopausal women and those with osteoporosis. Women receiving estrogen had a 50% reduced vertebral fracture occurrence compared with those receiving placebo (Lindsay et al., 1980). However, data from Women’s Health Initiative showing an increased risk of breast cancer and cardiovascular disease have dramatically dampened enthusiasm for the long-term use of estrogen (Rossouw et al., 2002). Therefore, ultra-low-dose estrogens are being explored and appear to have effects on bone turnover and bone mass consistent with active Antiresorptive medications (Bagi et al., 1997). Long-term safety and efficacy against fractures are unknown at this time.

Many women who have first-degree relatives with breast cancer will not consider estrogen. For them, selective estrogen receptor modulators (SERM) such as raloxifene offer an alternative. SERMs are “designer” drugs that activate the estrogen receptor and have estrogenic agonist effects on bone turnover and bone mass (Scott et al., 1999). However, SERMs are shown to increase the risk of thromboembolic disease, and lead to hot flushes in some patients, which may limit their use.

**Bisphosphonates:** Osteoclast endocytosis of bisphosphonate from the bone surface leads to FPPS (Farnesyl Pyrophosphate Synthetase) inhibition and osteoclast apoptosis. The bisphosphonate class of drugs is approved for the treatment of prevention of osteoporosis. Two of these agents, alendronate and risedronate, are currently approved and marketed for osteoporosis prevention and treatment. A third oral agent, ibandronate, is currently being studied in a novel once-monthly regimen for osteoporosis; and an intravenous bisphosphonate, zoledronic acid, is being studied for possible once-yearly treatment in women with established osteoporosis.
All bisphosphonates are retained over time in the skeleton and may exert long-term effects. The biological half-lives of bisphosphonates may vary and depend on the rate of bone turnover, potency, and binding affinity of each bisphosphonate to bone. A prolonged half-life may be beneficial, because it may prolong a residual effect of the agent after discontinuation. On the other hand, prolonged marked suppression of bone turnover may have a theoretical influence on the ability to repair bone microdamage. The optimal duration of bisphosphonate treatment is unknown.

Alendronate (Fosamax): in clinical trials, alendronate has been shown to reduce the risk of new spinal and hip fractures by 50%. Gastrointestinal problems, such as nausea, acid reflux symptoms, and constipation, are the most common side effects. To eliminate these side effects, it is encouraged that the medication be taken first thing in the morning with large amount of water and not lie down or eat for 30 minutes. Some patients find it difficult drug compliance. This medication is usually taken daily or once a week (Black et al., 2005).

Risedronate (Actonel): Results from a recent study showed that daily risedronate use can lead to a significant reduction in new vertebral fracture (62%) and multiple new vertebral fractures (90%) (Siris et al., 2008) in postmenopausal women with osteoporosis, compared with a placebo group. Gastrointestinal upset is its most common side effect. Women with severe kidney impairment should avoid this drug.

Zoledronate (Reclast): this is a new and powerful intravenous bisphosphonate that is given once a year (Lambrinoudaki et al., 2008). This can be especially beneficial for patients who cannot tolerate oral bisphosphonates or are having difficulty with complying with the required regular dosing of oral medications. Its side effect is still unknown.
Ibandronate (Boniva): this drug is the most recently FDA-approved bisphosphonate to prevent or treat osteoporosis in menopausal women. Ibandronate has been proven effective in inhibiting bone resorption at considerably lower doses than other bisphosphonates (Mühlbauer et al., 1991, Monier-Faugere et al., 1999). Following long-term treatment with ibandronate, bone mass, strength and architecture were maintained, or even improved (Bauss and Dempster, 2007). The antiresorptive effects of ibandronate will be thoroughly discussed in the Chapter 3-5.

Calcitonins: Calcitonin binds to the calcitonin receptor (CTR) and results in activation of multiple pathways including cAMP-PKA, PKC and intracellular calcium, which lead to the inhibition of osteoclast resorption. A nasal spray containing calcitonin (200 IU/day) was approved in the United States in 1995 for the treatment of osteoporosis in late postmenopausal women. Calcitonin is a polypeptide hormone normally produced by the thyroid gland. It can be either injected or administered intranasally. Although the effect of calcitonin is considered weaker as compare to bisphosphonate, it is generally very well tolerated, with rhinitis as the only common adverse event.

2.1.2 Anabolic agent

Teriparatide (recombinant human parathyroid hormone): Teriparatide is the only anabolic osteoporosis medication. Parathyroid hormone (PTH) is naturally an 84-amino-acid polypeptide. In 2002 the FDA approved recombinant PTH (1-34) made by Lilly with the brand name Forteo for the treatment of osteoporosis. PTH binds to osteoblasts and stimulates osteoblasts to increase their expression of RANKL (Receptor activator of nuclear factor kappa-B ligand) and inhibits their expression of Osteoprotegerin (OPG). OPG binds to RANKL and blocks it from interacting with RANK, a receptor for RANKL.
The binding of RANKL to RANK stimulates these osteoclast precursors to fuse, forming new osteoclasts, which ultimately enhances bone resorption and formation. All trials show that PTH is highly effective at increasing bone mineral density in the spine and throughout the skeleton. Biopsy and bone turnover results indicate that it works by stimulating new bone formation on trabecular, endocortical, and periosteal bone surfaces by preferentially simulating osteoblastic activity over osteoclastic activity (Dempster et al., 2001).

PTH currently must be administered by subcutaneous injection, although alternative modes of delivery are being investigated. The FDA has approved the use of Teriparatide for 2 years in postmenopausal women and men at high risk for fracture. It is contraindicated in patients with Paget’s disease of the bone or patients with bone metastases or preexisting hypocalcaemia. The most common side effects associated with teriparatide include dizziness and leg cramps. The anabolic effects of PTH will be thoroughly discussed in the Chapter 3-5.

2.1.3 Combination treatment

Whether the concurrent use of antiresorptive and anabolic agents would produce an additive effect by reducing fracture risks is still controversial. For instance, Black et al. (2003) and Finkelstein et al. (2003) have reported that a combined therapy of PTH (1-84) and alendronate has no synergistic effect on postmenopausal women or aged men, in terms of changes in BMD. It was concluded that bisphosphonate (alendronate) impairs the ability of parathyroid hormone to increase the BMD at the lumbar spine and the femoral neck. However, a recent ovariectomized rodent study discovered that the effect of combined administration of alendronate and PTH (1-34) on bone strength is
synergistic in the lumbar vertebra and additive in the femur (Johnston et al., 2007). The table 2.2 below lists several important studies exploring the effect of combination therapy.
Table 2.2. Literature review on different combination treatments of anabolic and antiresorptive drugs (Ratio=Anabolic:Antiresorptive)

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Group</th>
<th>Ratio</th>
<th>Regimen</th>
<th>Duration</th>
<th>Species/Gender</th>
<th>Methods</th>
<th>Combination effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>2003</td>
<td>PTH (1-84) alendronate</td>
<td>1:100</td>
<td>daily</td>
<td>12m</td>
<td>Human/Female</td>
<td>DXA/qCT/markers</td>
<td>No synergistic effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH (1-84)+alendronate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finkelstein</td>
<td>2003</td>
<td>PTH (1-34) alendronate</td>
<td>1:250</td>
<td>daily</td>
<td>24m</td>
<td>Human/Male</td>
<td>DXA/qCT/marker</td>
<td>No synergistic effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH (1-34)+alendronate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cosman</td>
<td>2005</td>
<td>alendronate PTH (1-34)+alendronate daily</td>
<td>1:400</td>
<td>daily</td>
<td>15m</td>
<td>Human/Female</td>
<td>markers/DXA</td>
<td>No synergistic effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH (1-34)+alendronate cyclic</td>
<td></td>
<td>per 3m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cosman</td>
<td>2009</td>
<td>PTH (1-34) alendronate</td>
<td>1:0.69</td>
<td>daily</td>
<td>12m</td>
<td>Human/Female</td>
<td>DXA</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH (1-34)+zoledronate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samadfar</td>
<td>2007</td>
<td>PTH (1-34) alendronate</td>
<td>1:0.2</td>
<td>daily</td>
<td>2m</td>
<td>Mice/male</td>
<td>densitometer/3 pt/histology/marker</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH (1-34)+alendronate</td>
<td></td>
<td>weekly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Johnston</td>
<td>2007</td>
<td>PTH (1-34) alendronate</td>
<td>1:0.3</td>
<td>daily</td>
<td>2m</td>
<td>Mice/Female</td>
<td>DXA/qCT/marker/4 pt/lumbar compression</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH (1-34)+alendronate</td>
<td></td>
<td>weekly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclic: PTH+alendronate</td>
<td></td>
<td>daily</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2 Biophysical interventions

2.2.1 Exercise

Even though a range of effective drug treatments are available on prescription, there are people who cannot tolerate or have low rates of long-term adherence of these drug treatments. Thus there is always great interest in any “non drug therapies” for people with osteoporosis, though it must be recognized that these are not always supported by good research to prove that they reduce the fracture risk. Many patients are encouraged to exercise their musculoskeletal system, as bone mineral density can be improved by moderate to high intensity weight-bearing physical activity (Bischoff-Ferrari, 2011). Postmenopausal women who initiate weight-bearing exercises and/or muscle-strengthening exercises are proven to help prevent bone loss. A meta-analysis of exercise programs indicates that exercisers can gain about 1% bone mineral density vs. their non-exercised peers over 1 year (Wallace and Cumming, 2000). If that bone density effect continues to be addictive each year, it would ultimately produce very substantial effects. In another aspect, exercise also benefit on neuromuscular function, coordination, balance, and strength, thus reduce the risk of failing. There are different types of exercises which are proven effective. Walking modestly increases the load on the skeleton above gravity and, and, therefore, this type of exercise has proved to be less effective in preventing osteoporosis. This exercise intervention is not specifically designed to maximize loading forces to mechanically stress bone and induce changes in BMD, therefore, interventions that combine aerobic training with other forms of exercise that provide adequate skeletal loading may have greater benefits in improving bone mass among older adults. On the other hand, strength exercise seems to be a powerful stimulus for improving and
maintaining bone mass during the ageing process. In literature, the best improvements seem to be achieved through strength training of high-loading intensities with 3 sessions per week and 2–3 sets per session (Zehnacker et al., 2007). Although significant effects can be observed after 4 or 6 months in some locations of the body, the efficacy of the training programme is greater when it extends for at least 1 year. Multi-component exercise programmes of strength, aerobic, high-impact and/or weight-bearing training as well as WBV alone or in combination with exercise, may help to increase or at least prevent bone mass decline with ageing, especially in postmenopausal women.

2.2.2 Whole-body vibration

Not all elderly are able to undertake these high-intensity or weight-bearing exercise. One possible alternative intervention is WBV treatment which is performed in a low-impact manner with the possibility of producing similar results as the physical exercises (Prisby et al., 2008, Wysocki et al., 2011). Recently it has been considered a possible therapy for mitigating bone loss and improving muscle performance. In animal studies, vibration increases the anabolic (bone building) activity of bone tissue, as well as bone area and density (Rubin et al., 2001, Xie et al., 2006, Judex et al., 2007). However, the mechanism is not well understood (Rittweger et al., 2010). In WBV therapy, forced sinusoidal oscillations produced by motors beneath a mechanical platform transfer energy to a human body on the machine. It is believed that WBV induced fast but short stretches in muscles and tendon fibers causing increase in muscle power and strength. The increased muscle and ligament strength also places the skeleton under greater stress and bone responds to this by becoming stronger (Torvinen et al., 2002, Tanaka et al., 2003, Kerschan-Schindl et al., 2001). In sum, WBV aims to increase muscle strength with an
additional beneficial effect on bone. The vibration is transmitted via a platform on which the person usually stands and it is of reasonably high magnitude (0.3-0.8 g) and frequency (20-50 Hz) defined by International Organization for Standardization (ISO).

Table 2.3 shows different protocols of WBV treatment and their effects on the bone properties of the animals. Rubin et al. (2002) had demonstrated an osteogenic potential of WBV by a study in which the hind legs of ovine were subjected to mechanical vibration for 1 year (30 Hz and 0.3g acceleration, 20min/day) resulting in a 32% higher trabecular bone volume fraction compared to control. Later on, few ovariectomized rodent studies have examined the effect of WBV on osteoporosis specifically. Oxlund et al. (2003) found that WBV at 45 Hz, 3.0g for 30 min/ day increased periosteal bone formation rate after 90 days. In another study, cortical and medullary areas in WBV group were significantly larger than ovariectomized group after 8 weeks of treatment (30 Hz, 3g, 20min/day) (Rubinacci et al., 2008). However, there are other researcher who claimed that no substantial effects of 6 weeks of WBV treatment (90 Hz, 0.3g, 40min/day) on tibial bone microstructure and strength in ovariectomized rats were found (Brouwers et al., 2010). Similarly, van der Jagt et al. (2012) could not demonstrate any significant effects of 10 weeks WBV (45 Hz, 0.5g, 16min/day) on bone loss as a consequence of ovariectomy in rats. These studies thus suggest that WBV might have an osteogenic effect in ovariectomized animals, but that its effect varies with vibration characteristics (intensity, magnitude and duration etc.). The optimal protocol of whole-body vibration is still under research. In most of the studies listed below, WBV treatment showed positive results, in terms of increment in trabecular bone quantity, bone mineral density. Others showed either negative effects or none observable effects. Furthermore, how WBV on
ovariectomized rat progresses over long time (more than 12 weeks) has seldom been documented. It is of great importance since WBV is often suggested as a life-long intervention for osteoporotic patients.
Table 2.3. Literature review on whole body vibration therapy (↑: increase, ↓: decrease)

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Species</th>
<th>Parameters</th>
<th>Durations</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>flieger</td>
<td>1997</td>
<td>OVX, wistar rats</td>
<td>50Hz, 2g</td>
<td>30min, 60 days</td>
<td>↑ BMD; ↑ ultimate strength. No influence on SHM + vibration</td>
</tr>
<tr>
<td>Rubin</td>
<td>2002</td>
<td>Sheep</td>
<td>30 Hz, 0.3g</td>
<td>20min, 365 days</td>
<td>↑ BMD; ↑ trabecular formation; ↑ trabecular thickness</td>
</tr>
<tr>
<td>oxlund</td>
<td>2003</td>
<td>OVX, wistar rats</td>
<td>17 Hz, 0.5g/30Hz, 1.5g/45 Hz, 3.0g</td>
<td>30min, 90 days</td>
<td>↑ bone formation rate; ↓ endocortical resorption</td>
</tr>
<tr>
<td>Christiansen</td>
<td>2006</td>
<td>Old C57BL/6 mice</td>
<td>45 Hz, 0.1g/0.3g/1.0 g</td>
<td>15 min, 35 days</td>
<td>↑ trabecular bone volume in 0.1 and 1.0 g groups; ↓ marrow oestrogenic potential in 0.3 g group</td>
</tr>
<tr>
<td>Rubinacci</td>
<td>2008</td>
<td>OVX, SD rats</td>
<td>30 Hz, 0.6g/3g</td>
<td>20 min, 40 days</td>
<td>No observable cortical bone response at 0.6g; ↑ periosteal apposition and endosteal resorption at 3g.</td>
</tr>
<tr>
<td>Sehmisch</td>
<td>2009</td>
<td>OVX, SD Rats</td>
<td>90 Hz, unknown g</td>
<td>30 min, 35 days</td>
<td>↑ bone formation; ↓ trabecular microstructure</td>
</tr>
<tr>
<td>Brouwers</td>
<td>2010</td>
<td>OVX, Wistar rats</td>
<td>90 Hz, 0.3g</td>
<td>40 min, 42 days</td>
<td>No changes in trabecular and cortical structure in tibia</td>
</tr>
<tr>
<td>Wenger</td>
<td>2010</td>
<td>Old C57BL/6 mice</td>
<td>32 Hz, 0.5g/32 Hz, 1.5g</td>
<td>30min, 60 days</td>
<td>↑ femoral neck BMD in 0.5g group; ↓ femoral diaphysis BMD in 1.5g; ↑ stiffness and ↑ strength in the radius in 0.5g and 1.5g; ↓ BV/TV mean values compared to controls. No distinction between 0.5g and 1.5g.</td>
</tr>
<tr>
<td>Shi</td>
<td>2010</td>
<td>Fracture, OVX, SD rats</td>
<td>35 Hz, 0.3g</td>
<td>20 min, 40 days</td>
<td>↑ fracture healing, particularly the early phase; ↑ mineralization and remodelling</td>
</tr>
<tr>
<td>Tezval</td>
<td>2011</td>
<td>OVX, SD Rats</td>
<td>90 Hz, 3.9g</td>
<td>15 min, 35 days</td>
<td>↑ strength in femur; ↑ trabecular density and thickness. ↑ rate of bone formation in the periosteal femur</td>
</tr>
<tr>
<td>van der Jagt</td>
<td>2011</td>
<td>OVX, wistar rats</td>
<td>45 Hz, 0.5g</td>
<td>16 min, 50 days</td>
<td>no improvements in bone architecture WBV ↓ OVX-induced weight gain</td>
</tr>
</tbody>
</table>
2.3 Ovariectomized rat model

The ovariectomized female rat has been extensively used to study osteoporosis. The striking resemblance of OVX rat to humans with respect to estrogen deficiency related pathophysiology – i.e., increase in bone turnover, bone loss, and prevention of such by estrogen replacement make the OVX rat a gold standard model of osteoporosis. Since the OVX rat model effectively demonstrated the skeletal response of known agents such as estrogen and PTH in a fashion similar to that in postmenopausal women, it is therefore used for evaluation of new therapies for both prevention and treatment of osteoporosis (Kharode et al., 2008).

In this study, bilateral ovariectomy was performed as follows:

1. Anesthetize rats with an intraperitoneal injection of standard rat anesthesia (45 mg/kg ketamine, 8.5 mg/kg, xylazine, and 1.5 mg/kg acepromazine,) from Animal Holding Unit, National University of Singapore.

2. Shave the fur over the dorsal lumbar area; disinfect the skin with Betadine followed by an alcohol rinse for three times.

3. Make a 2-cm skin incision along the dorsal midline (near to the last rib) and through the abdominal musculature.

4. Gently grasp the ovarian fat pad using forceps, expose and remove the ovary (Fig. 2.1).
Fig. 2.1 incision site and exposure of ovary for ovariectomy in rats.

