Muscular Strength Training Modifies Regulation of Bone Remodeling: Inferences from Serum Biomarkers in Young Women

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Muscular Strength Training Modifies Regulation of Bone Remodeling: Inferences from Serum Biomarkers in Young Women

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(**ABSTRACT**)

Biochemical markers of bone turnover allow inference of the events occurring at the bone tissue level and may detect changes in bone cell activity earlier than densitometric technologies. Serum concentrations of receptor activator for nuclear factor kappa-beta ligand (RANKL), osteoprotegerin (OPG), osteocalcin, and N-telopeptide (NTx) were measured in women aged 20 $\pm$ 1.5 years (mean $\pm$ SD) who underwent 32 weeks of unilateral isokinetic concentric or eccentric muscular strength training. Changes in serum biomarkers were compared with changes in arm and leg flexor and extensor muscle strength. Dual X-ray absorptiometry (DXA) measures of bone mineral density (BMD) and bone mineral content (BMC) of the total forearm, total tibia, and total body also were assessed. The mean serum OPG concentration increased from 4.6 $\pm$ 1.9 pmol/L to 5.2 $\pm$ 2.1 pmol/L ($\uparrow$14.9 %, mean $\pm$ SD; $p = 0.05$, $n = 20$) following long-term isokinetic exercise training that also increased elbow extensor and knee flexor muscular strength ($p \leq 0.05$) and total forearm BMD ($p = 0.04$). The ratio of OPG/RANKL also increased over the course of the study ($p = 0.045$). Serum concentrations of other measured bone biomarkers did not change during training. Serum concentrations of OPG, a suppressor of osteoclastogenesis, increased with high-load muscular strength training that led to local increases in muscle strength and BMD. These adaptations may represent an exercise-mediated suppression of osteoclast differentiation and activity. The central role of the RANKL-OPG cytokine system in the regulation of bone cell biology is well established. Further research is needed to confirm the efficacy of using serum OPG and RANKL as biomarkers of bone cell metabolism in healthy populations undergoing long-term exercise interventions.

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CHAPTER 1

Introduction

Stress fractures may occur in as many as 14% of female military recruits (Beck, et al., 2000). Stress fractures result in lost duty time and impaired military readiness. It is imperative that the cause and consequences of stress injuries be identified and prevention strategies developed in order to sustain a healthy and medically protected military force. The etiology of stress fractures is multifactorial and includes both extrinsic and intrinsic risk factors. Extrinsic factors include running surface, footwear, and physical training parameters. Intrinsic factors involve physiological factors such as muscular strength and endurance and mechanical factors including skeletal alignment, body size, bone density, and bone turnover rate (Lauder, et al., 2000). Bone turnover or remodeling is the mechanism by which the adult skeleton controls both the composition of bone and the adaptation of bone stimulated by the mechanical environment. The elucidation of interventions, which can cause alterations in the rate of bone turnover and result in increased bone formation, can lead to a better understanding of how remodeling may be affected by various forms of exercise-induced loading during physical training. This knowledge may lead to improved exercise program design that favors more effective bone remodeling and improved bone health.

Under normal physiological circumstances, bone is able to sense its mechanical environment and adapt to it. Wolff's law states that bone accommodates to loads imposed on it by altering its mass and distribution of mass (Rubin and Lanyon, 1985). Bone strain arising from mechanical loading stimulates the remodeling process, which results in bone better suited to withstand the applied load. Frost's mechanostat theory

of bone adaptation to strain puts forth the concept that when bone cells are in equilibrium with the surrounding mechanical environment, a basal/physiological level of stress exists. However, a minimum effective strain exists which results in an increase in bone formation entailing an upregulation of osteoblast activity and net matrix deposition (Basso and Heersche, 2002). Remodeling commences five days after stimulation (the initiation of altered strain pattern or magnitude) and consists of a two to three week period of osteoclastic tunneling (resorption) followed by two to three months of osteoblastic formation (Rosen, 2003).