5. Stitch the muscle with 4.0 absorbable sutures and use stainless steel wound clips to close the skin incision.

6. Sham operation was performed using above steps but without removing the ovaries.

It was observed in literature that the 3 weeks post-ovariectomy osteoporosis development period is adequate for establishing a significant trabecular bone loss in mature Sprague-Dawley rats (Boyd et al., 2006, Bauss et al., 2002).
CHAPTER 3

IBANDRONATE DOES NOT BLOCK
THE ANABOLIC EFFECTS OF PTH
3.1 Introduction

In the previous chapter, different medical treatments were characterized. The first goal of this dissertation was to study the density and microarchitectural changes in ovariectomized rat bone given treatment of anabolic, antiresorptive and concurrent treatment using BMD and μCT measurements. PTH and ibandronate were chosen due to several features: As mentioned in Chapter 2, PTH has been shown to have an anabolic effect on bone structural properties in animal models (Iida-Klein et al., 2007) and humans (Black et al., 2003, Black et al., 2005). It increases bone strength primarily by stimulating bone formation. Alternatively, third generation nitrogen-containing bisphosphonates such as ibandronate and zoledronate have been successfully used to prevent fracture by suppressing bone resorption (Fleisch, 1996, Miller et al., 2005, Lambrinoudaki et al., 2008). Ibandronate has been proven effective in inhibiting bone resorption at considerably lower doses than other bisphosphonates in both rats (Mühlbauer et al., 1991) and ovariohysterectomized dogs (Monier-Faugere et al., 1999). Following long-term treatment with ibandronate, bone mass, strength and architecture were maintained, or even improved (Bauss and Dempster, 2007).

Whether the concurrent use of antiresorptive agent and PTH would produce an additive effect by reducing fracture risks is still controversial. Several studies had been tabulated in Chapter 1. In two clinical studies published in 2003, bisphosphonate was found to impair the ability of PTH to increase the BMD at lumbar spine and femoral neck (Black et al., 2003, Finkelstein et al., 2003). They claimed that this effect may be attributable to an attenuation of parathyroid hormone-induced stimulation of bone formation by bisphosphonate (alendronate). Nevertheless, additive effects of the same drug
combination were observed in other studies (Johnston et al., 2007, Samadfam et al., 2007, Cosman et al., 2009). In order to assure the efficacious use of pharmaceutical materials, it is essential to investigate the interaction and the net effect of PTH with newly approved bisphosphonates. From another aspect, it provides a basic understanding on drug combinations for future consequential or alternative administration. As stated in Chapter 1, we hypothesized that the combination therapy with PTH (1-34) and ibandronate would offer an advantage over monotherapy, in terms of bone mineral density combined with microarchitectural changes (Campbell et al., 2008) and eventually bone strength. The weekly low dosages of both PTH and Ibandronate adopted in this study was selected respectively from individual dosing tests, and also served to circumvent the negative side effects present in higher doses (Gittens et al., 2004, Bauss et al., 2002). To test this hypothesis, we compared the microarchitectural and mechanical changes of OVX rat tibiae in the following three treatment groups: PTH (1-34) alone, ibandronate alone and combined administration of PTH (1-34) and ibandronate.

### 3.2 Methods

As described in Chapter 1, overall design, proximal tibia bone and rat serum will be used to establish the OVX model with drug treatments. Every two weeks, two animals from SHM and OVX and three from each treatment groups were euthanized by carbon-dioxide asphyxiation. Both right and left tibia bones were harvested, wrapped in 0.9% saline soaked guaze and stored at -20°C until used for the experiments. From the study of osteoporotic bone fracture using rat models, the majority of fractures were observed at the metaphysis of long bones (Stürmer et al., 2006) where a rapid deterioration of trabecular bone occurs (Boyd et al., 2006). Thus, the trabecular-abundant metaphyseal
tibia is a suitable region to determine the degree of osteoporosis by bending tests (Stürmer et al., 2006, Stürmer et al., 2010) and CT analyses of trabecular bone (Boyd et al., 2006) in ovariectomized rat models.

**Micro-computed tomography (µCT)**

The metaphysis region (Fig.3.1 and Fig.3.3A) of the right proximal tibia was scanned ex vivo in an upright position with a source to object distance (SOD) of 41 mm and a source to image distance (SID) of 339 mm (Teo et al., 2006) using a SMX-100CT µCT scanner (Shimadzu, Kyoto, Japan). A 3.29 mm-thick volume of interest (VOI, 200 slices) was obtained starting from 1 mm distal to the proximal growth plate (Campbell et al., 2008). The resultant grayscale images obtained had an isotropic voxel size of 16.47 µm from cone-beam reconstruction (46 kV, 49 µA, scaling coefficient of 50 and averaged 3 times).

A semi-automated contouring method was employed to select trabecular from cortical bone (Bouxsein et al., 2010). In this method, an irregular, anatomic region of interest is drawn manually a few voxels away from the endocortical surface (Fig. 3.1). The drawback of the semi-automated contouring method is that it is dependent on the user’s judgment and the region can vary among users.

**Fig.3.1.** Contouring method used to delineate the trabecular bone region. Left: x-ray image with ROI (red) in the center; Right: binary image with black region as the selected ROI.
The grayscale images were segmented using an global threshold of 15.0 % of the maximal grayscale value (Laib et al., 2000) using the CT Analyzer software (Skyscan, Phil Salmon). Three-dimensional analysis (Teo et al., 2006) were used to assess bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), structural model index (SMI) and trabecular porosity (Tb.Po) for the same VOI. Tb.Po calculates the percentage of all the pores (closed and open) within the volume of interest. It is also referred to total porosity which is inversely related to bone volume fraction.

*Peripheral quantitative computed tomography (pQCT)*

The pQCT scans were carried out with a pixel size of 0.1 mm resolution and a slice thickness of 0.5 mm using a StraTEC’s XCT machine (Research SA+, StraTEC Medizintechnik, GmbH, Pforzheim, Germany). A scout view was performed prior to the actual scan to enable exact positioning of the bone specimens. The region of interest selected was similar to that used for µCT where the primary effect of osteoporosis was expected to be significant. Three types of volumetric bone mineral density (vBMD) were obtained, i.e. mean BMD, trabecular density (Tb.BMD) and cortical density (Ct.BMD). The thresholds used for separating soft tissue from bone and sub-cortical from trabecular bones were 280 mg/cm$^3$ and 550 mg/cm$^3$ respectively. The mineral density measurements from pQCT were taken from 5 adjacent slices within the region of interest for each tibia sample, i.e. we measured BMD of 10 slices for each rat and reported the mean values of each region of interest. Besides BMD measurement, we have conducted Strength-Strain Indices with respect to Y axis (SSIy) to assess the difference in mechanical property of
COM group which showed the similar BMD values to the IBN group (Kokorogiannis et al., 2009). The SSI is related to both geometrical properties and cortical density (Ward et al., 2005) (Fig. 3.2).

![Fig.3.2. A schematic diagram to show the calculation of stability index SSI.](image)

\[
SSI = \sum_{i=0}^{n} r_i^2 a \left( \frac{CD}{ND} \right) / r_{max} \quad 3.1
\]

Where \( r_{max} \) is the distance of voxel from centre; CD is the apparent cortical (bone) density; ND is the normal (cortical bone) density (1200 mg/cm\(^3\)); \( r_i \) is the pixel position from the centre; \( a \) is the area of a pixel.

**Atypical three point bending test for tibia**

A newly developed testing protocol was adopted for the 3-point bending test of metaphyseal tibia (Stürmer et al., 2006). A micro-testing machine (Instron 5848, Norwood, MA, USA) with a measuring range from 2 N to 1000 N at a precision of 0.2% of the load was used. The speed of the feed motion was 5 mm/s with 5% strain rate and the automatic switch-off pressure was set at 300 N. The experiment was programmed to stop in case of a strength drop of >20N or a linear displacement of >2mm to avoid shattering the tibia specimens. Maximum load (\( F_{max} \)), yield load (\( F_y \)) and stiffness (\( S \))
were recorded using ‘Merlin’ software. The yield load was determined by the 0.2% offset method.

The tibia were thawed and continuously moistened with isotonic saline solution during the test. Each tibia was placed with the three-point contact on the aluminium base, consisting of an aluminium block (30 mm wide, 14 mm high, 65 mm long) with the three rounded edge free notches (1 mm deep; 2, 3, or 4 mm in diameter) on top (Fig. 3.3B). The end of the dorsal proximal tibia (the two condyles) was placed in one of the notches (a in Fig. 3.3B). Because of its curved shape, the posterior distal diaphysis was rested on the other side of base (b in Fig. 3.3B). During the breaking test, the proximal tibia could not slip due to the notch on the base, but it was able to lengthen along the diaphyseal axis. The tip of the stamp consisted of an axle-led aluminium roller (8 mm high, 8 mm wide, 8 mm in diameter). A 2 mm-wide and 1 mm-deep circular notch with rounded edges was located in the center of the roller (c in Fig. 3.3B). The roller axle was fixed in a U-shaped support, which was connected to the micro tester by an aluminium stem. This positioning of the tibia with the custom-made jig ensured its stability during the 3-point bending test (Stürmer et al., 2006).

The movable base was placed so that the distance between the proximal end of the tibia (with epiphysis removed) and the center of the roller stamp was 3 mm throughout the test to achieve a consistent loading scenario. The stamp was driven down to the ventral metaphysis of the tibia until the primary strength of 1 N was reached. After a final visual check of the correct tibia position, the breaking test was started.
Fig. 3.3 The tibia specimen in the testing position. (A) μCT rendered image of rat tibia. (B) Details of the three-point bending test consisting of the aluminum base and the roller stamp.
**Biomarkers of bone turnover**

At week 0, 6 and 12, 300 μL of blood were collected from the lateral tail vein after overnight fasting of the rats, 5 days after drug administration. The serum was separated by centrifugation and stored at -20°C until analysis. Serum levels of bone formation markers, procollagen type 1 N-terminal propeptide (P1NP), and bone resorption markers, C-terminal cross-linked telopeptides of type I collagen (CTX), were assessed using enzyme-linked immunosorbant assay (Immunodiagnostic Systems Ltd, UK) (Schaller et al., 2004, Rissanen et al., 2008) for control, standard and duplicate tests.

**Statistical Analysis**

One-way analysis of variance (ANOVA) was performed by time to determine significant difference between different groups for each time point. Similarly, groups were evaluated for significant differences over the entire period. Mixed-model and Bonferroni test were adopted. The analysis has taken into account that two bones are from the same rat sample, using rat as the random factor. With preliminary data, t-test analysis to calculate sample size of this study was performed and the statistical significance was assumed at p < 0.05.

### 3.3 Results

**Animal weights**

At week 0 of ovariectomy surgery, there was no significant difference in average weight among all the testing groups. However, at week 4, the average weight of OVX group was significantly higher than that of the SHM group (338.8±18.1 and 294.3±16.6 g respectively, p=0.040). There was a general trend of longitudinal increase in the body weight for all the groups, with OVX showing significant weight gain (p=0.009) by week 2 after surgery compared to SHM at week 8 (p=0.007). For treatment groups, weight gain
for animals given PTH, IBN and combined treatment became obvious by 6 weeks (p<0.0005), 4 weeks (p=0.006) and 2 weeks (p=0.038) respectively. At the end of study, the overall increase in average weight was 29.3% (p=0.013), 54.9%, 46.7%, 52.3% and 69.5% (all p<0.0005) for SHM, OVX, PTH, IBN and COM groups respectively.

Micro-computed tomography

Three dimensional rendering of the tibial metaphyseal region is shown in Fig. 3.4 and the corresponding changes in the structural indices determined from μCT scans are shown in Fig. 3.5. Within 6 to 8 weeks of OVX surgery, trabecular bone micro-architecture of the OVX group exhibited significant difference from the SHM group in all the measured indices (BV/TV, Tb.Th, Tb.Sp, Tb.N, SMI and Tb.Po). This is in agreement with previous reports (Campbell et al., 2008) on the early detrimental effects of estrogen deficiency on trabecular architecture of rats. The results revealed that instead of trabecular thinning, the OVX rats are losing whole trabeculae.
Fig. 3.4. Visualization of bone loss in a transaction of the tibial metaphysis of an rat from each group at the time of surgery, and two, six, ten and twelve weeks following surgery.
Fig. 3.5. Changes in trabecular morphology measured by μCT. Top row: Bone Volume fraction (BV/TV), Structure Model Index (SMI) and Trabecular Porosity (Tb.Po). Bottom row: Trabecular Thickness (Tb.Th), Trabecular Separation (Tb.Sp) and Trabecular Number (Tb.N).

The anabolic effect of PTH was apparent on the trabecular architecture. PTH showed significant difference from the OVX group in Tb.N and Tb.Sp at week 8 (+43.2%, and -33.4%, p<0.05) and week 10 (+61.3% and -40.5%, p<0.05) of treatment respectively. At week 12, PTH showed higher Tb.N (+71.6%, p=0.024) and lower Tb.Sp (-31.8%, p=0.006), as compared to the OVX group. We also observed significant increase in BV/TV (+81.8%, p=0.008), with a drop in Tb.Po (-16.2%, p=0.008) compared to the OVX group at week 10 and 12.

It has been shown that reduction in bone volume fraction (BV/TV), loss of trabecular number (Tb.N) and increased trabecular separation (Tb.Sp) were all prevented by ibandronate treatment in an OVX rat model (Bauss et al., 2002). In this study,
ibandronate’s ability to preserve bone microarchitecture was observed earlier than PTH. After week 6, IBN exhibited significant difference from the OVX group in terms of BV/TV (+82.9%, p=0.009), SMI (-53.5%, p=0.009), Tb.Po (-19.8%, p=0.009) and Tb.N (+75.2%, p=0.016). Tb.Sp decreased significantly compared to OVX group after week 8 (-38.8%, p=0.015). At the end of the study, BV/TV and Tb.N increased (+78.2%, p=0.012) and (+76.9%, p=0.013), while SMI, Tb.Po and Tb.Sp decreased (-21.4%, p=0.013), (-15.3%, p=0.012) and (-50.4%, p<0.001) respectively as compared to the OVX group.

The combined COM treatment showed a significant increase in BV/TV (+76.2%, p=0.01) and Tb.N (+67.5%, p=0.021) as well as a significantly lower Tb.Po (-18.2%, p=0.010) and SMI (-41.1%, p=0.037) as compared to OVX group after week 6.

**Mechanical Testing**

The results of maximum load ($F_{\text{max}}$), yield load ($F_y$) and stiffness ($S$) of different groups from three point bending tests are provided in Table 3.1. Due to the growth of the rats involved in this study, $F_{\text{max}}$ of SHM group increased from $61.2 \pm 21.7$ N in week 0 to $104.0 \pm 11.0$ N at week 12 (+69.9%). However, there was no significant change in the OVX group from week 2 ($49.4 \pm 11.8$ N) to week 12 ($48.9 \pm 12.1$ N). There was a significant difference between SHM and OVX groups from the second week after surgery, indicating the development of osteoporosis. Within the treatment group, in week 8 and 12, $F_{\text{max}}$ of COM group are significantly higher than that of the IBN or the PTH group. However, in week 10, the COM group only shown significant higher $F_{\text{max}}$ than PTH group. At week 12, the percentage increase from OVX in the COM group (168%) was greater than the sum of the effects of PTH alone (59%) and IBN alone (77%) in $F_{\text{max}}$. 
Table 3.1. Mechanical property changes of animal groups measured by 3-point bending test. Group averages are represented as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Weeks after surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>F&lt;sub&gt;max&lt;/sub&gt; (N)</strong></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>61.2 ± 21.7</td>
</tr>
<tr>
<td>OVX</td>
<td>49.4 ± 11.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PTH</td>
<td>64.4 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IBN</td>
<td>65.0 ± 13.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>COM</td>
<td>81.8 ± 3.4</td>
</tr>
<tr>
<td><strong>F&lt;sub&gt;y&lt;/sub&gt; (N)</strong></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>42.4 ± 20.4</td>
</tr>
<tr>
<td>OVX</td>
<td>41.9 ± 20.8</td>
</tr>
<tr>
<td>PTH</td>
<td>38.8 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IBN</td>
<td>41.7 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>COM</td>
<td>58.5 ± 15.4</td>
</tr>
<tr>
<td><strong>S (N/mm)</strong></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>184.8 ± 19.4</td>
</tr>
<tr>
<td>OVX</td>
<td>111.7 ± 35.7</td>
</tr>
<tr>
<td>PTH</td>
<td>163.9 ± 47.7</td>
</tr>
<tr>
<td>IBN</td>
<td>158.1 ± 41.2</td>
</tr>
<tr>
<td>COM</td>
<td>147.8 ± 10.1</td>
</tr>
</tbody>
</table>

a. Significant difference from SHM (p<0.05)
b. Significant difference from OVX (p<0.05)
c. Significant difference from PTH (p<0.05)
d. Significant difference from IBN (p<0.05)
There was a significant difference in $F_y$ between SHM and OVX groups in week 12 ($p=0.048$). $F_y$ in the OVX group decreased by 18.2% from the surgery to week 12, while a sharp increase was observed in the PTH, IBN and COM groups at week 12 (36.6%, 67.7% and 126.8% respectively).

From the day of surgery to the end of 12th week, stiffness in OVX has decreased by 38.9%. On the other hand, in the SHM group, stiffness was relatively stable, from 184.8 ± 19.4 N/mm to 206.7 ± 31.2 N/mm (Table 3.1). From the 10th week, significant difference was seen between the SHM and OVX groups ($p=0.045$). At the end of the experiment at week 12, within the three treatment groups the only observed significant difference was the COM group compared to OVX ($p=0.010$).

*Peripheral quantitative computed tomography (pQCT)*

The mean BMD, trabecular density (Tb.BMD) and cortical density (Ct.BMD) from pQCT scans for all the groups at different time points are provided in Table 3.2. One way ANOVA of the mean BMD of different groups revealed a significant difference between OVX (-26.0%, $p<0.001$) and SHM groups, starting from week 4 to the end of experiment, revealing the successful establishment of our ovariectomized rat model. From week 8 onwards, COM (+18.4%, $p=0.015$) and IBN (+21.3%, $p=0.006$) groups showed a significant difference from OVX group. At week 10, mean BMD of all treatment groups, including PTH (+16.2%, $p<0.001$) group, were significantly higher than those of the OVX group, which demonstrates the effectiveness of PTH and/or IBN treatments. Within the treatment groups, COM ($p<0.001$) group had a significantly higher mean BMD value than PTH groups. At week 12, the final week of our experiment, no significant difference was found among COM, IBN and SHM groups in terms of mean BMD.
OVX exhibited a significantly lower Tb.BMD (-27.6%, p=0.002) than SHM from week 2 onwards. The IBN and COM groups showed significantly higher Tb.BMD (+47.5%, p<0.001 and +34.0%, p=0.011) than OVX group from week 8. At the end of experiment (week 12), all three treatment groups showed significantly higher Tb.BMD (p<0.05) with the IBN group showing the highest value among all treatment groups. We also observed a plateau in Tb.BMD for OVX and PTH from week 6 onwards.

The Ct.BMD of all the rat groups showed an increasing trend with time, indicating the continual cortical bone growth. Ct.BMD of the treatment groups (IBN, PTH, COM) (p<0.05) was significantly higher than of OVX at week 8. However, after week 8, the Ct.BMD of IBN reached a plateau whereas Ct.BMD of PTH and COM groups reached the plateau after week 10.
Table 3.2. Density changes of animal groups measured by pQCT. Group averages are represented as mean ± SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weeks after surgery</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean BMD (mg/cm³)</td>
<td>SHM</td>
<td>631 ± 16</td>
<td>743 ± 14</td>
<td>743 ± 20 (^b)</td>
<td>749 ± 9 (^b)</td>
<td>786 ± 70 (^b)</td>
<td>750 ± 22 (^b)</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>513 ± 24</td>
<td>550 ± 23 (^a)</td>
<td>584 ± 93 (^a)</td>
<td>592 ± 14 (^a)</td>
<td>573 ± 4 (^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>617 ± 25</td>
<td>647 ± 38 (^a)</td>
<td>666 ± 29 (^a, b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>632 ± 9</td>
<td>718 ± 49 (^b)</td>
<td>675 ± 18 (^a, b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>653 ± 63</td>
<td>701 ± 39 (^b)</td>
<td>721 ± 23 (^b, c, d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb.BMD (mg/cm³)</td>
<td>SHM</td>
<td>356 ± 19</td>
<td>445 ± 13 (^b)</td>
<td>430 ± 9 (^b)</td>
<td>392 ± 18 (^b)</td>
<td>456 ± 18 (^b)</td>
<td>392 ± 59 (^b)</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>322 ± 20 (^a)</td>
<td>273 ± 27 (^a)</td>
<td>193 ± 19 (^a)</td>
<td>200 ± 13 (^a)</td>
<td>190 ± 15 (^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>212 ± 13 (^a)</td>
<td>231 ± 20 (^a)</td>
<td>218 ± 22 (^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>255 ± 47 (^a)</td>
<td>295 ± 27 (^a, b, c)</td>
<td>281 ± 34 (^a, b, c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>256 ± 56 (^a)</td>
<td>268 ± 50 (^a, b)</td>
<td>244 ± 30 (^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.BMD (mg/cm³)</td>
<td>SHM</td>
<td>1043 ± 3</td>
<td>1038 ± 8</td>
<td>1061 ± 28</td>
<td>1095 ± 26</td>
<td>1090 ± 13</td>
<td>1113 ± 65</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1026 ± 26</td>
<td>1064 ± 24</td>
<td>1095 ± 30</td>
<td>1110 ± 33</td>
<td>1132 ± 23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>1124 ± 29</td>
<td>1156 ± 21 (^a, b)</td>
<td>1174 ± 13 (^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>1127 ± 23</td>
<td>1161 ± 30 (^a, b)</td>
<td>1153 ± 15 (^a, b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>1132 ± 19</td>
<td>1158 ± 10 (^a, b)</td>
<td>1182 ± 5 (^a, b)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Significant difference from SHM (p<0.05)  
\(^b\) Significant difference from OVX (p<0.05)  
\(^c\) Significant difference from PTH (p<0.01)  
\(^d\) Significant difference from IBN (p<0.01)
At the 12th week, SSIIy values of all groups are presented as follow: SHM = 6.10±0.53 mm$^3$, OVX = 4.44±0.59mm$^3$, PTH = 5.43±0.88mm$^3$, IBN = 5.70±0.38mm$^3$, COM = 7.36 ±0.79 mm$^3$. The SSIIy of COM group were significantly higher than IBN (p=0.007), PTH (p=0.001) and OVX (p<0.001) groups. The percentage increase from OVX in the COM group (66%) was greater than the sum of the effects of PTH alone (22%) and IBN alone (28%) in SSIIy. As shown in Fig 3.6, the significance and trend is in accordance with $F_{max}$ results ($R^2=0.8033$, p<0.001).

![Graph showing positive linear correlation between maximum load ($F_{max}$) and strength-strain index to the y axis (SSIIy).](image)

**Biomarkers of bone turnover**

The serum levels of bone formation (P1NP) and bone resorption (CTX) biomarkers at 0, 6 and 12 weeks of different rat groups are provided (Table 3.3).
At week 0, the serum P1NP concentration of different groups did not show any significant difference. However, at the end of week 12, COM and PTH showed significantly higher (p<0.001) levels of P1NP than the rest of the groups. Moreover, COM showed 71% significant higher concentration of P1NP than the PTH group (p<0.001). The percentage increase from OVX in the COM group (543%) was much greater than the sum of the effects of PTH alone (276%) and IBN alone (-20%) in bone formation marker, at week 12.

At week 0, the serum CTX levels of different groups did not show any significant difference. At the end of week 12, a serum CTX level of the OVX group was 113.2% (p<0.05) higher than that of the SHM group, indicating higher bone resorption in the OVX group than in the SHM group. PTH did not show any significant difference from the OVX group. IBN and COM showed a significant decrease in the serum CTX levels as compared to the OVX group, suggesting that ibandronate alone or its concurrent administration with PTH decreases bone resorption.
Table 3.3 Serum levels of bone formation (P1NP) and resorption (CTX) markers. Average concentration is expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Week(s) after surgery</th>
<th>0</th>
<th>6</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1NP (µg/L)</td>
<td>SHM</td>
<td>19.9 ± 11.6</td>
<td>14.4 ± 7.2</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>17.3 ± 7.9</td>
<td>7.8 ± 1.1</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>19.6 ± 11.9</td>
<td>10.3 ± 0.7</td>
<td>28.6 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>13.3 ± 2.1</td>
<td>9.3 ± 2.3</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>21.6 ± 3.1</td>
<td>12.9 ± 1.8</td>
<td>48.9 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>CTX (µg/L)</td>
<td>SHM</td>
<td>21.4 ± 0.1</td>
<td>28.3 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>21.6 ± 3.0</td>
<td>43.2 ± 13.9</td>
<td>32.4 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>23.3 ± 9.0</td>
<td>36.6 ± 7.4</td>
<td>41.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>20.5 ± 3.8</td>
<td>29.8 ± 11.0</td>
<td>14.3 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>26.2 ± 0.2</td>
<td>14.1 ± 1.6</td>
<td>17.5 ± 3.6</td>
</tr>
</tbody>
</table>

a. Significant difference from SHM (p<0.05)
b. Significant difference from OVX (p<0.05)
c. Significant difference from PTH (p<0.01)
d. Significant difference from IBN (p<0.01)
3.4 Discussion

This chapter investigated the effects of ovariectomy induced osteoporosis and the efficacy of the combination therapy of anabolic (PTH) and anti-resorptive (ibandronate) drugs in terms of morphological and biomechanical analysis. By using the OVX rat model, the changes in bone quality and the efficacy of concurrent treatments were assessed through μCT, pQCT, biomarkers and three-point bending tests. The SHM group served as a negative control to illustrate normal bone growth over the course of the experiment. The OVX group was a positive control group to study changes occurring during the development osteoporotic condition following the ovariectomy and to compare the effect of various treatments. Differences between OVX and SHM have been well-characterized from previous studies using μCT (Boyd et al., 2006, Bauss et al., 2002), three point bending test (Stürmer et al., 2006). The effect of individual treatment such as anti-resorptive treatments: tiludronate (Barbier et al., 1999), ibandronate (Bauss et al., 2002), have also been extensively studied. Fewer animals were allocated to the OVX and SHM group in this study due to the existing results on those groups. In phase 1 study (Chapter 3, 4, 5), relatively young rats (12 weeks-old) were used for OVX surgery. There are other studies which successfully used 3 months-old rats to investigate the effects of estrodiol, testosterone and raloxifene in fracture healing during early osteoporosis (Stürmer et al., 2006). Moreover, the effect of vibrational stimulation to prevent bone loss in the OVX model were thoroughly investigated using 3-months-old rats (Flieger et al., 1998, Rubinacci et al., 2008, Sehmisch et al., 2009). Unlike the rabbit osteoporosis model, the skeletons of aged rats do not achieve full skeletal maturity and have very low rates of remodeling in the cortical bone. Therefore, the trabecular-rich VOI in the proximal
metaphysis of rat tibiae are appropriate to study the efficacy of the anti-resorptive and/or anabolic drugs in OVX rat model.

Microstructural and BMD changes in OVX and SHM

The most significant reduction occurred in bone volume fraction (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) during the first 6-8 weeks after OVX surgery. The trabecular struts became more rod-like and the bone material within the VOI becomes significantly porous. These results are consistent with previous studies which reported that irreversible microarchitectural changes happen within the first 8 to 12 weeks after the ovariectomy (Campbell et al., 2008, Boyd et al., 2006). BV/TV for the SHM group used in this study is comparatively higher than in previous studies due to the fact that we used 12 weeks-old mature virgin rats as compared to 6-8 month-old retired breeders. We observed 57% reduction in BV/TV while previous studies reported a 30-40% reduction within the same period of 8 weeks post-ovariectomy (Campbell et al., 2008).

Bone loss resulting from estrogen deficiency in younger rats is proportionally more drastic, mainly because younger rats have denser trabecular bone at the beginning. Plateaus were observed from 8 weeks onwards for structure model index (SMI), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular density (Tb.BMD) for OVX, as previously described by Campbell et al (2008). The leveling off of microarchitectural changes was observed even in the treatment groups. Thus, drastic structural changes following estrogen deficiency and the leveling off in microarchitectural indices illustrate the importance of early treatment in osteoporosis in order to prevent further deterioration in bone.

Beneficial effects of ibandronate
Ibandronate inhibits bone resorption and formation by reducing the bone turnover rate (Bauss and Dempster, 2007), which helps protect the trabecular microstructure from the deleterious effects of bone resorption. Between week 2 and 4 weeks after administration, the anti-resorptive effect of ibandronate was apparent in the higher BV/TV and Tb.N and lower SMI, Tb.Sp and Tb.Po as compared to OVX group (Fig. 3.5). The beneficial effect of ibandronate was also observed from pQCT, which showed a significant increase in Tb.BMD after 4 weeks of administration (Table 3.2). Smith et al. also reported a similar effect of ibandronate in preventing bone loss and maintaining bone strength in ovariectomized cynomolgus monkeys (Smith et al., 2003). Even though the positive effects of ibandronate could be observed as early as 2-4 weeks after administration, the duration was limited as indicated in the plateau of μCT and pQCT indices.

**Beneficial effects of PTH**

PTH stimulates bone formation and also bone resorption, effectively increasing bone remodeling rate. The net result can produce a catabolic or anabolic effect on trabecular bone depending on dosage and frequency of administration (Frolik et al., 2003). The comparative higher PTH to ibandronate ratio and intermittent PTH administration used in our study exerted a net anabolic effect on BV/TV, Tb.Sp and Tb. Po from the 10th week after OVX surgery. Effects of ibandronate on the structural indices were observed as early as the 2nd week after the administration of the drug. The fact that the effect of PTH was significant only after 6 weeks in rats suggests that ibandronate was more effective at preventing the early deterioration of trabecular bone than PTH at similar dosage.

**Beneficial effects of combination treatment**
Previous studies using alendronate have shown that it may negatively reduce PTH’s anabolic effects on bone tissues (Black et al., 2003, Finkelstein et al., 2003). In our study of using the combined PTH and ibandronate treatment, however, significant increase in BV/TV and Tb.N while achieving lower SMI and Tb.Po were observed from the 2nd week after treatment initiation as compared to individual treatments. The non-blocking effect of the concurrent treatments could also be observed in the mean BMD of the pQCT results (Table 3.2). This suggests no reduction in PTH’s anabolic effects when combined with ibandronate administration.

Mechanical testing result

The results from three-point bending tests were compared to pQCT and µCT analysis to evaluate the mechanical property change of the rat tibiae, corresponding to those of structural changes. Tibiae treated with either drug or their combination had significantly higher $F_{\text{max}}$ than OVX rats. Among treatment groups, a significant improvement on $F_{\text{max}}$ was observed in the COM group at the end of the study. This means that the combined treatment would allow the bone to withstand higher load before fracturing compared to mono treatments. This phenomenon is consistent with the higher BMD and Ct.BMD in the combined drug treated group as obtained from pQCT. Large standard deviation of $F_y$ in the COM group resulted in the loss of statistical significance when compared to other treatment groups. This made it difficult to draw a conclusion on early stage microfracture in response to each individual treatment. Among treatment groups, COM is the only group which showed a significantly higher stiffness as compared to OVX, with the value approaching those of the SHM group. It is worth noticing that the sliding support at the distal end would possibly introduce an additional friction moment which lies in a
direction to increase the force of breaking. However, the moment here is negligible in group comparison.

**Biomarker result**

From the biomarker results, we could see that PTH group had significantly higher (p<0.001) levels of P1NP than OVX group whereas CTX levels of PTH did not show any significant difference from OVX group. This shows that PTH treatment resulted in higher bone formation, but had no influence on bone resorption. Meanwhile, IBN group had significantly lower (p=0.015) levels of CTX compared to the OVX group but the P1NP levels of IBN did not show any significant difference from OVX group. This shows that ibandronate treatment resulted in reduced levels of bone resorption, but had no influence on bone formation. Overall, the mono-treatment PTH group showed only increased bone formation and the IBN group showed only decreased bone resorption, as is consistent with previous study (Black et al., 2008). On the other hand, the combined treatment group (COM) showed significantly higher (p<0.001) levels of P1NP as well as lower (p=0.040) levels of CTX as compared to OVX group. This shows that the concurrent treatment has an added advantage on bone mass as it results in the increase in bone formation as well as a significant decrease in bone resorption.

Interestingly, the CTX levels of COM group did not significantly differ from IBN, whereas the P1NP levels of COM were significantly higher (p<0.001) than the PTH group. This shows that PTH has very little or no effect on the action of ibandronate, whereas concurrent administration of ibandronate has a positive effect of inducing the action of PTH, thereby increasing bone formation.

*Comparison with literature: drug dosage*
As a counter additive effect on concurrent administration of both anabolic and anti-resorptive drugs was shown in previous studies (Black et al., 2003, Garcés and García, 2006, Finkelstein et al., 2003), a partial positive additive effect was observed in $F_{\text{max}}$, SS$	ext{I}y$ and P1NP results in this study. To our knowledge, this is the first study to assess concurrent administration of ibandronate (third generation bisphosphonates) and PTH in an OVX rat model. Most of the previous studies that analysed the additive effect of the anabolic and anti-resorptive treatments used the second generation bisphosphonates (alendronate) along with PTH (Black et al., 2003, Cosman et al., 1998, Garcés and García, 2006, Finkelstein et al., 2003, Johnston et al., 2007, Rittmaster et al., 2000). While other bisphosphonates require frequent administration to maintain their beneficial effects, Bauss et al. (2002) have shown that effect of ibandronate is accumulative and independent on the frequency of drug administration. The truncated parathyroid hormone (1-34) used in this study was different from full intact parathyroid hormone (1-84). It is speculated that the presence or the absence of the C-terminal region of the PTH molecule can affect the expression of a biological response (Yamamoto et al., 1994). Since there is no previous study of using PTH and ibandronate on SD rats to follow the used dosage, thus, based on previous study on the dose dependent effect of PTH (SD rats) (Gittens et al., 2004) and ibandronate (Wistar rats) (Bauss et al., 2002), we adopted a relatively high dosage ratio of PTH to ibandronate via weekly administration compared to the prevalent therapies for the OVX model (Johnston et al., 2007, Samadfam et al., 2007). We believe the effect of a drug to those two rats should be quite similar in physical characteristic, which helped our decision of choosing drug dosage based on their body weight. The use of weekly low dosages of both PTH and ibandronate (with higher PTH to ibandronate ratio) still
provided benefits while avoiding negative consequences associated with higher dosages (Sato et al., 2002).

Despite encouraging results, higher dosage and extended duration of exogenous PTH (1-34) treatment have been reported to produce undesirable side effects (Sato et al., 2002). A daily treatment of only 8 µg/kg PTH (1-34) for one year in OVX rats can result in an 11% increase in cortical bone brittleness. Also, PTH (1-34) therapy has been linked with hypercalcemia both in animals and in clinical studies (Crandall, 2002). Headaches, nausea and back pain were reported with clinical PTH (1-34) therapy. Thus, we reduced the PTH (1-34) dosing frequency from daily to weekly as a means of circumventing some of these adverse effects. The PTH (1-34) dosage and frequency we adopted are in accordance with a comparative study by Gittens et al (2004). The comparison between the data in their experiment and the current study suggested that these low doses, 28-40 µg/kg over 4 weeks, may be close to the threshold for effective dosage for bone mass accretion in the OVX rat model. Also, it has been reported that weekly administration of PTH increases bone mass as much as daily administration of PTH (Uzawa, 2007).

Additionally, the high dosage used in previous studies is not clinically relevant. The clinical dose of PTH (1-34) used for osteoporosis therapy is 20 µg/day (Finkelstein et al., 2003, Komatsu et al., 2009). In rats, PTH (1-34) doses of 5 µg/kg/day result in 3 times the systemic exposure seen with 20 µg/day in patients (Co Ela, 2002). However, prior fracture studies in rats have used doses of PTH(1-34) as high as 200 µg/kg/day (Cipriano et al., 2009), calling into question the clinical relevance of the dosage used. These data underscore the importance of conducting clinical studies to evaluate the optimized dosage of PTH in bone regeneration (Komatsu et al., 2009).
The mean BMD results were in agreement with the three-point bending results in terms of $F_{\text{max}}$, $F_y$ and $S$, suggesting that BMD measurements can still be used to predict mechanical properties of bone. The lack of sufficient statistical significance in our three point bending test may be due to the following reasons. The rats used in this study were considerably young and their skeleton still growing. Also, three different types of fractures (the metaphyseal oblique fracture, the Y-fracture, and the condyle fracture) observed by Stürmer et al (2006) may exist in all animal groups, but were not taken into account in this study due to limited sample size.