Microcracking may occur as a consequence of reduced tissue resistance to strain following the development of remodeling-related bone porosity or bone resorption spaces (Burr, 1997). If microdamage is too extensive to be successfully repaired by remodeling, and repetitive loading continues, a stress fracture may result. Stress fractures are a form of fatigue damage resulting from repetitive subclinical fracture threshold stresses applied to the skeleton.

Whether the remodeling process is stimulated more by the accumulation of bone matrix microcracks, or by the transduction of mechanical load signals to the bone matrix, remains controversial (Beck, 1998). Transduction involves the transmission of load induced biophysical signals (substrate deformation, fluid flow, or electrokinetic effect) to a cell, and ultimately throughout the cellular network, eventually effecting bone remodeling (Donahue, 1998). The majority of researchers hypothesize that osteocytes are involved in mechanotransduction. Osteocytes are osteoblasts, which have been trapped in the bone matrix after matrix formation and mineralization. Osteocytes have numerous long processes that are in contact with the processes of other

osteocytes or with cells lining the bone surface. Osteocyte processes form a network of thin canaliculi, which permeate the entire bone matrix. Deformation (strain) of bone by loading causes flow of interstitial fluid through the lacunar-canalicular network. Osteocytes are sensitive to relatively small extracellular fluid shear stresses acting on membranes of their osteocyte processes. The osteocytic response to strain includes an increase in the production of nitric oxide, an inhibitor of osteoclast resorption, and an increase in the release of prostaglandins that stimulate osteoblast formation (Owan, et al., 1997). Osteocytes may also produce biochemical messengers that cause a signal to dissipate through the osteocytic network to the bone surface, where an osteoblast recruitment stimulus is created. The nature of this recruitment signal may be an electrical current or ion transport (Huiskes, et al., 2000). Though the mechanism by which signals are integrated and amplified within the osteocyte-osteoblastic network is unclear, gap junctions (membrane-spanning channels that allow passage of small molecules from one cell to another) may communicate the load-induced signals (Donahue, 1998).

Mechanostimulation and the corresponding mechanotransduction of load-induced signals eventually lead to bone adaptation. Turner has discussed three rules for bone adaptation to mechanical stimuli: (1) bone adaptation is driven by dynamic, rather than static loading; (2) only a short duration of mechanical loading is necessary to initiate an adaptive response; and (3) bone cells accommodate to a customary mechanical loading environment, making them less responsive to routine loading signals (Turner, 1998). Several characteristics of dynamic strain have been identified as contributors to the magnitude of bone adaptation. Animal studies have shown that bone formation is

proportional to peak strain magnitude caused by a dynamic mechanical loading stimulus (Rosen, et al., 1997). Strain rate has also been found to play a role in influencing the adaptive remodeling response (Hsieh and Turner, 2001). Finally, changes in strain distribution are likely to be potent osteogenic stimuli. Lanyon has proposed that the more unusual the strain distribution, the more potent the osteogenic potential (Lanyon, 1996).

The ultimate mechanical strain magnitude, rate, directional movement, and duration needed to cause bone biophysical transduction, changes in bone cell metabolism, and eventual bone composition alterations have remained elusive. However, strength training may be an effective type of exercise for several reasons. First, strength training imposes loads on bone that are dynamic, large in strain magnitude, and irregularly distributed. Second, cross-sectional studies have shown that young female strength-trained athletes have greater bone mineral density (BMD) than endurance–trained athletes and untrained subjects (Davee, et al., 1990, Heinonen, et al., 1995). Finally, muscle strength and muscle mass have been positively correlated with BMD and bone mineral content (BMC) both within groups of young female athletes and within the general female population (Nichols, et al., 1995, Snow-Harter, et al., 1992). Weight training involves both eccentric and concentric muscle contractions. Both eccentric and concentric muscle training have been shown to increase muscle strength (Hawkins, et al., 1999). However, eccentric muscle contraction has been shown to produce substantially greater peak loading forces than concentric muscle contraction (Tesch, et al., 1990). The superior osteogenic potential of eccentric contraction was demonstrated in one study where 18 weeks of isokinetic eccentric training of the knee resulted in an

increase (+ 3.9%) in mid-femur segment BMD (Hawkins, et al., 1999). Thus, eccentric muscle training has the potential to cause substantial changes in bone cell metabolism leading to significant bone adaptations.