SSI can predict maximum load

Besides BMD measurement, we have conducted Strength-Strain Indices (SSI) calculation to understand the combined treatment effect of COM group which showed the similar BMD values to IBN group. Since SSI is related to both geometrical properties and cortical density, the axial SS$I_y$ predicts the bending strength with respect to the Y axis (the longitudinal axis of rat tibiae). At the 12th week of study, The SS$I_y$ of COM group were significantly higher than IBN ($p=0.007$), PTH ($p=0.001$) and OVX ($p<0.001$) groups. The percentage increase from OVX in the COM group was greater than the sum of the effects of PTH alone and IBN alone for SS$I_y$ and $F_{\text{max}}$, indicating an additive effect. As shown in Fig.3.6, the significance and trend is in accordance with $F_{\text{max}}$ results ($R^2=0.8033$, $p<0.001$). A recent study shows that SS$I_y$ presents a significantly positive correlation with maximum load with a correlation value $R=0.846$ ($P<0.001$) (Kokoroghiannis et al., 2009). Since SS$I_y$ served as a good predictor of $F_{\text{max}}$, the SS$I_y$ values from this study could be served as an explanation to the discrepancy between BMD and mechanical testing result.
**Important factor in drug synergy: Dosage and ratio**

As discussed, we have conducted a pilot study with a relatively low dosage and weekly administration of ibandronate and PTH. A higher ratio of anabolic to anti-resorptive agent is less likely to inhibit the bone formation function by PTH, which could account for the lack of blocking effect by ibandronate used in this study. It also provides a possible explanation of why an anti-resorptive agent (ibandronate) is more effective than an anabolic agent (PTH) on BMD and mechanical properties. Although the synergistic pathway of the two drugs is still unknown in the current study, we believe the partial beneficial effect is attributed to the right drug dosage and ratio so that the effect of PTH on bone density can be optimized when a potent anti-resorptive agent is used either concurrently or alternatively. In previous rodent studies, the effect of concurrent treatment of PTH and bisphosphonate was additive at drug ratios of 1:0.3 (Johnston et al., 2007) and 1:0.2 (Samadfam et al., 2007). On the other hand, negative / blocking effects from alendronate occurred at dose ratios of 1:100 (Black et al., 2003) and 1:250 (Finkelstein et al., 2003) in clinical trials. In this study, we used a PTH to ibandronate ratio of 1:0.7 according to tests done by previous studies (Bauss and Dempster, 2007, Gittens et al., 2004, Bauss et al., 2002). A threshold ratio of anabolic and anti-resorptive agents would be postulated in this OVX model. If the dosage of bisphosphonate went beyond the threshold, no beneficial effect would be observed. Below this threshold ratio, an increase of PTH dosage might lead to an increase in beneficial effect correspondingly (Cosman et al., 2005). Series of ratio dependency experiments and histomorphometric studies would be conducted subsequently to better illustrate the mechanism of these effects. Apart from dose and ratio, other factors such as rodent appendicular bone effect,
nature of drug time course (daily, cyclic, alternation therapies, etc.) could also be possible factors that affect the results of this combination therapy.

3.5 Conclusion

- Compared to the previous studies which showed impedance from bisphosphonates in combination therapy with PTH, this study suggested that ibandronate does not block the anabolic effects of PTH in ovariectomized rat tibiae. Maximum load, Strength-Strain Indices and bone formation serum markers of COM group are significantly higher compared to both mono therapy groups.

- The effect may be attributed to the proper ratio of anabolic and anti-resorptive drugs combined with the appropriate course of treatment. Further exploration of these findings would allow us to optimize the ratio between PTH and ibandronate and maximize the beneficial effects of the concurrent administration of the two drugs in the treatment of osteoporosis.
CHAPTER 4

ALTERATIONS OF BONE VISCOSITY AND GEOMETRY UPON DRUG TREATMENTS
4.1 Introduction

In Chapter 3, combination therapy of PTH and ibandronate has an additive effect on rat tibia in terms of several traditional bone quality parameters. However, the osteoporotic drug effects on nano-level viscoelastic properties were seldom investigated. Nano viscoelasticity of bone, which has been of recent concern, mainly arises from the the natural viscoelastic response of collagen fiber as a polymer (Ammann et al., 2007, Garner et al., 2000, Brennan et al., 2009). During daily life, time-dependent viscoelastic deformation occurs in bone as the primary function of skeleton is to bear long-term load from bodyweight and muscular activity (Kim et al., 2010, Garner et al., 2000). For a better understanding of the pathogenesis and drug efficacy in osteoporosis, timely investigation into bone nano viscoelasticity is necessary.

In this chapter, nanoindentation tests were performed on individual trabeculae and cortex of the distal metaphyseal femur. A novel nanoindentation creep test (Kim et al., 2010) was adopted to calculate the changes in bone viscosity \( \eta \), accompanying with ovariectomy and drug administration. In comparison and explanation, possible concomitant alterations in macro bone geometry, microarchitecture and traditional BMD measurements will also be studied in rat femur. As stated in Chapter 1, it is hypothesized that osteoporotic bone properties would be influenced by mono or concurrent treatments in various aspects.

4.2 Methods

As described in Chapter 1, overall design, distal femur bone is used to characterize the nano-level viscoelastic change.

*Micro-computed tomography (\( \mu \)CT)*
Due to several limitations, the previous μCT (SMX-100CT scanner, Shimadzu, Kyoto, Japan) is no longer accessible. Thus another μCT machine (Skyscan 1076 scanner, Skyscan, Belgium) is adopted here to measure 3D microstructure of distal femur. Difference exists in the two μCT machines used in this thesis. Data acquisition from 360° around the long axis of the bone sample is required in order to provide a three-dimensional rendering of the region of interest for further analysis. With the sample positioned in between the x-rays source and detector, there are two ways to accomplish this. The bone sample has to either rotate on its long axis or the x-rays source and detector revolve perpendicularly to this axis. The Shimadzu scanner uses the first method while the Skyscan machine uses the second. It is not encouraged to directly compare the results from two different methods of scanning. However, group comparison within one approach would be still valid.

Scanning parameters adopted in this Chapter were also modified according to the machine. The metaphysis region of the distal femur was scanned ex-vivo in an upright position with a source to object distance (SOD) of 121 mm and a source to camera distance (SID) of 161 mm using a Skyscan 1076 μCT scanner. A 3.6 mm-thick volume of interest (VOI, 200 CT slices) was selected 1mm above distal growth plate (Fig. 4.2) (Campbell et al., 2008). The resultant grayscale images obtained had an isotropic voxel size of 17.75 µm from cone-beam reconstruction (100 kV, 100 µA, using a 0.5 mm Al filter and averaged 3 times). Semi-automated contouring method was again employed to select trabecular from cortical bone. The grayscale images were segmented using the same threshold of 15% of the maximal grayscale value as last Chapter (Laib et al., 2000). Bone volume ratio (BV/TV), bone surface to volume ratio (BS/BV), trabecular separation
(Tb.Sp), trabecular number (Tb.N), structural model index (SMI), trabecular thickness (Tb.Th) and cortical porosity (Ct.Po) were assessed for the same VOI using the CT Analyser program (Phil Salmon, Skyscan). 3D visualization of the VOI using adaptive rendering algorithm was done with the ANT program (Skyscan, Belgium).

**Peripheral quantitative computed tomography (pQCT)**

The pQCT scans were carried out with the same parameters described in Chapter 3. The mineral density measurements from pQCT were taken from 5 adjacent slices (inter slice distance: 0.75mm) at the VOI for each femur sample -- 1mm above distal growth plate (Fig. 4.3). Same as Chapter 3, three types of volumetric bone mineral density (vBMD) and SSIy were measured. Besides, cortical bone area (Ct.Ar), cortical bone thickness (Ct.Th), periosteal perimeter (Ps.Pm), endocortical perimeter (Ec.Pm), cross-sectional moment of inertia (CSMI) and were specifically determined at the VOI in this Chapter.

**Nanoindentation testing**

Harvested rat femurs were positioned vertically and embedded in epoxy resin. Over-night hardened samples were then metallographically cut to create a smooth surface 1 mm above growth plate, perpendicular to the long axis of the bone. Silicon carbide paper of grit size 320, 500, 1200, 4000 were consequently used to grind the sample surface. Further polishing was done by microcloths with alumina powder of grit size 3µm and 1µm. Immediately after 16 hours of rehydration with 0.9% saline, the samples were subjected to nanoindentation tests using G200 Nanoindenter (Agilent Technologies Inc., Chandler, AZ) at room temperature (26ºC). A Berkovich diamond indenter was used for all measurements. A total of 30 indents were produced on each sample, four bone samples per group. For cortical indentation, 20 indents were evenly distributed on the
interstitial bone from medial-lateral area. According to our preliminary test result, this area of bone is matured and the value obtained is relatively stable. For trabecular indentation, 10 indents were placed in the center of 10 single trabeculae. Data of indents from the same sample were averaged and the mean value was used in statistical analysis. In this study, Continuous Stiffness Measurement (CSM) and basic creep tests were paired to obtain $E$, $H$ and $\eta$, in a single set of test.

**Continuous Stiffness Measurement (CSM):** $H$ and $E$ were continuously obtained in a range of depth. It is accomplished by imposing a small, sinusoidally varying signal on top of a DC signal that drives the motion of the indenter. This allows the measurement of contact stiffness ($S$) at any point along the loading curve and not just at the point of unloading as in conventional measurements (Li and Bhushan, 2002). Superimposed upon the quasi-static loading segment was a small oscillating force at 45 Hz. The amplitude of the oscillating force, $F_o$, was continually adjusted in order to maintain the amplitude of the resulting displacement oscillation at $z_o = 2$ nm. $S$ was determined continuously during loading from the amplitude ratio $F_o/z_o$:

$$S = \left| \frac{1}{F_o \cos \phi - (K_s - m\omega^2)} - \frac{1}{K_f} \right|^{-1}$$  \hspace{1cm} 4.1

Where $\phi$ is a phase angular lag between the oscillatory force (with frequency $\omega$) and displacement; $K_f$ and $K_s$ are the stiffness of load-frame and support springs; $m$ is mass of the indenter. These parameters can be determined by calibration of the system. $E$ and $H$ were then calculated using this continuous measure of $S$.

$$E = \frac{(\sqrt{\pi} - 5) S}{2\beta \sqrt{A}}$$  \hspace{1cm} 4.2

and
\[ H = \frac{P}{A} \]

\( P \) is the load applied to the test surface, and \( A \) is the projected contact area at that load which can be determined from calibration. \( B \) is a constant that depends only on the geometry of the indenter.

For the purpose of eliminating surface roughness interference in shallow indentation, all measurements were taken at the depth range of 800-1000 nm. \( E \) and \( H \) were determined separately for cortex and trabeculae as cortical elastic modulus \((E_c)\), trabecular elastic modulus \((E_t)\), cortical hardness \((H_c)\) and trabecular hardness \((H_t)\).

**Basic XP creep**: This test was adopted to determine the indentation viscosity \( \eta \). After the indenter tip reached the designated displacement, it was held at the maximum load (6 mN) for 100 s (Fig. 4.1).

![Fig. 4.1](image_url)

Fig. 4.1. Left: A typical indentation load-displacement curve with holding period indicated by arrow; Right: Non-linear regression curve fitting of displacement-time data in calculation of \( \eta \) \((R^2 > 0.99)\).

The indenter tip was then unloaded to 10% of the maximum load and held another 50 s for thermal drift measurement. Creep displacement-time curve (the holding period) was
fitted using the three element Voigt model as given in Eq. 4.1. \( \eta \) is computed based on the curve fitting of creep displacement by non-linear regression.

\[
h^2(t) = \left(\frac{\pi}{2}\right)P_{\text{max}} \cot \alpha \left[1 - e^{-t(E_2/\eta)}\right] \]

where, \( h(t) \) is the indentation creep displacement as a function of time, \( \alpha \) is an equivalent cone semi-angle (70.3°) to the face angle of the Berkovich indenter (65.27°), \( E_2 \) is an elastic element of the Voigt model and \( P_{\text{max}} \) is the peak load.

**Scanning electron microscopy (SEM)**

After nanoindentation, the samples were polished and etched in a hydrogen peroxide solution (35% H\textsubscript{2}O\textsubscript{2}) at 40 oC for two hours. The etched specimens were then dehydrated immersing in different concentrations (50%, 70%, 90% and 100%) of ethanol solution. After being glued on to stubs for SEM, the samples were coated with a thin gold layer. Cortical and trabecular bone hydroxyapatite and collagen were examined by scanning electron microscopy (FE-SEM, Philips XL series, Netherlands).

**Statistical Analysis**

Statistical analyses were carried out using the SPSS v16 software and the results are reported as mean ± SD (standard deviation). One-way ANOVA tests were performed by time to determine significant difference between different groups for each time point. Similarly, one-way ANOVA tests were also performed by groups to determine significant difference within one group over the time period. Bonferroni corrections were used for all the comparisons. Statistical significance was assumed at \( p < 0.05 \). Correlation of \( \eta \) versus \( E_c \) at week 12 was measured using regression analysis.
4.3 Results

Microarchitectural changes in trabecular bone

Three dimensional μCT rendered images of distal femur region of all the groups at week 12 are shown in Fig. 4.2. Corresponding changes in the structural indices are represented in Fig. 4.4A.

![3D μCT images of distal femur](image)

Fig. 4.2. μCT rendered image of rat tibia showing the volume of interest (VOI) (left); Visualization of bone loss in a transverse section of the femur metaphysis of a rat from each group at week 12 (right), showing prominent trabecular bone loss in OVX group and positive effects of drugs in negating the bone loss.

Starting from week 8 post-surgery, trabecular bone microarchitecture of OVX group exhibited significant differences from SHM group in all the measured structural indices. The effects of IBN and COM treatments in restoring bone microarchitectural indices were observed earlier than that of PTH group. Starting from week 8 of our study, IBN group began to exhibit a significant deviation from OVX group in BV/TV (+69.7%, p = 0.041), Tb.N (+40.5%, p = 0.006), Tb.Sp (-34.9%, p = 0.007) and Ct.Po (-21.7%, p = 0.032).
In COM group, significant differences from OVX group also occurred as early as week 8 in terms of Tb.N (+31.5%, p = 0.023), and Tb.Sp (-12.0%, p = 0.035). No significant difference was observed between PTH and OVX groups in any of these parameters before week 8. At the end of week 12, IBN showed higher BV/TV (+116.4%, p = 0.006) and Tb.N (+78.1%, p < 0.001), and lower BS/BV (-27.5%, p = 0.002), Tb.Sp (-49.2%, p = 0.016) and Ct.Po (-27.9%, p < 0.001) as compared to OVX group. COM also showed higher BV/TV (+120.6%, p = 0.005), Tb.N (+68.0%, p = 0.023) and lower SMI (-35.3%, p = 0.049), BS/BV (-30.4%, p = 0.001), Tb.Sp (-37.5%, p = 0.042) and Ct.Po (-28.9%, p = 0.015) relative to OVX group. Significant increase in Tb.N (+49.7%, p = 0.012) was observed in PTH group as compared to OVX group at week 12. Among the treatment groups, a few significant differences were observed. At week 8, IBN group showed a higher Tb.N than PTH group (p = 0.050), and a significantly lower Tb.Sp than PTH (p = 0.004) and COM group (p = 0.035). At week 12, PTH group had higher trabecular BS/BV value than both IBN (p = 0.009) and COM groups (p = 0.004).

Densitometric changes of cortical and trabecular bone

pQCT yielded cross-sectional images of five adjacent slices at the VOI of one femur from each group are presented in Fig. 4.3. OVX bone specifically showed a less dense trabecular mesh and a larger hollow bone marrow region. This is in contrast to the healthy state as shown by SHM bone where the bone is highly dense and rich in trabeculae. The loss of trabecular bone caused by ovariectomy was observed to be largely reversed in COM and IBN groups, partially restored in PTH group.
Fig. 4.3. Five adjacent pQCT slices from the femur metaphysis of each group at week 12. There was evidence of great trabecular bone loss in the OVX group specimens showing a larger bone marrow area with less dense (red/yellow) trabecular mesh as opposed to highly dense (cyan/white) trabecular bone mesh of SHM specimens. Compared to OVX, trabecular bone was better preserved in PTH, IBN and COM specimens along the longitudinal axis.

The measured value of mean BMD, Tb.BMD and Ct. BMD are represented in Fig. 4.4B. At week 4 post-surgery, a significant decrease in mean BMD (p < 0.001) was observed in OVX group as compared to SHM. Significant elevation of mean BMD were found in week 8 IBN (p < 0.001) and COM (p < 0.001) groups as compared to both PTH and OVX groups. At week 12, mean BMD of COM group approached to that of SHM group (p = 1.000) and was higher than other treatment groups (p < 0.001 as compared to PTH and IBN). As compared to week 8, there was an observed mean BMD reduction of IBN
group at the week 12, resulting in significantly lower mean BMD values as compared with SHM (p = 0.002) and COM groups (p < 0.001). At the end of the study, the percentage increment from OVX group was greater in COM group (+36.3%) than the sum effect of PTH (+6.2%) alone and IBN (+23.0%) alone in mean BMD.
Fig. 4.4. Changes in morphology and volumetric bone mineral density measured by (A) μCT: bone surface to volume ratio (BS/BV), bone volume ratio (BV/TV), structural model index (SMI), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th) and cortical porosity (Ct.Po); and (B) pQCT: mean BMD, trabecular density (Tb.BMD) and cortical density (Ct.BMD).
In Tb.BMD value, ovariectomy-induced deterioration was observed in OVX group starting from week 4. At weeks 8 and 12, IBN and COM groups had significantly higher Tb.BMD value as compared to OVX and PTH groups (p < 0.001). SHM group showed the highest Tb.BMD (p < 0.001) at the end of 12 weeks. In Ct. BMD, OVX and treatment groups showed an increasing trend with time, suggesting continual mineral deposition in cortical bone. SHM group showed the lowest Ct.BMD (p < 0.010) from week 8 onwards. However, there was no significant difference in Ct.BMD between treatment groups throughout the study.

*Geometric and morphological changes of bone*

Geometrical parameter changes of cortical metaphyseal femur of different groups were measured (Table 4.1). Statistical significance between SHM and OVX was observed as early as week 4 in endocortical perimeter (p = 0.048). At the end of week 12, all the geometrical cortical parameters except CSMI showed significant difference between SHM and OVX. Although OVX had significant increment in both Ps.Pm (p = 0.001) and Ec.Pm (p < 0.001) as compared to SHM, the net result was a reduced Ct.Ar (p = 0.037) and Ct.Th (p = 0.001). Within treatment groups, COM group showed significantly larger Ct.Ar and Ct. Th than PTH (p = 0.001 and 0.007 respectively) and IBN bone (p = 0.003 and 0.034 respectively). In Ps.Pm, both COM and PTH group were larger than SHM bone (p = 0.003 and p = 0.015 respectively). In Ec.Pm, PTH and IBN were larger than SHM (p < 0.001 and p = 0.022 respectively) and were similar to OVX (p > 0.100 for both). However, COM group had much smaller Ec.Pm as compared to OVX (p = 0.020) and was comparable to SHM (p = 0.105). In CSMI and SSIy analysis, COM was the largest among all five groups (p < 0.050 for all comparisons).
Table 4.1. Geometrical parameters changes of different groups measured by pQCT. Group averages are represented as mean ± SD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct.Ar (mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHM</td>
<td>6.19 ± 0.76</td>
<td>8.47 ± 1.27</td>
<td>8.10 ± 0.74</td>
<td>7.45 ± 0.43 b</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>6.74 ± 0.73</td>
<td>7.28 ± 0.79</td>
<td>6.55 ± 0.45 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>7.10 ± 0.42</td>
<td>6.79 ± 0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>7.73 ± 0.19</td>
<td>6.91 ± 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>8.42 ± 0.15 b, c</td>
<td>8.12 ± 0.62 b, c, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.Th (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHM</td>
<td>0.50 ± 0.05</td>
<td>0.72 ± 0.26</td>
<td>0.65 ± 0.10 c</td>
<td>0.62 ± 0.04 b, c</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0.50 ± 0.08</td>
<td>0.54 ± 0.05</td>
<td>0.50 ± 0.06 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>0.50 ± 0.04 a</td>
<td>0.52 ± 0.03 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>0.58 ± 0.05</td>
<td>0.54 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>0.57 ± 0.02</td>
<td>0.63 ± 0.05 b, c, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ps.Pm (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHM</td>
<td>13.91 ± 1.41</td>
<td>14.36 ± 0.40</td>
<td>14.65 ± 0.48</td>
<td>14.04 ± 0.48 b, c</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>15.27 ± 1.14</td>
<td>15.10 ± 0.48</td>
<td>14.97 ± 0.33 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>15.72 ± 0.75</td>
<td>14.75 ± 0.31 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>15.16 ± 0.91</td>
<td>14.54 ± 0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>16.55 ± 0.35 a, b, d</td>
<td>14.87 ± 0.23 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ec.Pm (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHM</td>
<td>10.75 ± 1.51</td>
<td>9.79 ± 1.95 b</td>
<td>10.59 ± 1.07 c</td>
<td>10.17 ± 0.64 b, c, d</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>12.15 ± 1.48 a</td>
<td>11.69 ± 0.24</td>
<td>11.89 ± 0.60 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>12.55 ± 0.93 a</td>
<td>11.54 ± 0.27 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>11.50 ± 1.21</td>
<td>11.16 ± 0.52 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>12.97 ± 0.48 a</td>
<td>10.93 ± 0.21 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHM</td>
<td>OVX</td>
<td>PTH</td>
<td>IBN</td>
<td>COM</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>CSMI (mm$^4$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>9.01 ± 2.56</td>
<td>10.62 ± 0.95</td>
<td>13.49 ± 0.89</td>
<td>9.46 ± 1.25</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>11.19 ± 2.25</td>
<td>11.25 ± 1.80</td>
<td>9.82 ± 0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>12.45 ± 1.99</td>
<td>10.03 ± 1.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBN</td>
<td>12.18 ± 2.56</td>
<td>9.89 ± 0.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COM</td>
<td>16.18 ± 2.00 $^{b,c,d}$</td>
<td>11.82 ± 0.54 $^{a,b,c,d}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSIy (mm$^4$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>6.03 ± 1.33</td>
<td>7.53 ± 0.73</td>
<td>7.45 ± 0.19</td>
<td>7.67 ± 0.48 $^{b,c}$</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>6.73 ± 0.79</td>
<td>7.00 ± 0.40</td>
<td>6.35 ± 0.42 $^{a,d}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>7.65 ± 0.72</td>
<td>6.65 ± 0.80 $^{a}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBN</td>
<td>7.88 ± 0.42</td>
<td>7.33 ± 0.24 $^{b}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COM</td>
<td>9.00 ± 0.77 $^{a,b,c,d}$</td>
<td>8.68 ± 0.50 $^{a,b,c,d}$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ct.Ar = cortical area, Ct.Th = cortical thickness, Ps.Pm = periosteal perimeter, Ec.Pm = endocortical perimeter, CSMI = cross-section moment of inertia, SSIy = Y axis Strength-Strain Indices

$^a$ Significant difference from SHM (p<0.05);
$^b$ Significant difference from OVX (p<0.05)
$^c$ Significant difference from PTH (p<0.05)
$^d$ Significant difference from IBN (p<0.05)
Fig. 4.5 shows the SEM images of the cortical bone of femur metaphysis for all five groups. The cortical bone of SHM group consisted of densely packed nanosized hydroxyapatite particles, which seemed to grow tightly on the collagen fiber matrix. However, the OVX group had a much looser structure with numerous nanovoids and micropores. Since secondary osteon structure is not fully developed in rat skeleton, these nanovoids and micropores are the osteocytes residues which exist in primary osteon structure. Ibandronate and PTH had different effects on bone microstructure as seen from the SEM images. PTH group had a porous bone structure, with visible microfracture at the edge of narrow micropores. IBN group showed a denser structure but still with rounded pores. COM group had a less porous dense structure, where hydroxyapatite seemed to have embedded richly on aligned collagen fiber matrix. Fig. 4.6 shows the SEM images of the trabecular bone of femur metaphysis for all five groups. SHM group showed a uniform aligned compact structure. However, the differences between the treatment groups are not observable. Scale-like fibrils or minerals are randomly distributed on the bone matrix. It supports the results of $E_i$ and $H_i$ which showed no significant differences between different groups.
Fig. 4.5. SEM surface images of the cortical bone of femur metaphysis from different groups of rat, taken at 10,000X magnification.
Fig. 4.6. SEM surface images of the trabecular bone of femur metaphysis from different groups of rat, taken at 10,000X magnification.
Viscoelastic analysis of cortical and trabecular bone

Table 4.2 shows the average values of $E_c$, $E_t$, $H_c$, $H_t$ and $\eta$ of all the groups during the course of the study. $E_c$ showed significant difference between OVX and SHM groups, starting from week 4 (-21.6%, $p < 0.001$). From week 8 onwards, $E_c$ of all the treatment groups were significantly higher than the OVX group ($p < 0.001$). At the end of week 12, $E_c$ of PTH ($p < 0.001$), IBN ($p = 0.001$) and COM ($p < 0.001$) were significantly higher than that of SHM group. Statistically significant difference was not observed in the $E_t$ between any of the groups at any time point. $H_c$ of OVX and SHM group started to show a difference from week 4 ($p = 0.047$) to week 8 ($p = 0.009$). At week 8, COM group was the only treatment group which had significantly higher $H_c$ as compared to OVX ($p = 0.012$). This significant difference between COM and OVX was also observed at week 12 ($p < 0.001$). $H_c$ of PTH and IBN groups steadily increased and presented significant increment from OVX group (PTH: $p < 0.001$; IBN: $p = 0.035$) at week 12. In $H_t$ analysis, IBN and COM groups demonstrated significantly higher value than OVX group at the end of the study (IBN: $p = 0.020$; COM: $p = 0.032$).
Table 4.2. Viscoelastic property changes of different groups measured by nanoindentation. Group averages are represented as mean ± SD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group</th>
<th>Weeks after surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$E_c$ (GPa)</td>
<td>SHM</td>
<td>16.74 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>14.01 ± 1.08 $^a$</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>17.52 ± 1.36 $^b$</td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>17.30 ± 0.80 $^b$</td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>17.61 ± 0.87 $^b$</td>
</tr>
<tr>
<td>$E_t$ (GPa)</td>
<td>SHM</td>
<td>13.21 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>12.75 ± 1.12</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>13.28 ± 1.14</td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>13.10 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>13.45 ± 1.23</td>
</tr>
<tr>
<td>$H_c$ (GPa)</td>
<td>SHM</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0.45 ± 0.06 $^a$</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>0.52 ± 0.05 $^b$</td>
</tr>
<tr>
<td>$H_t$ (GPa)</td>
<td>SHM</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>0.48 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>IBN</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>COM</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>$\eta$</td>
<td>(GPa S)</td>
<td>SHM</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$18743 \pm 4264$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$13326 \pm 2807^a$</td>
</tr>
</tbody>
</table>

$E_c$ = cortical elastic modulus, $E_t$ = trabecular elastic modulus, $H_c$ = cortical hardness, $H_t$ = trabecular hardness, $\eta$ = viscosity

\textsuperscript{a} Significant difference from SHM (p<0.05); \textsuperscript{b} Significant difference from OVX (p<0.05); \textsuperscript{c} Significant difference from PTH (p<0.05); \textsuperscript{d} Significant difference from IBN (p<0.05)
Starting from week 4, significant difference between $\eta$ of OVX and SHM group was observed ($p < 0.001$). $\eta$ of OVX group decreased from week 0 to week 4 (-28.9%, $p = 0.011$). From week 8 onwards, $\eta$ of IBN, PTH and COM groups started to be significantly different from OVX group (PTH: +14.8%, $p = 0.009$; IBN: +22.3%, $p < 0.001$; COM: +35.3%, $p < 0.001$). At week 8, COM group had significantly higher $\eta$ value compared with PTH group ($p = 0.044$) but not with IBN group ($p = 0.110$). At week 12, $\eta$ of COM group was closer to SHM group ($p = 0.153$), and significantly higher than the other two mono treatment groups (PTH: $p < 0.001$; IBN: $p = 0.014$). Correlation of $\eta$ versus $E_c$ was studied (Fig. 4.7). Each data point in the figure represents one indentation set on cortical bone in week 12 analysis. Power-law regression trend line was plotted individually for each group and the $r^2$ value of each group is represented in the figure.
Fig. 4.7. Power-law regression analysis of the relationship between viscosity ($\eta$) and cortical elastic modulus ($E_c$) for five different groups at week 12.

Equations for each group:

OVX: $y = 23.402x^{2.527}$

SHM: $y = 68.774x^{2.1332}$

PTH: $y = 356.44x^{1.4337}$

IBN: $y = 99.914x^{1.9016}$

COM: $y = 559.49x^{1.3348}$
4.4 Discussion

In this chapter, we mainly analyzed the effect of PTH, ibandronate and their combination therapy on several parameters of bone property (microarchitecture, geometry and viscoelasticity) in ovariectomized rat femur. To our knowledge, this is the first study that analyzed the viscosity change associated with ovariectomy and administration of drugs. The implications from the observed changes in different aspects of bone quality are complicated and will be discussed individually.

Trabecular bone quality

In trabecular bone architecture and density analysis, both μCT and pQCT results have revealed the successful establishment of osteoporotic condition in the femur of OVX rat model. Significant reduction occurred in BV/TV, Tb.N and Tb.BMD as soon as 4 weeks after ovariectomy surgery. SMI, Tb.Sp and BS/BV results showed that the trabecular struts became more rod-like, mean distance between trabeculae and the strut surface area have proportionally increased in the OVX group as compared to SHM group. These results are consistent with Chapter 3 which also found irreversible microarchitectural changes happen within the first 8 to 12 weeks after the ovariectomy in rat tibia. On the other hand, treatment groups tended to restore the trabecular microarchitecture closer to SHM level. Ibandronate is known to reduce bone turnover rate, mostly by inhibiting bone resorption, which helps to protect the trabecular microstructure from the deleterious effect of bone resorption (Bauss and Dempster, 2007). At the end of the study, IBN and COM groups presented better therapeutic efficacy in restoring trabecular architecture (BV/TV, BS/BV, Tb. Sp) and BMDs (mean BMD and Tb. BMD) as compared to PTH group. Although PTH is known to stimulate bone formation and resorption (Black et al.,
2008), the PTH group in this study only yielded an increase in Tb.N at the week 12. This could be due to the weekly low dosage of PTH (10μg/kg body weight) that we have chosen. According to literature and the previous chapter, the concurrent administration can yield a catabolic or anabolic effect on trabecular bone depending on dosage and frequency of administration (Frolik et al., 2003, Poole and Reeve, 2005, Yang et al., 2012).

*Cortical bone quality*

Effects on cross-sectional geometry of cortical bone occurred at a later time period as compared to trabecular density and architecture. OVX group had both larger Ps.Pm and Ec.Pm than SHM, with a net result of significant reduction in Ct.Ar and Ct.Th. This revealed ovariectomy-promoted periosteal bone formation and endocortical resorption, in order to compensate the great loss in trabecular density and architecture (Bagi et al., 1997). Similarly, PTH treated group showed significantly increased Ps.Pm and Ec.Pm than SHM at week 12. These geometric changes are in accordance with previous bone histomorphometry research (Okimoto et al., 1998), which had also observed that weekly hPTH injections at dose of 10 or 90 μg/kg body weight promoted bone formation at femoral periosteal envelope and also increased bone marrow area. On the contrary, IBN group in this study showed a comparable periosteal perimeter to SHM, and a smaller endocortical perimeter as compared to OVX. This preserving effect was also observed in other bisphosphonate (i.e., tiludronate, residronate) treated SD rats long bones (Ohnishi et al., 1997, Iwamoto et al., 2006). The Ps.Pm of COM group was observed significantly larger than SHM, and comparable with OVX bone which has the largest value. In addition, the Ec.Pm of COM group was significantly smaller than OVX, and comparable
with SHM bone value which is the smallest. Consequently, Ct.Ar and Ct.Th of COM group was the largest among treatment and OVX groups. Thus, COM group followed neither of the trends observed in monotherapy, but a net gain of bone quantity which would have been resulted from increased periosteal bone formation by its PTH component and retarded endocortical bone resorption by its ibandronate component. This observed additive increase in the cortical bone quantity was reflected in increased CSMI and SSiy, which tightly correlates with bone strength (Kokoroghiiannis et al., 2009). Thus combined therapy offers an added advantage than the mono-therapy in preserving cortical bone geometry.

*Nano E and H changes*

In $E$ and $H$ analysis, there were more observable changes in cortical bone than trabecular bone. Ovariectomy was found to decrease the $E_c$ and $H_c$ of OVX group significantly as compared to SHM group as early as 4 weeks post-surgery. At the end of the study, IBN, PTH and COM groups showed increased $E_c$, $H_c$ as compared to OVX. However, $E_t$ and $H_t$ of PTH group remained the same as OVX group. These results were similar to literature which observed that $H_c$ was found to be positively influenced by PTH effect, whereas $H_t$ showed significant decrease (Brennan et al., 2009). It is speculated that the remodeling process stimulated by PTH in the trabecular bone leads to a substantial deposition of new bone, reducing the hardness of the bone until the tissue matures. Accordingly, in our study, a significant increase in trabecular bone number was also observed in PTH group, indicating the formation of new trabecular bone without positive changes in $H_t$. On the contrary, with the participation of ibandronate, $H_t$ of IBN and COM group was positively influenced as compared to OVX group. One possible explanation
would be that the more effective stimulation of type I collagen maturation by bisphosphonates can increase the hardness of the newly formed bone as compared with PTH alone (Garnero et al., 2008, Boivin and Meunier, 2002). The degree of osteoporosis and drug efficacy was often determined at trabecular bone abundant region, rather than the cortical bone, density being the key parameter (Stürmer et al., 2006). Accordingly, in our study, trabecular bone density showed significant difference between OVX and treatment groups whereas cortical density did not. However, the intrinsic properties like elastic modulus, hardness, viscosity as well as geometric parameters of cortical bone in week 12 treated rats were significantly different from OVX group, revealing therapeutic effects on cortical bone. This indicates the importance to assess the effect of osteoporosis and associated treatments on cortical bone quality.

Correlation between $E$ and $\eta$

It was reported in literature that cortical viscosity had a strong positive power-law correlation with $E_c$ (Kim et al., 2010). Our result partially supports this claim (Fig. 4.7). The positive power-law correlation between $\eta$ and $E_c$ existed within each group individually. However, the increases in $\eta$ in different groups were not correlated to the increases in $E_c$ in same proportion. Firstly, as can be observed from the Fig. 4.7, the treatment groups were inclined towards the right side of the graph due to the sharp increase in $E_c$ as compared to both OVX and SHM group. On the other hand, the increments in $\eta$ of treatment groups were comparatively moderate. Thus, 8 weeks of drug administration to ovariectomized rats was more influential in restoring $E_c$ than $\eta$ at the femur metaphysis. One possible explanation would be the selective modification of bone quality by the drugs or the rates at which these drugs may act on bone remodeling,
resulting in rapid changes in $E_c$ and delayed changes in $\eta$ (Brennan et al., 2009). Secondly, as compared to OVX group, $E_c$ of IBN, PTH and COM groups increased by 28.2%, 31.6% and 30% respectively, and $\eta$ of IBN, PTH and COM groups increased by 27.4%, 20.3% and 38.8% respectively. Concurrent administration of drugs restored $\eta$ of the bone better than other two groups whereas their effects on $E_c$ were similar. PTH was observed to be less effective in restoring $\eta$ of the cortical bone. As discussed earlier, it is suggested that active bone formation induced by both ovariectomy and the PTH may result in increased number of newly formed bone and cement lines with less matured collagen (Brennan et al., 2009, Garnero et al., 2008). This can cause more creep deformation under constant load and therefore a lower $\eta$ in rat bones treated with PTH only.