The measurement of biomarkers of bone metabolism is one way of monitoring changes in bone cell activity. Biomarker assays rely on the measurement, in serum or urine, of bone matrix protein enzymes synthesized by osteoblasts or osteoclasts that spill over into the body fluids, or on the measurement of osteoclast-generated degradation of products of the bone matrix itself (Watts, 1999). Biochemical markers of bone turnover provide minimally invasive measures that reflect the cellular events in bone and may detect changes in bone cell activities that precede densitometric changes.

Osteocalcin is a biomarker utilized to monitor bone formation. Osteocalcin is a 49-residue polypeptide and is a specific product of the mature osteoblast. The actual function of osteocalcin is unknown; however, it is thought to have a role in the organization of the extracellular matrix and the regulation of osteoblast function during the mineralization phase of bone formation (Lee, et al., 2000). The majority of osteocalcin is secreted by osteoblasts and subsequently deposited in the bone matrix. Serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite within the matrix. Intact osteocalcin molecule comprises 1/3rd of the osteocalcin present in serum. Another third is comprised of a large n-terminal mid-molecule fragment. The remaining serum osteocalcin consists of several other smaller osteocalcin fragments (Garnero, et al., 1994). Biochemical assays measuring both intact osteocalcin and the n-terminal mid-molecule fragment of osteocalcin have been

shown to be more stable and reproducible than those measuring intact osteocalcin alone.

The findings of studies utilizing osteocalcin to monitor changes in bone cell metabolism during long-term exercise interventions have been equivocal. Some study protocols have shown a significant elevation in osteocalcin, some a significant decline in osteocalcin concentrations, and some no difference at all. Reasons for the disparities in study outcomes may include exercise intervention inequalities and disparities in the age and sex of study subjects. Serum concentrations of osteocalcin and other bone biomarkers demonstrate intra-subject biological variability and are affected by acute changes in circulatory dilution, transient plasma fluid shifts, and circulatory clearance factors that modify blood concentration. Thus, blood concentrations of osteocalcin can only provide inference of events at the bone tissue level.

During the resorption phase of remodeling, osteoclasts acidify and dissolve the inorganic phase of bone and subsequently degrade the organic component of bone with either activated collagenase or cathepsins. The lysosomal protease Cathepsin K is considered to be among the more important of the cathepsins (Teitelbaum, 2000). In the process of protease degradation, amino- and carboxy-terminal fragments of collagen are released with cross-links attached. These fragments are called telopeptides. Antibodies have been raised against telopeptides and immunoassays have been created to measure both C-terminal telopeptides (CTx) and N-terminal telopeptides (NTx) in human urine and serum (Watts, 1999). The NTx assays provide a specific indicator of the current level of bone resorption.

Serum and urinary NTx have been used extensively as markers of bone resorption during therapeutic interventional studies. Additionally, several studies have been conducted, which monitored urinary NTx changes during long-term exercise interventions (Eliakim, et al., 1997, Ryan, et al., 1998). Serum NTx has not been monitored during exercise intervention studies in either gender or any age group; however, it has excellent potential as a marker of resorption during long-term exercise.