Collagen matrix change

Mechanical performance of bone is determined by the type and arrangement of different structural elements. Hard minerals, as reflected by BMD, contribute to a high strength and stiffness to resist compressive stresses and fracture, while compliant collagen fibers provide high toughness and viscosity to retard fracture propagation under shear or tensile stress (Chang et al., 2011). Based on limited literature, we speculated that the concurrent administration of ibandronate and PTH may offer an advantage of promoted bone formation by PTH and an effective secondary maturation of collagen matrix lengthened by ibandronate (Brennan et al., 2009, Kim et al., 2010, Boivin and Meunier, 2002, Garnero et al., 2008). This assumption of matrix changes was further tested using SEM surface imaging of cortical bone. The SEM images of the experimental groups implied distinct drug effects on cortical bone matrix and their ability to retard fracture
propagation. The bone structure of SHM group consisted of densely packed hydroxyapatite crystals, which contributes to bone strength and stiffness. Osteoporosis can cause porous and randomly aligned matrix structure with poor viscoelastic properties (reduced $\eta$), as observed in OVX rats. Therefore, microcrack formation and rapid propagation are more likely to occur. The ovariectomy induced deterioration of structure was alleviated by administration of drugs. Although PTH showed tighter alignment of collagen matrix resulting in an increased $\eta$, microcracks were also observed which can affect the ability of bone to resist fracture propagation. Porous structure in OVX and PTH treated groups indicates more active remodeling. IBN showed tighter alignment of collagen with lesser pores than OVX group, resulting in an increased $\eta$ in IBN group. Combined treatment was observed to have an aligned collagen matrix structure with dense hydroxyapatite crystals which was similar to that of SHM group. The denser collagen structure corresponds to higher $\eta$ values observed in COM and SHM groups. Thus, the changes in the collagen matrix structure as observed from SEM images complies with corresponding changes in $\eta$ of the bone indicating that $\eta$ can be a potential indicator of bone quality. These observations from SEM corresponded to the Ct.Po result, in which OVX and PTH group presented a higher Ct.Po than other two treatment groups, whilst SHM group has the lowest value. In addition, according to previous chapter, the serum collagen biomarker (CTX and P1NP) analysis had demonstrated that the PTH treatment increased both collagen synthesis and degradation, whereas ibandronate treatment decreased bone collagen degradation only. However, the combined treatment group exerted an increase in collagen formation as well as a significant decrease in degradation. Once the balance has been disrupted, the
feature and alignment of collagen fiber and hydroxyapatite crystal properties are also expected to change in treatment condition, which correlates with mechanical performance of bone by its viscoelasticity to retard crack propagation (Chang et al., 2011). Overall, we speculate that combined effect of ibandronate and hPTH on collagen formation and degradation in COM group could contribute to the distinct matrix structure as compared to other monotherapy groups. We do, however, recognize several limitations of our work. First, there are discrepancies between rat and human cortical bone, i.e., the absence of harvesian remodeling in rats (Turner et al., 2001). Thus the results observed in this study may not have good compliance with clinical observations. Nevertheless, this study would shed light on possible viscosity changes associated with osteoporosis and drug administration. Although the rats used in our study are matured but young (3 months old), treatment efficacy on early osteoporosis phase can be successfully established using them (Stürmer et al., 2006, Bagi et al., 1997, Sehmisch et al., 2009). Secondly, it is important to note that our data may not translate to combination therapy of PTH with bisphosphonates other than ibandronate.

4.5 Conclusion

- To conclude, PTH, ibandronate and their concurrent treatment were found to partially reverse the ovariectomy induced deteriorations in both trabecular and cortical bone. Different treatments had selective effects, especially in preserving geometric and nano-level viscoelastic properties of the cortical bone.
- Similar to the observations in proximal tibia (Chapter 3), the concurrent administration of PTH and ibandronate offered an added advantage in mean BMD and SSIy of distal femur. Furthermore, it and had a combined positive effect in
preserving cortical bone geometry (Ct.Ar, Ct.Th) resulting from a promoted periosteal formation by PTH and a decreased endocortical resorption by ibandronate.

- A newer bone quality determinant, viscosity $\eta$, has also been studied. Prominent increment in $\eta$ was observed in concurrent treatment group in accordance with a denser alignment of collagen fibers and hydroxyapatite crystals with fewer pores. A larger scale study is needed to further characterize the relationship between bone viscosity and micro-fracture propagation.
CHAPTER 5

POSITIVE CORRELATIONS BETWEEN LOSS TANGENT AND ULTIMATE STRENGTH
5.1 Introduction

In the Chapter 4, nanoindentation was used to investigate osteoporosis and treatment induced changes in bone nano-level viscoelasticity. In order to further explore the relationship of bone strength and macro-level viscoelastic property (loss tangent), DMA and mechanical test was mainly used in this chapter. Literature studies (1.2.2) suggested that the macro viscoelastic behavior of bone may have a pronounced effect on mechanical performance of the bone under dynamic loading. As stated in Chapter 1, it is hypothesized that osteoporotic bone macro-viscoelastic properties would be influenced by mono or concurrent treatments.

5.2 Methods

As described in Chapter 1, overall design, diaphyseal femur bone is used here to characterize the macro-level viscoelastic change.

*Peripheral quantitative computed tomography (pQCT)*

All specimens were scanned by pQCT with the same parameters described in Chapter 3. The region of interest for cortical BMD, bone mineral content (BMC) measurement selected was 14-16mm above distal condyle, which covers mid-shaft of each femur. For the slice at 15 mm from the distal end, periosteal perimeter (Ps.Pm), endocrotical perimeter (Ec.Pm), cortical area (Ct.Ar), cortical thickness (Ct.Th), cross-sectional moment of inertia (CSMI) and Strength-Strain Indices (SSIy) on the frontal plane were measured.

*Three point bending test of femur*

The mechanical properties of the right femur were measured using three-point bending test. A micro-testing machine (Instron 5848, Norwood, MA, USA) with a measuring
range from 2 N to 500 N at a precision of 0.2% of the load was used. The bending load was applied at 15mm from the proximal end of femur on the posterior surface at a speed of 0.05mm/s until fracture, with the anterior surface of the femur face down on supports separated by 15mm (shown in Fig. 5.1). The experiment was programmed to stop in case of a strength drop of >20N or a linear displacement of >2mm to avoid shattering the tibia specimens. The ultimate forces, stiffness (slope of the load-displacement curve) were measured as whole bone mechanical properties. The intrinsic biomechanical properties such as ultimate stress was calculated using the formula

$$\sigma_u = \frac{F_u (Lc/4I)}{c}$$  \hspace{1cm} 5.1

where, $F_u$ is ultimate force and $L$ is the span of the support points (15mm). $c$ is the half-width of mid-shaft in the load direction. $I$ is the cross-sectional moment of inertia. $c$ and $I$ are generated from pQCT pictogram.

Fig.5.1. A standard femur diaphysis three point bending test configuration.

**Dynamic mechanical analyses**

Dynamic mechanical analysis assesses viscoelastic properties of bone by applying oscillating stress $\sigma$ with angular frequency $\omega$ to a material,

$$\sigma = \sigma_0 \cos \omega t$$  \hspace{1cm} 5.2
Resulting strain $\varepsilon$ was recorded,

$$\varepsilon = \varepsilon_0 \cos (\omega t - \delta)$$  \hspace{1cm} 5.3

where $\delta$ is the phase angle between stress $\sigma$ and strain $\varepsilon$ in response to a sinusoidal stress $\sigma(t)$ as shown in Fig. 5.2.

![Diagram showing sinusoidal stress applied to linear viscoelastic material, resulting in strain and phase lag $\delta$.](image)

Fig.5.2. Sinusoidal stress $\sigma$ applied to linear viscoelastic material, resulting in strain $\varepsilon$ and phase lag $\delta$.

The complex modulus of the material under these conditions, $E^*$ can be defined as

$$E^* = E' + iE''$$  \hspace{1cm} 5.4

where $E'$ is the real or storage modulus (approximated to the Young's modulus)

$$E' = (\sigma_0/\varepsilon_0) \cos \delta$$  \hspace{1cm} 5.5

and $E''$ is the loss modulus:

$$E'' = (\sigma_0/\varepsilon_0) \sin \delta$$  \hspace{1cm} 5.6

The loss tangent, or $\tan \delta$, is defined as

$$\tan \delta = E''/E'$$  \hspace{1cm} 5.7
tan δ indicates the bone’s ability to dissipate energy as relative to the energy which it stored. DMA assumes that bone is a linearly viscoelastic material. The result is used for the purpose of group comparison to assess treatment efficacy.

In this study, the sample preparation was shown schematically in Fig. 5.3. 36 beam-shaped bone specimens (11.5 x 1.4 x 0.3 mm) were cut out from left femur posterior region under cold water irrigation using a diamond saw (Buehler, Lake Bluff, IL). The specimens’ dimensions are given in Table 5.1. A dynamic mechanical analyzer (DMA 8000, PerkinElmer, USA) was used to acquire tan δ, $E'$ and $E''$ of cortical femur bone in a single cantilever bending mode with 5mm span at 37°C water bath. Each beam was tested in a posterior face up orientation. A static load of 500mN and dynamic load of 500mN was applied in frequency scan from 0.1 to 10 Hz, 25 points per decade.
Fig. 5.3. DMA sample preparation procedure. (A) Anatomical position of the sample: femur diaphysis; (B) Specimen was cut along longitudinal axis and posterior site of the femur; (C) Actual size of a typical sample; (D) Testing position on the DMA clamp.
Table 5.1. Dimensions of rat cortical bone specimens. The span length for all the specimens was 5mm. (mean ± standard deviations).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total length (mm)</th>
<th>Width (mm)</th>
<th>Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHM</td>
<td>11.48 ± 0.24</td>
<td>1.42 ± 0.03</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>OVX</td>
<td>11.84 ± 0.06</td>
<td>1.41 ± 0.04</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>PTH</td>
<td>11.52 ± 0.15</td>
<td>1.41 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>IBN</td>
<td>11.56 ± 0.32</td>
<td>1.42 ± 0.03</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>COM</td>
<td>11.86 ± 0.12</td>
<td>1.41 ± 0.02</td>
<td>0.33 ± 0.03</td>
</tr>
</tbody>
</table>

\( ^a \) Significant difference from SHM (p<0.05)
\( ^b \) Significant difference from OVX (p<0.05)
\( ^c \) Significant difference from PTH (p<0.05)
\( ^d \) Significant difference from IBN (p<0.05)

**Statistical analysis**

For sample dimension, mechanical, geometric and BMD results, statistical analyses were carried out using the SPSS v16 software and the results are reported as mean ± SD (standard deviation). First, General linear model (GLM) repeated measures was used to analyze the effect of different frequencies and treatment groups on tan δ. Frequency was set as a within-subjects factor, while group was set as a between-subjects factor. The test of within-subjects effects showed that there is no main effect of frequency factor (p = 0.109). Thus tan δ at each frequency was taken as an independent measurement in the later correlation analysis. In fact, constant relationship or trend between tan δ and frequency was never observed in literature using bony material (Garner et al., 2000; Les et al., 2005). The tests of between-subjects effects showed that there is a significant difference between groups (p = 0.017). Thus, one-way ANOVA tests were performed to determine significant difference between different groups. Bonferroni corrections were used for all the comparisons. Statistical significance was assumed at p < 0.05. Similarly,
mean values of \( E' \) and \( \tan\delta \) at frequency 0.1, 0.3, 0.6, 0.9, 1, 3, 6, 9 Hz were used as dependent variables in a series of one-way ANOVA, examining the effect of treatment.

In correlation analysis, predictor variables were defined as storage modulus \( E' \), \( \tan\delta \) at frequency 0.1, 0.3, 0.6, 0.9, 1, 3, 6, 9 Hz, cortical BMD and cortical area. Strength variables were defined as the ultimate force, stiffness and ultimate stress. A non-parametric Spearman rank-order correlation was performed between the potential predictors and the strength variables.

5.3 Results

Densitometric and geometric changes of cortical bone by pQCT

pQCT yielded cross-sectional images of one femur from each group are presented in Fig. 5.4. The images were taken at 15mm below distal end for each femur bone. Cortical BMD, BMC and geometrical parameter changes of cortical diaphyseal femur of different groups were measured (Table 5.2). There was no significant difference in Ct.BMD between all groups. However, COM group was significantly larger than OVX, PTH and IBN group in Ct.BMC analysis (\( p = 0.035 \), 0.038 and 0.012 respectively). In terms of geometry, COM group showed significant larger Ct.Ar and Ct.Th than OVX (\( p = 0.038 \) and 0.025), PTH (\( p = 0.037 \) and 0.009) and IBN bone (\( p = 0.023 \) and 0.015). SHM showed a significantly larger SSIy than OVX bone (\( p = 0.047 \)).

Fig.5.4. Visualization of bone geometry in a transverse section of the femur diaphysis of a rat from each group yielded by pQCT.
Table 5.2. Parameters by pQCT analysis of mid-shaft femoral cross-sectional diaphysis (mean ± standard deviations).

<table>
<thead>
<tr>
<th>Group</th>
<th>SHM</th>
<th>OVX</th>
<th>PTH</th>
<th>IBN</th>
<th>COM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.t. Ar (mm²)</td>
<td>6.00 ± 0.09</td>
<td>5.85 ± 0.19</td>
<td>5.83 ± 0.23</td>
<td>5.85 ± 0.39</td>
<td>6.16 ± 0.24 b,c,d</td>
</tr>
<tr>
<td>C.t. Th (mm)</td>
<td>0.64 ± 0.01</td>
<td>0.64 ± 0.02</td>
<td>0.64 ± 0.02</td>
<td>0.65 ± 0.03</td>
<td>0.68 ± 0.03 a,b,c,d</td>
</tr>
<tr>
<td>Ps. Pm (mm)</td>
<td>11.40 ± 0.06</td>
<td>11.09 ± 0.26</td>
<td>11.15 ± 0.37</td>
<td>11.10 ± 0.78</td>
<td>11.21 ± 0.17</td>
</tr>
<tr>
<td>Ec. Pm (mm)</td>
<td>7.39 ± 0.06</td>
<td>7.03 ± 0.33</td>
<td>7.14 ± 0.44</td>
<td>7.04 ± 0.91</td>
<td>6.95 ± 0.28</td>
</tr>
<tr>
<td>SSIy</td>
<td>4.70 ± 0.21 b</td>
<td>4.36 ± 0.21 a</td>
<td>4.36 ± 0.36</td>
<td>4.46 ± 0.50</td>
<td>4.55 ± 0.24</td>
</tr>
<tr>
<td>C.t. BMD (mg/cm³)</td>
<td>1370.13 ± 11.25</td>
<td>1371.37 ± 4.69</td>
<td>1373.64 ± 16.48</td>
<td>1368.31 ± 10.01</td>
<td>1370.55 ± 8.12</td>
</tr>
<tr>
<td>C.t. BMC (mg/mm)</td>
<td>8.23 ± 0.18</td>
<td>8.03 ± 0.27</td>
<td>8.01 ± 0.26</td>
<td>7.96 ± 0.53</td>
<td>8.44 ± 0.34 b,c,d</td>
</tr>
</tbody>
</table>

Ct.Ar = cortical area, C.t.Th = cortical thickness, Ps.Pm = periosteal perimeter, Ec.Pm = endocortical perimeter, SSIy = Y axis Strength-Strain Indices, C.t.BMD = cortical bone mineral density, C.t.BMC = cortical bone mineral content.

a Significant difference from SHM (p<0.05)
b Significant difference from OVX (p<0.05)
c Significant difference from PTH (p<0.05)
d Significant difference from IBN (p<0.05)
Three point bending test

The results of ultimate force, ultimate stress, stiffness and total femur length of different groups from three point bending tests are provided in Table 5.3. No difference was observed in femur length. However, there was a significant difference between SHM and OVX in ultimate force and stiffness (p = 0.001). Ultimate force and stiffness has been greatly decreased (-13.89% and -15.17% respectively) due to the ovariectomy surgery. SHM group has the highest ultimate force as compared to others (p < 0.050). Within the treatment groups, COM has significantly the highest ultimate force compared with PTH (p = 0.048) and IBN (p = 0.044) groups. In addition, COM group has higher stiffness as relative to OVX and IBN (p = 0.034 and 0.035 respectively), but not PTH (p = 0.105). In ultimate stress analysis, COM has a lower value than IBN (p = 0.035).

Viscoelastic analysis of cortical bone by DMA
**Table 5.3.** Mechanical properties (mean ± standard deviations).

<table>
<thead>
<tr>
<th>Group</th>
<th>SHM</th>
<th>OVX</th>
<th>PTH</th>
<th>IBN</th>
<th>COM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate force (N)</td>
<td>133.91 ± 7.71&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>115.31 ± 8.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>119.45 ± 8.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118.53 ± 8.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>129.40 ± 2.97&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ultimate stress (MPa)</td>
<td>229.80 ± 39.16</td>
<td>230.86 ± 9.02</td>
<td>223.75 ± 27.40</td>
<td>246.41 ± 17.50</td>
<td>205.99 ± 19.72&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>358.72 ± 40.65&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>304.31 ± 44.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>321.36 ± 37.45</td>
<td>304.80 ± 25.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>361.11 ± 32.19&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Femur length (mm)</td>
<td>36.40 ± 0.36</td>
<td>37.12 ± 1.30</td>
<td>37.03 ± 0.52</td>
<td>36.57 ± 0.83</td>
<td>36.71 ± 1.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant difference from SHM (p<0.05)

<sup>b</sup> Significant difference from OVX (p<0.05)

<sup>c</sup> Significant difference from PTH (p<0.05)

<sup>d</sup> Significant difference from IBN (p<0.05)
Fig. 5.5 and 5.6 shows the tanδ and $E'$ over 0.1 – 10 Hz frequency range with respect to different groups of rats. At frequencies lower than 6 Hz, tanδ of SHM bone was significantly the highest (p < 0.050) and OVX bone was the lowest in all five groups (p < 0.050). Among treatment groups, at 0.3 Hz, COM group has significantly higher value than the other mono therapy groups (p < 0.050). Furthermore, at 0.1 Hz COM group has higher tanδ than PTH group only (p = 0.018), whilst at 0.6 and 1 Hz, COM group was only significantly higher than IBN group (p = 0.035 and 0.018). At other specific frequency determined (0.9, 3, 6, 9 Hz), there was no significant difference in tanδ between treatment groups. In statistical analysis of $E'$, SHM was significantly higher than OVX and PTH group (p < 0.005), and COM group has shown the largest $E'$ among all the groups (p < 0.050) at all frequencies.

Fig. 5.5. The mean tanδ of rat femur diaphysis cortical bone for different groups; error bars represent the standard deviation.
Correlation analysis

Spearman rank-order correlations between the potential predictors and the strength variables were determined (Table 5.4). Storage modulus $E'$ and cortical area was positively associated with ultimate force. None of the predictor variables were significantly correlated with the ultimate stress. Cortical BMD was associated with stiffness of the bone. It was found that at frequency range of $0.9 - 6 \text{ Hz}$, $\tan \delta$ was positively associated with ultimate force. Furthermore, at $3 \text{ Hz}$, $\tan \delta$ was also associated with bone stiffness.

Fig. 5.6. The mean storage modulus ($E'$) of rat femur diaphysis cortical bone for different groups; error bars represent the standard deviation.
Table 5.4 Spearman rank-order correlations between viscoelasticity, density, bone area and macro mechanical properties.

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>Maco mechanical properties</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant values in bold type</td>
<td>Ultimate force</td>
<td>Stiffness</td>
</tr>
<tr>
<td></td>
<td>0.517</td>
<td>0.261</td>
</tr>
<tr>
<td></td>
<td>0.010</td>
<td>0.218</td>
</tr>
<tr>
<td>0.1Hz</td>
<td>0.394</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td>0.070</td>
<td>0.202</td>
</tr>
<tr>
<td>0.3Hz</td>
<td>0.344</td>
<td>0.230</td>
</tr>
<tr>
<td></td>
<td>0.117</td>
<td>0.304</td>
</tr>
<tr>
<td>0.6Hz</td>
<td>0.399</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>0.066</td>
<td>0.543</td>
</tr>
<tr>
<td>Tan δ</td>
<td>0.534</td>
<td>0.273</td>
</tr>
<tr>
<td></td>
<td>0.010</td>
<td>0.219</td>
</tr>
<tr>
<td>1Hz</td>
<td>0.537</td>
<td>0.410</td>
</tr>
<tr>
<td></td>
<td>0.010</td>
<td>0.058</td>
</tr>
<tr>
<td>3Hz</td>
<td>0.547</td>
<td>0.437</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.042</td>
</tr>
<tr>
<td>6Hz</td>
<td>0.493</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>0.646</td>
</tr>
<tr>
<td>9Hz</td>
<td>0.411</td>
<td>0.072</td>
</tr>
<tr>
<td></td>
<td>0.057</td>
<td>0.750</td>
</tr>
<tr>
<td>Density</td>
<td>0.003</td>
<td>0.530</td>
</tr>
<tr>
<td>Cortical</td>
<td>0.990</td>
<td>0.011</td>
</tr>
<tr>
<td>Area</td>
<td>0.587</td>
<td>0.367</td>
</tr>
<tr>
<td>Cortical</td>
<td>0.003</td>
<td>0.077</td>
</tr>
<tr>
<td>Area</td>
<td>0.003</td>
<td>0.077</td>
</tr>
</tbody>
</table>
5.4 Discussion

This chapter demonstrated the effect of PTH, ibandronate and their combination therapy on several determinants of bone property (macro-viscoelasticity, geometry and BMD) in ovariectomized rat femur diaphysis. These three parameters are independent and their correlations with macro bone strength were further characterized using statistical analysis. To our knowledge, this is the first study that analyzed the viscoelastic change on OVX rat bone associated with administration of drugs using DMA.

Comparison of DMA result with literature

Cortical bone is a viscoelastic material. The strain-rate dependence of cortical bone can account for up to a fifth of the strength of the human femur during high speed loading (Courtney et al., 1994). DMA has been demonstrated as a useful tool to investigate bone mechanical properties. It has been proved to be sensitive in distinguishing different physiological sites, directions, different nutrition and diseased conditions of the bone tested (Abdel-Wahab et al., 2011, Chang et al., 2011, Les et al., 2005, Les et al., 2004). In comparison, the tanδ measured in our different treatment groups were in the same range as the bone samples with similar dimension reported in literature (tanδ = 0.035-0.1) (Abdel-Wahab et al., 2011, Yamashita et al., 2002, Garner et al., 2000). It is close to that of the glassy state of polymers (around 0.1) and higher than that of metals (10^-3 or less).

Les et al. (2005) has reported that long-term ovariectomy decreases ovine compact bone viscoelasticity using DMA. The storage modulus and tanδ was greatly decreased at high frequencies in the ovariectomized ovine (5.2% and 83% respectively). In our study, the storage modulus and tanδ of OVX rat femur was 21.21% and 34.98% lower than SHM significantly at 1 Hz, which is more comparable to the changes in ovariectomized
C57BL/6 black mice (23.3% reduction in storage modulus) (Chang et al., 2011). The reduction in storage modulus and tanδ indicates the ovariectomized rat femur is less efficient at damping oscillatory stresses.

**Viscoelasticity is more sensitive than BMD in detecting osteoporotic change**

According to literature (Les et al. 2005), energy dissipation during an oscillatory stress (ΔW/W) is related to δ (Currey, 1988):

\[
\frac{\Delta W}{W} = 2\pi \sin \delta
\]

Thus, at 1 Hz, the bone sample from our SHM rats was theoretically able to absorb and subsequently dissipate 127% as much energy in damping alone as bone sample from the OVX rats. It has been proven that the relationship between ash density \(\rho_a\) and the energy to yield \(U_Y\) is an exponential one (Les et al., 1994):

\[
U_Y = 1.05 \rho_a^{1.83}
\]

Then this loss of energy absorptive capability at high frequencies might be equivalent to that seen in a 13% loss of ash density in a monotonic test. However, BMD changes were not seen between groups in our study. Deteriorations in viscoelastic properties which unaccompanied by reduction in mineral density could potentially alter bone mechanical performance, leading to an increase in fracture risk that would be invisible to clinical screen (Les et al., 2005). Similarly, a recent bone fragility study has shown significant reductions in storage modulus and tanδ in chronic kidney disease rat femur compared with control group, however without any difference in BMD (Iwasaki et al., 2011).

**Drugs/nutrition can potentially restore macro-viscoelasticity**

Nutrition supplements and hormone were found to restore bone viscoelasticity from a diseased state. Recently, Chang et al. (2011) has revealed an effective inhibition of
osteoporosis in fermented milk supplemented mice assessed by DMA test. The storage modulus of bone in ovariectomized mice markedly dropped to 1833 MPa as compared to sham operated mice (2125 MPa). However, in fermented milk supplemented mice, the storage modulus was as high as 2377 MPa at the end of their study (Chang et al., 2011).

In our study, although different trends for storage modulus and tan\(\delta\) were observed for different treatment groups with a frequency increase, COM group has both larger storage modulus and tan\(\delta\) than the other two mono therapy groups.

**Geometry change**

Treatment effects on cross-sectional geometry of femur diaphysis were also examined. It is worth to notice that while Ct.Th of SHM and OVX bone are similar, Ps.Pm and Ec.Pm of SHM were larger than OVX bone as observed in Fig. 5.4. It is suggested in literature that with a constant cortical thickness, increasing the diameter of cortex would result in an increased strength (Davison et al., 2006). This finding was proved by our mechanical test that SHM bone has a significant larger ultimate force than OVX bone. Comparing treatment groups, although not significant, the Ps.Pm of COM group was observed larger than OVX, whilst the Ec.Pm of COM group was smaller than OVX. Consequently, Ct.Ar and Ct.Th of COM group was the significantly larger than treatment and OVX groups. Combination therapy has offered a net gain of bone quantity which would have been resulted from increased periosteal bone formation and retarded endocortical bone resorption. A larger diameter of bone with thick cortices is ideal for strength and fracture avoidance (Davison et al., 2006). The observed additive effect on the cortical bone geometry was in accordance with mechanical test, in which COM has the highest
ultimate force among treatment groups. In such a distinctive geometric change in drug treated OVX bone, BMC would be more accurate in prediction of overall bone strength.

**Correlation between predictor and bone strength**

Strength of bone is dependent on two main individual physical factors: quantity and quality (Chesnut et al., 2001). Bone quantity consists of density and size, which together form bone mass. Bone quality consists of structural and material properties. In this study, several distinctive surrogates of quantity and quality – BMD, bone area and viscoelasticity, and their correlation with bone strength were examined (Table 5.4). Not surprisingly, the measured BMD, which reflects hard mineral, was positively associated with bone stiffness. However, there was no significant correlation between BMD and ultimate force in this study. Storage modulus (analogous to Young’s modulus in a monotonic test) and cortical bone area were found positively correlated with ultimate force. Interestingly, it was observed that over 0.9-6 Hz tanδ was also correlated well with ultimate force, which covers normal daily activity frequency range (Les et al. 2005, Garner et al., 2000). It was widely reported in bovine and human bone studies that a relatively minimum in tanδ over a frequency range predominantly contained in normal activities. However, the tanδ trends over 0.1-10 Hz in our study were relatively constant. This absence of minimum tanδ was also found in ovine sample (Les et al. 2005). Different animal model, age or testing condition would possibly attribute to the different trend observed. In further study, a wider range of testing frequency would be suggested to investigate tanδ change. There were several limitations of this work. First, there are discrepancies between rat and larger mammalian animal cortical bone with matured harvesian system. Thus the results observed in this study may not have good compliance
with observations on larger animals. Nevertheless, this study would shed light on possible viscosity changes associated with osteoporosis and drug administration.

5.5 Conclusion

- In diaphyseal femur, combination treatment of PTH and ibandronate was demonstrated to have a better therapeutic effect on bone than mono therapy, in terms of a higher ultimate force, larger cortical area and increased $E'$ and tan $\delta$.
- These changes accompanying ovariectomy and treatment would be ignored in clinical screening by an unchanged BMD.
- It is the first time that positive correlations between tan $\delta$ and ultimate strength of the bone were observed during normal daily activity frequency range (0.9-6 Hz).
- DMA was demonstrated as a useful tool to assess osteoporotic drug efficacy.
CHAPTER 6

LONG-TERM WHOLE BODY VIBRATION IMPAIRS

OSTEOPOROTIC BONE QUALITY
6.1 Introduction

In Chapter 3- 5, we have studied the drug effect in osteoporosis treatment in terms of bone mineral density, microstructure, geometry and viscoelasticity. The techniques we have been practiced for the previous work were optimally developed in OVX rat model. Now it is the time to further apply these methods on to the other aspect of treatment – whole-body vibration (WBV) therapy. As stated in Chapter 1, we first hypothesized that the long-term use (20 weeks) of WBV in ovariectomized rat model can still alleviate the bone loss due to osteoporosis. Secondly, according to Wolff’s law, bone remodeling occurs in response to the mechanical stimulus acting on it. The rat skeleton which received WBV therapy was expected to response and damp oscillatory stress more efficiently. Thus, the whole bone DMA was used here to measure macro-level viscoelasticity. WBV may affect the skeleton in different magnitude with respect to their different physiological function (weight bearing/non-weight bearing), shape (long/short/flat/irregular) and site. WBV has been associated especially with low back pain (Lings et al., 2000, Tiemessen et al., 2008). Thus different bone types (tibia, femur and spine) were included in this study to thoroughly examine the effect of long-term WBV.

6.2 Methods

As described in Chapter 1, overall design, proximal tibia and spine bone are used to establish the OVX model with WBV therapy, and diaphyseal femur bone is used to characterize the macro-level viscoelastic change. Starting from 4th week post-surgery, 0.3g, 30 Hz WBV was administered to VIB group, 20 min/day for 16 weeks (as shown in the Fig. 6.1). A box with lid was used to constrain the rat and to ensure that four limbs are
on the platform to receive the whole body vibration. Vertical sinusoidal oscillation was produced by motors beneath the platform and transmitted to the rat body. The SHM and OVX rats were placed on the vibration platform without vibration for the same amount of time as the VIB group. At weeks 20, animals were euthanized by carbon-dioxide asphyxiation. Femur, tibia and lumbar vertebral bones were harvested, wrapped in 0.9% saline soaked gauze and stored at -20°C until they were used for the experiments.

![Image of rat on vibration platform](image1)

**Fig. 6.1.** 20 min/day, 0.3g, 30 Hz WBV was administered to the VIB group

**Peripheral quantitative computed tomography (pQCT) analysis**

For the longitudinal study, in vivo PQCT scanning was performed every 4 weeks to examine the progress of osteoporosis and treatment effect. Fig. 6.2 shows the operating position of a rat in an in vivo scanning. Only rat tibia was scanned due to several technical constraints (platform size and rat weight). During the 1 hour scanning process, light anesthesia (Ketamine 37.5mg/ml, Xylazine 5mg/ml, 0.2ml/body weight) was administered to the rats.
Fig. 6.2. In vivo pQCT scans were performed under anesthesia at week 4, 8, 12 and 16.

For the end point (week 20) ex vivo scanning, the harvested tibiae and vertebrae were thawed to room temperature and scanned with the same parameters as previous Chapters. The region of interest for tibiae was the metaphysis region of the proximal tibia. For vertebrae the region of interest was lumbar vertebrae L3. mean BMD, Tb.BMD, Ct.BMD and BMC were obtained. The mineral density measurements were taken from five adjacent slices within the region of interest for each tibiae sample and three adjacent slices within the region of interest for each vertebra sample.

Compression Test

After the end point ex vivo pQCT scanning, L3 vertebra was immediately prepared for compression testing. The vertebral pedicles were dissected out with care to avoid any damage to the cortical shell. Using a low-speed precision saw (Isomet, Buehler, Lake Bluff, IL, USA) under constant water irrigation, the cranial and caudal ends including the growth plate were removed to obtain planoparallel surface. After sawing, the vertebral height was measured using calliper and found to be approximately 4mm. The test
specimen was mounted between two aluminium plates. The mechanical resistance to failure was tested using a micro-testing machine (Instron 5848, Norwood, MA, USA) with a measuring range from 2N to 1000N at a precision of 0.2% of the load was used. The deformation rate was applied in the craniocaudal direction and set at 0.033mm/second (Ikeda et al., 2001, Barak et al., 2010). Load-deformation curves were recorded continuously. The specimens were loaded to failure and the whole process would be automatically terminated when the load reached 200N (Fig. 6.3). From the load-deformation curve, maximum load, stiffness and energy dissipation (work to fracture) was obtained.

Fig.6.3. Vertebra sample placed on the compression test device

**Whole bone dynamic mechanical analysis (DMA)**

Femur bone was used in DMA test. DMA has been used to characterize cortical bone chip viscoelastic properties in Chapter 5. Here in this Chapter, the configuration is modified for conducting the whole bone DMA. Before the analysis, the thickness and
width at the centre of each femur were measured. Then the femur bone sample was then placed in a dynamic mechanical analysis device (DMA 8000, PerkinElmer, Norwalk, CT) at 37 °C water bath in a 3-point bending configuration. Fig. 6.4 shows one sample in the testing position. Frequencies of scanning ranged from 0.1 to 10 Hz. The test was conducted under displacement control. Storage modulus $E'$ and tan $\delta$ were measured for each sample.

Fig. 6.4. Femur sample placed on the DMA device with 3 point bending configuration

Statistical Analysis

Statistical analyses were carried out using the SPSS v16 software and the results are reported as mean ± SD (standard deviation). One-way ANOVA tests were performed by time to determine significant difference between different groups for each time point. Bonferroni corrections were used for all the comparisons. Statistical significance was assumed at $p < 0.05$. 
6.3 Results

Animals

There was no significant difference in the weight of the rats in each group at week 0. However, at the end of the experiment, there were significant differences in amount of weight gain between SHM and OVX group ($p = 0.012$) as well as VIB and OVX group ($p = 0.037$). Compared with the baseline measurement, OVX group had the highest weight gain (47.75%), followed by VIB group (26.40%). SHM group had the lowest weight gain (15.54%) (Fig. 6.5).

Fig.6.5. Percentage change in body weight. Mean value with standard deviation error bar were presented for each group. *: $p < 0.05$ from OVX. †: $p < 0.01$ from OVX

Densitometric changes of trabecular and cortical bone

Both in vivo and ex vivo pQCT rendered images were shown in Fig. 6.6. Highly densed bone tissue were shown in cyan and white color, whereas less densed bone tissue were presented in yellow and red. According to Fig. 6.6., SHM was rich in trabecular bone in
both tibia metaphysis and lumbar. This is in contrast to the VIB and OVX bones where the bone is less densed and poor in trabeculae connection.

Fig.6.6. Visualization of bone loss in (A) longitudinal in vivo PQCT image of tibial metaphysis at week 4, 8, 12 and 16; (B) ex vivo PQCT image of tibial metaphysis at end point week 20; (C) ex vivo PQCT image of lumbar vertebral L3 from each group.

Table 6.1 showed the quantitative changes in BMD and BMC in different groups at week 4, 8, 12, 16 and 20. Between OVX and SHM group, there was significant difference in tibia mean BMD starting from week 4 after surgery till the end of the study. At the end of week 20, OVX and SHM had significant differences in tibia mean BMD (p = 0.007), tibia Tb.BMD (p < 0.001), and lumbar mean BMD (p < 0.001). This is in accordance with the previous studies which had shown an ovariectomy induced significant trabecular bone loss in rat (Kharode et al., 2008).
Table 6.1. BMD and BMC changes of different groups measured by pQCT. Group averages are represented as mean ± SD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group</th>
<th>Tibia</th>
<th>Spine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weeks after surgery</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>mean BMD (mg/cm$^3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>651.48 ± 6.76</td>
<td>645.50 ± 16.27</td>
<td><strong>640.56 ± 20.74</strong></td>
</tr>
<tr>
<td>OVX</td>
<td>620.25 ± 12.41</td>
<td>608.50 ± 23.73</td>
<td>598.33 ± 10.42</td>
</tr>
<tr>
<td>VIB</td>
<td>614.37 ± 10.23</td>
<td>591.43 ± 25.50</td>
<td>598.10 ± 7.07</td>
</tr>
<tr>
<td>Tb.BMD (mg/cm$^3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>244.22 ± 15.14</td>
<td>264.53 ± 25.02</td>
<td><strong>296.87 ± 10.23</strong></td>
</tr>
<tr>
<td>OVX</td>
<td>234.57 ± 26.34</td>
<td>231.33 ± 16.16</td>
<td>209.33 ± 32.25</td>
</tr>
<tr>
<td>VIB</td>
<td>232.33 ± 18.77</td>
<td>244.22 ± 15.14</td>
<td>215.02 ± 14.14</td>
</tr>
<tr>
<td>Ct.BMD (mg/cm$^3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>1097.44 ± 11.39</td>
<td>1098.20 ± 31.11</td>
<td>1120.37 ± 27.49</td>
</tr>
<tr>
<td>OVX</td>
<td>1081.65 ± 15.60</td>
<td>1099.67 ± 23.71</td>
<td>1139.21 ± 21.36</td>
</tr>
<tr>
<td>VIB</td>
<td>1099.67 ± 28.36</td>
<td>1128.25 ± 24.79</td>
<td>1088.75 ± 64.03</td>
</tr>
<tr>
<td>BMC (mg/mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHM</td>
<td>13.36 ± 0.43</td>
<td>13.42 ± 0.59</td>
<td>13.39 ± 0.81</td>
</tr>
<tr>
<td>OVX</td>
<td>13.19 ± 0.25</td>
<td>12.91 ± 0.52</td>
<td>13.15 ± 0.17</td>
</tr>
<tr>
<td>VIB</td>
<td>13.07 ± 0.65</td>
<td>12.57 ± 1.05</td>
<td><strong>12.35 ± 0.49</strong></td>
</tr>
</tbody>
</table>

Bold: Significant difference from OVX (p<0.05)
In the comparison between OVX and VIB tibia bone, there were no significant differences in tibia mean BMD and tibia Tb.BMD (p > 0.05). In addition, there was a significant reduction in tibia BMC of VIB group as compared to OVX (p < 0.001). Furthermore, VIB vertebrae bone has significantly lower mean BMD, Tb.BMD and BMC than OVX group (p < 0.001, p = 0.028 and p = 0.005 respectively).