Receptor Activator for Nuclear Factor Kappa-Beta (NF-kB) Ligand (RANKL), receptor activator of (NF-kB) (RANK), and osteoprotegerin (OPG) comprise a cytokine network, which acts as a key regulator of bone metabolism and osteoclast biology. Receptor Activator for Nuclear Factor Kappa-Beta Ligand is produced by osteoblastic lineage cells and activated T cells. Receptor Activator for Nuclear Factor Kappa-Beta Ligand stimulates its specific receptor RANK, which is expressed by progenitor and mature osteoclasts. Receptor Activator for Nuclear Factor Kappa-Beta activation by RANKL initiates intracellular signaling cascades that involve Nuclear Factor Kappa Beta (NF-kB) and Jun N-terminal Kinase (JNK) (Schoppet, et al., 2002). Receptor Activator for Nuclear Factor Kappa-Beta Ligand promotes osteoclast formation, fusion, differentiation, activation, and survival, which all lead to enhanced bone resorption. Osteoprotegerin is a glycoprotein produced by osteoblasts and acts as a decoy receptor for RANKL. The effects of OPG are opposite to those of RANKL. Osteoprotegerin actions include; the inhibition of differentiation and fusion of osteoclastic precursor cells, suppression of osteoclast activation, and promotion of osteoclast apoptosis (Udagawa, et al., 2000).

Changes in serum RANKL have been studied during pharmacological trials, but not during long-term exercise interventions. Though the effects of mechanical stimulation *in vivo* remain unknown, mechanical strain has been shown to down-regulate RANKL messenger ribonucleic acid (mRNA) and to inhibit osteoclast formation *in vitro* (Rubin et al. 2002). The revelation that changes in RANKL mRNA occur during short-term stress interventions *in vitro* may indicate a potential use of serum RANKL assays to monitor changes in human bone metabolism in vivo during long-term exercise interventions.

The discovery that OPG acts to inhibit the binding of RANKL to RANK and leads to an inhibition of osteoclast formation, activation, and survival, has caused the scientific community to take a great interest in the glycoprotein. Serum OPG concentrations have been extensively used to monitor changes in bone metabolism occurring during therapeutic interventions. Serum OPG has not been utilized to monitor changes during long- or short-term exercise interventions; however, OPG mRNA levels have been shown to increase with force application *in vitro* (Kobayashi, et al., 2000). Because changes in OPG have been observed *in vitro* during application of tensional force, and serum OPG has been shown to be a useful marker for the inhibition of osteoclast activity during therapeutic interventions, serum OPG may prove to be an excellent method for monitoring changes in bone metabolism during exercise interventions.

The simultaneous evaluation of RANKL, OPG, OC, and NTx from serum samples may provide a more complete perspective of regulatory changes in bone cell metabolism that occur following long-term exercise interventions. This information

may enhance the understanding of how to manipulate the factors of exercise training needed to optimize bone mineral density (BMD), bone mineral content (BMC), and bone strength. The purpose of the current study was to determine if long-term mechanical stimulation of bone during eccentric and concentric isokinetic strength training in young military recruit-aged women caused contemporaneous changes in osteoblast and osteoclast functional activity.

Statement of Problem

Long-term isokinetic concentric and eccentric exercise training have the potential to cause alterations in the rate of bone turnover and result in increased bone formation. The measurement of biomarkers of bone metabolism is one method of monitoring changes in bone cell activity. Receptor Activator for Nuclear Factor Kappa-Beta (NF-kB) Ligand, RANK, and OPG recently have been identified as key controllers of bone metabolism and osteoclast biology. Osteoprotegerin and RANKL have never been used as biomarkers of metabolism during exercise interventions, but have excellent potential as indices of changes in bone cell activity. Osteocalcin and NTx have been utilized extensively as markers of bone turnover during exercise intervention studies. The simultaneous evaluation of RANKL, OPG, osteocalcin, and NTx from serum samples may provide a new and more complete perspective of regulatory and metabolic changes that occur in bone cell metabolism during and following long-term exercise interventions.