Vertebrae Compression Testing

Fig. 6.7 shows the results of the L3 vertebral compression test, in terms of maximum load, stiffness and energy dissipation. SHM group has the highest value of maximum load followed by OVX then VIB groups. VIB vertebrae has significantly lower maximum load as compared to OVX group (p = 0.012). There was no significant difference found in stiffness between OVX and VIB (p = 0.081). However, VIB group was significantly less stiff as relative to SHM (p = 0.035). The difference in maximum load and stiffness between each group can be clearly observed from the typical load-displacement curve. Energy dissipation -- area under the curve, is also determined. Both OVX and VIB groups had significantly lower energy dissipation as compared to SHM group (p = 0.018 and 0.001 respectively).
Fig. 6.7. Maximum load, stiffness, typical load-displacement curve and energy to failure in compression test of L3 vertebrae. Mean value with standard deviation error bar were presented for each group. *p<0.05 versus OVX rats.

Dynamic mechanical analysis

Fig. 6.8 shows the whole bone DMA results of different rat groups. The averaged values and significance were measured at 1 Hz frequency (within daily activity frequency range). Although not significant, SHM bone has the highest $E'$, followed by VIB and OVX group. In tan $\delta$ analysis, SHM was found significantly higher than OVX and VIB group ($p = 0.036$), while there was no difference found between OVX and VIB ($p = 0.125$). In summary, 16 weeks of WBV therapy did not render any enhancement in storage modulus or damping efficiency (tan $\delta$) of the ovariectomized rat femur.
6.4 Discussion

Currently, low-impact WBV platform has been introduced commercially as it is claimed to reduce bone loss due to osteoporosis. In this chapter, the long-term effects of WBV were examined using in vivo pQCT scanning allowing longitudinal follow-up of the bone densitometry in proximal tibia of individual rats for 16 weeks treatment. In order to investigate the capability of mechanical stimuli, in the form of WBV treatment, to reinforce the integral damping efficiency of the treated bone when encountered with oscillatory stress, the whole bone DMA test were carried out on femur bones. In vertebrae of the OVX and VIB rats where severe bone loss was revealed by ex vivo pQCT scanning, compression test was conducted to further elucidate the probability of the spine fracture.

Justification of the 30Hz, 0.3g regimen

The optimal vibration protocol (frequency, amplitude) to prevent bone loss is not yet determined (Table 2.3). In this study, we adopted 30 Hz and 0.3g regimen in OVX rats due to several reasons. First, similar regimens in rodents (32-35 Hz, 0.3-0.5g) were
shown to have successfully increased bone mineralization and strength (Shi et al., 2010, Wenger et al., 2010). Second, ISO 2631 (1985) standard had defined a safety vibration range for human: 0.3-0.8 g in 20-50 Hz. Beyond this range, acute discomfort may arise from: 1. focal pain in muscle and joints, especially in spine; 2. Alterations in visual perception and tracking. Thus 30 Hz and 0.3g whole body vibration, which proven to be effective, was adopted commercially (Galileo®, WAVE® and Juvent®). We do notice that there were more positive results reported from literature with vibration protocols beyond 45 Hz and 1g in rodents (Sehmisch et al., 2009, Tezval et al., 2011, Brouwers et al., 2010, Christiansen and Silva, 2006, Van der Jagt et al., 2012, Flieger et al., 1998). Despite of the encouraging result, the protocols would never be applicable for human, calling into question the clinical relevance of these data.

**WBV reduced weight gain**

OVX rat has a significantly larger weight increase (47.75%) as compared to SHM rats (15.54%). The effect of ovariectomy on hyperphagia and subsequent fat tissue gain has been extensively studied (Jiang et al., 2008). However, the effect of WBV on reduction of weight gain (26.40%) due to ovariectomy was unexpected in the current study thus the fat tissue were not preserved. A recent study has also revealed a similar effect on weight gain in WBV rats (Van der Jagt et al., 2012). In their study, the averaged weight gain of OVX rats was 88.5 g compared to 62.7 g in the delayed vibration group (p = 0.03). One possible reason for reduction of weight gain can be the inhibition of terminal differentiation of adipocyte (fat cells) progenitor by loading stimuli. It was reported that in the process of differentiation, the PTH/PTHrP signaling pathway were affected
(Menuki et al., 2008). Hence, vibration treatments might be beneficial to reduce post-menopausal weight gain in human.

**WBV had no effect on long bones, but deteriorates lumbar bone quality**

In this study, the rats were given 0.3 g, 30 Hz and 20 min/day WBV therapy based on the protocol used by Rubin et al., (2002) which showed increased BMD and trabecular formation. However, according to pQCT results on rat tibia, there were no significant differences observed in mean BMD, Tb. BMD or Ct.BMD between OVX and VIB bones. However, BMC of the VIB tibia bone was significantly lesser than OVX which reveals a decrease in bone area of the VIB group. The current study shows that vibrations are not proven in alleviating the loss of bone caused by osteoporosis in rat tibia. It is in accordance with the experiment results from Brouwers et al (2010), in which vibration was not shown any sufficient preserving effect on the proximal and metaphysis of rat tibia. In vertebrae densitometry analysis, VIB group even experience significantly bone loss in mean BMD, Tb. BMD and BMC as compared to OVX vertebrae bone which can be visualized in Fig. 6.6. The result indicated that not only the unit density was greatly decreased, but also a reduction in amount of existing bone in vibration treated rat vertebrae. The reduced BMD and BMC in vertebrae of VIB rats were reflected by compression test result of L3. Bone samples from VIB group had comparable stiffness and energy dissipation ability as OVX, while its maximum load was even significantly lower than that of OVX group. It indicates that after long-term vibration treatment, the rat vertebrae may become less able to bear load when fracture. It is worth notice that in longitudinal study, although not significant, VIB tibia had comparably larger Tb. BMD than OVX at week 8.
Comparison with literature

The result is similar to a previous study done by Oxlund et al., (2003) which found that ovariectomized rats that receive WBV has a higher BMD in the femur and the tibia compared to non-vibrated controls after 5 weeks of WBV but not after 12 weeks. Therefore, WBV therapy may not be beneficial but even detrimental to the vertebrae BMD after 16 weeks of treatment with the current protocol. It was found in rabbit annulus cells that vibratory loading had suppressed several collagens which function in bone remodeling as well (Yamazaki et al., 2002). Thus, one possible explanation for why vibration had a negative effect on spine in our study would be the reduction in functional collagens. Western blot of collagens excreted from bone cells will be characterized in future work to further elucidate the mechanism. The discrepancy in our finding and other studies may attribute to different vibration parameters used (frequency, magnitude), or duration of the regimen. To date, the optimal protocol for WBV therapy has not been explored thoroughly. Since not all WBV regimens would alleviate the bone loss in osteoporosis, prescription or recommendation of such a therapy should be more careful and depending on follow-up response from the individuals.

6.5 Conclusion

- The WBV treatment can significantly reduce postmenopausal osteoporotic weight gain.
- In this pilot study, the effect of WBV treatment on ovariectomized rat bone was evaluated using in vivo and ex vivo BMD measurement, compression test and DMA analysis. While no beneficial effect of WBV was found in BMD of tibia, vertebrae BMD and maximum load was significantly reduced in WBV treated rats.
• Since the optimal parameters were still under research, WBV should not be recommended for long-term usage.
CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS
7.1 Conclusion

The primary objective of this study was to examine whether there is a beneficial effect of PTH and ibandronate combined therapy in the ovariectomized rat bone. A total of 11 indices showed a significant difference between sham and ovariectomized groups, suggesting the successful establishment of osteoporosis in the rat model. Maximum load, strength–strain indices (SSIy) and serum bone formation markers of combination group were significantly higher than both monotherapy groups. Compared to the previous studies (Finkelstein et al., 2003, Black et al., 2003) which showed impedance from bisphosphonates in combination therapy with PTH, our study revealed that ibandronate does not block the anabolic effects of PTH in ovariectomized rat tibiae. The additive results of combination therapy can be attributed at least partially to the proper ratio of the two drugs. With the proper ratio of anabolic and anti-resorptive drugs, the effect could be more pronounced. These findings provide valuable information regarding the potential of combination drug in the treatment of osteoporosis. However, it should be point out that, the most beneficial ratio of the two drugs was not optimized in this study. Further research is therefore needed to optimize the ratio between PTH and ibandronate and maximize the treatment effects of the concurrent administration.

Strength of bone is dependent on two main individual physical factors: quantity and quality (Chesnut et al., 2001). Bone quantity consists of density and size, which together form bone mass. Bone quality consists of structural and material properties. Thus, besides BMD, viscoelastic properties (viscosity, elasticity, tan δ etc.) of bone are also crucial determinants of bone strength. Viscoelastic response of ovariectomized rat bones and treatment effect was further examined using nanoindentation and DMA test. Firstly, in
the nanoindentation test, ovariectomy induced deterioration in viscosity and elastic modulus was observed in cortical bone. PTH, ibandronate or its concurrent treatment can effectively reverse ovariectomy induced deteriorations in both trabecular and cortical bone. Different drugs had selective effects especially in preserving geometric and viscoelastic properties of the bone. The concurrent administration of PTH and ibandronate was shown to offer an added advantage in preserving mean BMD and had a positive effect on cortical bone geometry, resulting from an increased periosteal formation and a decreased endocortical resorption. Viscosity ($\eta$) was prominently restored in combined treatment group. It is in accordance with an observed denser alignment of collagen fibers and hydroxylapatite crystals matrix with fewer pores, which may play an important role in hindering fracture propagation.

Secondly, DMA measurements on identical cortical samples from control and drug treated diaphyseal femur bone were conducted to compare with the bending test on the same region of interest. The BMD of diaphyseal cortical femur bone samples did not show any difference between groups, whereas combination treatment was demonstrated to have a better therapeutic effect on bone than mono therapy, in terms of a higher ultimate force, larger cortical area and increased $E'$ and tan $\delta$. This indicates that these viscoelastic changes accompanying ovariectomy and treatment would be ignored in clinical screening by an unchanged BMD value. In order to translate these unseen changes into fracture risk, BMD, bone area and viscoelasticity and their correlation with ultimate bone strength were further examined. Not surprisingly, the measured BMD, which reflects hard mineral, was positively associated with bone stiffness. However, there was no significant correlation between BMD and ultimate force in this study. $E'$
and cortical bone area were found positively correlated with ultimate force. Interestingly, it is the first time that positive correlations between tan δ and ultimate strength of the bone were observed during normal daily activity frequency range (0.9-6 Hz). Results from this study demonstrated that DMA is a useful tool to assess osteoporotic drug efficacy. The measured tan δ can be another possible surrogate for measuring bone strength.

A comparison of results from different bones between Chapter 3, 4, 5 by shared techniques would be meaningful since same group of rats was used:

**CTs:** BMD values and microarchitectural indices from distal femur and proximal tibia are similar since they both are metaphysis region of long bone. Geometrical change of cortical bone is more prominent in metaphysis than diaphysis region. The drop of Tb. BMD in ovariectomized rat bone is mainly due to the changes in Tb.N and Tb.Sp, not because of Tb.Th. This implies the disappearance of whole trabeculae.

**Mechanical and viscoelastic tests:** End point three-point bending of tibia and femur both showed more significant differences between groups in F$_{\text{max}}$ than stiffness. Treatments had effect on the fundamental nano-level viscoelastic properties (i.e., $\eta$) as well as the apparent macro-level viscoelasticity (tan $\delta$). BMD and mechanical properties are in consistency only in metaphysis which is abundant in trabecular bone.

In addition, BMD, mechanical and viscoelastic properties in WBV therapy treated ovariectomized rat were investigated in Chapter 6. Based on the reported fact that high bone formation rates in long bones and vertebrae of ovariectomized animals after WBV (Rubin et al., 2002, Oxlund et al., 2003, Sehmisch et al., 2009, Tezval et al., 2011, Wenger et al., 2010, Christiansen and Silva, 2006), we assume that these pronounce
effects of WBV would last for long-term usage. In this pilot study, an in vivo pQCT and whole bone DMA were adopted to explore the longitudinal and endpoint effects of WBV. However, no substantial effects resulting from 16 weeks of low-magnitude, high-frequency vibration treatment (30 Hz, 0.3 g) on tibia BMD and viscoelasticity were found. There are several possible explanations for the fact that this expectation was not met. It should be noted that although the effects of WBV on femoral bone microstructure in the study by Rubin et al (2002) were considerable, in general the effects of WBV on bone microstructure and BMD have shown to vary per site, species, and vibration parameters (magnitude, frequency, duration). In fact, the WBV treated rats have significantly lower lumbar BMD than non-treated group. Furthermore, maximum load of rat vertebrae was significantly reduced in WBV treated rats. The only beneficial effect from WBV treatment is that it significantly reduced postmenopausal osteoporotic weight gain. This result indicates that the prevalent vibration therapy may not provide a curative effect in all conditions; on the contrary, negative results may occur in certain WBV regimen. This finding reveals the need to reconsider using WBV in osteoporosis prevention and treatment. We conclude from our results that WBV should not be recommended as a safe treatment to all osteoporotic patients. Since the optimal parameters were still under research, WBV should not be recommended for long-term usage.

The overall results of the present study provide a better understanding of pathogenesis of osteoporosis, and present a novel evaluation of different osteoporotic medication efficacy (combination of drugs, as well as whole-body vibration) in bone viscoelasticity aspect. For the first time, it is observed that:
- Osteoporosis does not only change bone quantity and microstructure, but also affect nano and macro level bone viscoelasticity ($\eta$, $E'$ and tan $\delta$).
- This deterioration in bone viscoelasticity can be partially restored by different treatments.
- Additive therapeutic effect of combination treatment (PTH and ibandronate) in osteoporotic bone geometry, BMD and viscoelasticity.
- A positive correlation between tan $\delta$ and ultimate strength during normal daily activity frequency range (0.9-6 Hz).
- Viscoelasticity is a useful new tool to assess various treatment efficacies in osteoporotic rat model.
7.2 Future work

In methodology, complementary Atomic Force Microscopy (AFM) and Raman Spectroscopy (RS) could be used to complement the current measurements. Firstly, the roughness of our nanoindentation samples was not quantified. Preparation protocol of nanoindentation sample varies from one study to another. Thus different sample roughness was induced in each study. According to our preliminary trials, it could range from 100 to 300 nm depending on polishing conditions. However, sample roughness was seldom reported in literature. The absence of this parameter creates the difficulty in comparison between studies and reproducibility of the results. For example, if an indentation depth of 800 nm were reported, the real penetration into the bulk sample could range from 500 nm to 700 nm depending on preparation technique. With the taping mode of AFM, the sample roughness could be well quantified before each indentation. An AFM scanning on the residue after the indentation would also provide useful information on visualization and quantification of the micro damage occurred.

Secondly, characterization of tissue composition around each indentation residue by RS can be recommended. In Chapter 4, SEM was used to study the drug induced alterations in collagen alignment. However, whether the matrix composition has been changed is still unknown. In addition, the bone matrix composition differs on hierarchical nano level structures. Thus, instead of traditional element analysis of bulk bone, RS quantification of the local tissue composition around indentation residue should be more suitable.

OVX rat model has been widely used to investigate osteoporotic drug efficacy. Compare to larger animal model, it has advantages of short life-span, low cost, easy handling skill and less ethical issue evolved. However, there are discrepancies between rat and human
cortical bone, i.e., the absence of harvesian remodeling in rats (Turner et al., 2001). Thus
the results observed in this study may not have good compliance with clinical
observations. Animal models with well developed osteon structure, i.e., Rabbit, beagle
dog, ovine etc., would provide a better understanding of the drug pathogenesis.
Bibliography


Bischoff-Ferrari, H., 2011. Three steps to unbreakable bones report. International Osteoporosis Foundation


Fleisch, H., 1996. The bisphosphonate ibandronate, given daily as well as discontinuously, decreases bone resorption and increases calcium retention as assessed by 45Ca kinetics in the intact rat. Osteoporos Int 6, 166-170.


Hasegawa, Y., Schneider, P. and Reiners, C., 2001. Age, sex, and grip strength determine architectural bone parameters assessed by peripheral quantitative computed tomography (pQCT) at the human radius. J Biomech 34, 497-503.


Hope, J., 2012. Popular osteoporosis drug 'raises risk of sight problems in elderly'. mail online


Judex, S., Lei, X., Han, D., Rubin, C., 2007. Low-magnitude mechanical signals that stimulate bone formation in the ovariectomized rat are dependent on the applied frequency but not on the strain magnitude. J Biomech 40, 1333-1339.


Appendix

Vita

YANG Xiao received her Bachelor’s degree in biotechnology, school of science, Wuhan University of Technology, in 2008. Her bachelor thesis title is “Bio-competitive exclusion of thermophilic sulfate reducing bacteria (SRB) in oilfield flooding.” During 4 years of PhD candidature, she has further facilitate her knowledge with engineering fundamentals, including biomechanics, CT based technology and material viscoelasticity. And her current research work is mainly focused on osteoporosis and treatment induced bone property changes.

Publications arising from the thesis


Yang X., Muthukumaran P., Dasde S., Teoh S. H.and Lee T., Positive alterations of viscoelastic and geometric properties in ovariectomized rat femurs with concurrent administration of ibandronate and PTH. Bone, 52 (1), 308-317, 2013


Yang X. and Lee, T., Sixteen weeks of whole body vibration impairs mechanical property and BMC in ovariectomized rat vertebrae. (Prepared for submission)

Award

A pilot study: the synergetic effect of anti-resorptive and anabolic treatments on ovariectomized rats. The best oral presentation award, First Scientific Meeting of Asian Federation of Osteoporosis Societies (AFOS), 2009