Research Hypotheses

Thirty-two weeks of unilateral eccentric or concentric isokinetic muscular strength training will induce the following changes in bone and bone cell metabolism:

- Alterations will occur in osteoblast production of the osteoclast regulatory cytokines OPG and RANKL. Modifications in bone remodeling will be indicated by changes in and serum levels of intact and N-terminal mid-molecule fragment of osteocalcin and serum NTx levels.
- Percent change in the markers of bone metabolism will be related too the percent change in total body and regional bone mineral density and bone mineral content in trained limbs and the percent change in peak torque strength in trained limbs.
- Changes in bone biomarkers will predict changes in total body and regional bone mineral density and mineral content and muscle torque strength.

Significance of Study

Simultaneous evaluation of RANKL, OPG, osteocalcin, and NTx from serum samples may provide a more complete perspective of regulatory adaptations that occur in bone because of mechanical loading from muscular strength training interventions. This information may enhance understanding of how to manipulate the factors of exercise training needed to optimize bone cell activity, BMD, BMC, and bone strength.

Basic Assumptions

The investigator made the following assumptions in conducting this study:

1. All subjects performed to the best of their ability during training.

2. Volunteer subjects were representative of the population of military aged females.

3. All measures were performed and recorded accurately by the Biodex® System 3 dynamometer and the trained technicians.

4. Bone density and BMC were measured and recorded accurately by the QDR 4500 A Elite Fan Beam X-ray Bone Densitometer (Hologic, Bedford, MA) and the trained technician.

5. All biomarker assays were performed and recorded accurately. Assays utilized included sRANKL enzyme immunoassay (Biomedica, Windham, NH); OPG (Biomedica), Mid-Tact Human Osteocalcin enzyme immunoassay Kit (Biomedical Technologies Inc., Stoughton, MA), Osteomark NTx Serum (Osteomark, Seattle, WA), Stanbio Alkaline Phosphatase LiquiColor Procedure No. 2900 (Stanbio, Boerne, TX) modified by the addition of sodium hydroxide 0.25 moles (M), and Stanbio Total Calcium LiquiColor (Arsenazo III) Procedure No. 0155 (Stanbio). A trained technician accurately measured and recorded results from the spectrophotometer and enzyme immunoassay reader.

Delimitations

The investigator delimited the study through the following methods:

1. Eccentric and concentric isokinetic training was performed by only one arm and one leg.

2. Serum biomarkers were analyzed at only two time points during training.

3. Only ten eccentrically and ten concentrically trained individuals were included in the study.

4. Only participants who completed blood draws at 0 and 32 weeks were studied.

Limitations

Interpretation of the data was limited by the following:

1. Unilateral eccentric and concentric isokinetic training of only one arm and one leg caused a stimulus that affected a limited area of bone. Thus, the mechanical stimulus of this study may not have been robust enough to cause significant changes in bone cell metabolism.

2. Changes in bone cell metabolism may have occurred at various times after the stimulus was applied. Since biomarker levels were measured at only two time points, critical changes in bone cell activity that occurred at other time points during training may have been missed.

3. This study only included twenty subjects whom where either eccentrically or concentrically trained. Biomarkers measured in a larger number of subjects may have imparted different information about bone cell activity during muscular strength training.

4. Only subjects who completed blood draws at 0 and 32 weeks were included in this study. Many potential subjects failed to attend blood draw sessions; therefore, their data were not available for inclusion.

5. There was variance in subject compliance with training schedules. Subject attendance may have affected the frequency of mechanical stimulation applied to bone and concomitant changes in bone cell metabolism.

6. Biomarkers of bone turnover only measure overall changes in bone cell metabolism. Regional changes in bone turnover may have occurred that were not detected in total body biomarker measures.

Definition of Terms

- Apoptosis — Programmed cell death
- Carboxylation — The addition of a carboxyl group
- Equibiaxial strain — Uniform stretching that entails both bending and compressive forces
- Histomorphometry — The quantitative measurement of tissue
- Hydroxyapatite — A bone matrix crystal formed from calcium and phosphate
- Kinase — An enzyme that catalyzes the transfer of phosphate from ATP to an acceptor molecule
- Polypeptide — A union of two or more amino acid
- Mechanostimulation — Mechanical stimulation
- Mechanotransduction — The transduction of a mechanical stimulus throughout the bone matrix
- Morphometry — The measurement of forms of organisms
- Nadir — The lowest point
- Neoangiogenesis — Blood vessel development
- Remodeling — Bone change or growth which is the net effect of all appositional growth and bone resorption and occurs continually in life to adapt the skeletal elements to the changing forces applied by growth, muscular activity, gravity, or mechanical pressures
- Senescent — Growing old

List of Abbreviations

- Akt — Serine/threonine protein kinase
- BMD — Bone mineral density
- BMC — Bone mineral content
- BMP — Bone morphogenic protein
- cAMP — Cyclic adenosine-3’5’-monophosphate
- EMG — Electromyography
- JNK — Jun N-terminal kinase
- IL — Interleukin
- M-CSF — Macrophage colony stimulating factor
- nM BCE — Nanomoles bone collagen equivalents
- nM/mM Cr — Nanomoles/ millimoles Creatinine
- mRNA — Messenger ribonucleic acid
- NF-kB — Nuclear factor kappa-beta
- NTx — N-terminal telopeptide
- OPG — Osteoprotegerin
- PKB — Protein Kinase B
- PKA — cAMP –dependent protein kinase
- PTH — Parathyroid hormone
- PTH1R — Parathyroid-1 receptor
- RANK — Receptor activator for nuclear factor kappa-beta
- RANKL — Receptor activator for nuclear factor kappa-beta ligand
- TGF-b — Transforming growth factor beta

- TNF-a Tumor necrosis factor alpha
- TNF-b Tumor necrosis factor beta
- TNFR Tumor necrosis factor receptor
- TRAF Tumor necrosis factor receptor-associated factor

Summary

Bone health and the prevention of bone fractures continue to be of great concern for young military aged females participating in basic training. The elucidation of interventions, which can cause alterations in bone strength and protect against injury, is an important goal for military medicine and the community at large. Long-term isokinetic concentric and eccentric exercise training has the potential to cause alterations in the rate of bone turnover and result in increased bone formation.

The measurement of bone biomarkers is one method of monitoring changes in bone cell activity. Receptor activator for nuclear factor kappa-beta (NF-kB) Ligand (RANKL) and OPG have recently been identified as key controllers of bone metabolism and osteoclast biology. Osteoprotegerin and RANKL have never been used as biomarkers of bone metabolism during exercise interventions, but have excellent potential as indices of change. Osteocalcin and NTx have both been utilized extensively as markers of bone turnover. The simultaneous evaluation of RANKL, OPG, osteocalcin, and NTx from serum samples may provide a new and more complete perspective of regulatory and metabolic changes that occur in bone cells during and following long-term exercise interventions.

CHAPTER 2 – LITERATURE REVIEW

Introduction

The current chapter will review the available literature pertaining to the adaptations that occur in bone cell metabolism during mechanical stimulation and the use of biochemical markers of bone for the determination and evaluation of changes in bone cell activity. First, an overview of bone remodeling, mechanical simulation of bone remodeling, and mechanical signal transduction will be provided. Second, the bone related effects of weight training and isokinetic muscle contraction will be reviewed. Finally, an overview of relevant bone biomarkers and a discussion of studies utilizing biomarkers to monitor changes in bone during interventional studies will be conducted. Biomarkers of interest will include osteoprotegerin (OPG), receptor activator of nuclear factor kappa-beta (RANKL), osteocalcin, and N-terminal telopeptide (NTx).

Bone Remodeling

The primary function of bone is mechanical load bearing which is carried out by cortical bone throughout the skeleton and by peripheral cancellous bone. Additional bone functions, carried out mainly by central cancellous bones, include: participation in plasma calcium and other mineral homeostasis and the support of hematopoiesis (Parfitt, 2002). Bone remodeling allows for the alteration of essential mineral balance and provides a mechanism for healing of microcracks or for the replacement of hypermineralized bone (Burr, 1997, Martin, 2002, Noble, et al., 1997). Bone remodeling also provides a mechanism for the skeleton to adapt to its mechanical environment.

Remodeling is carried out by osteoblasts and osteoclasts. The primary function of osteoblasts is the synthesis of bone matrix on bone forming surfaces. Osteoblasts are also the principal cells that mediate the bone-forming processes and are indirectly responsible for regulating osteoclastic bone resorption through paracrine factors. Osteoclasts are multinucleated giant cells that specialize in bone resorption. Bone replacement is initiated by osteoclastic resorption followed by osteoblastic formation. Remodeling occurs in a sequence: a rapid two to three-week bone resorption phase followed by a slower two- to three- month bone formation phase (Rosen, 2003). Both of these processes are closely linked.

The basic unit for bone turnover can be called the "basic multicellular unit" usually abbreviated BMU. A fully developed BMU consists of a team of osteoclasts in front forming the cutting cone and a team of osteoblasts behind forming the closing cone (Parfitt, 1994). The BMU excavates and refills a tunnel through cortical bone or a trench across the surface of cancellous bone. A cortical BMU travels for about 4000 um at about 20 um/day taking about 200 days. A cancellous BMU travels about half this distance at about half the speed, taking about the same period (Parfitt, et al., 1996).

Basic multicellular unit target recognition, site selection and directional control probably involves the nearby network of osteocytes. There are several steps in the process of bone resorption. First, there is the digestion of the endosteal membrane by enzymes released from lining cells. Second, changes occur in lining cell morphology to expose the mineralized bone surface and angiogenesis (blood vessel development). Third, mononuclear osteoclast precursors exit out of the blood circulation at precisely the correct location, which are then attracted to the region of exposed mineral. Here, the

mononuclear osteoclasts fuse to form multinuclear osteoclasts. Next, there is assembly of a sufficient number of osteoclasts to form the cutting cone. Osteoclasts undergo apoptosis as the BMU progresses. Senescent osteoclasts are replaced by newly fused mononuclear osteoclasts. Eventually, the supply of osteoclast precursors is turned off, most likely because the stimulus for site-specific exit from the circulation is no longer turned on (Parfitt, 2002). The BMU stops moving forward and reaches the end of its life span when the cavity is refilled.

Mechanical Stimulation of Bone Remodeling

The adult skeleton is a dynamic organ that undergoes a constant process of remodeling. Under normal physiological circumstances, bone is able to sense its mechanical environment and adapt to it. Wolff's law states that bone accommodates to loads imposed on it by altering its mass and distribution of mass (Hortobagyi and Katch, 1990). Bone strain arising from mechanical loading stimulates the remodeling process, which results in bone better suited to withstand the applied load (Friedlander, et al., 1995).

Bone morphometry appears to have evolved to the extent that strains produced during normal, functional patterns of loading remain within the ranges of magnitude that are unlikely to produce tissue damage (Beck, et al., 2000). Bone exhibits an intrinsic ability to adapt to alterations in chronic loading patterns by modifying geometric and/or material properties to best withstand future loads of the same nature (Rubin and Lanyon, 1984, 1985). Frost's mechanostat theory of bone adaptation to strain puts forth the concept that when bone cells are in equilibrium with the surrounding mechanical environment, a basal/physiological level of stress [200-2500 microstrain (ue)] exists and there is no

formation of bone. However, a minimum effective strain exists, 2500 ue, which results in an increase in bone formation entailing an upregulation of osteoblast activity and net matrix deposition. Strains of > 5000 ue are considered to be pathological (Basso and Heersche, 2002, van der Meulen, et al., 1993). Frost suggested that the objective of adaptations in bone is to reduce the potential for strain-related tissue damage (Frost, 1987).

Dynamic Strain

Mechanical loading during physical activity results in internal forces (stress) and deformation (strain) (Bennell, et al., 1999). These stresses and strains are thought to provide the stimulus for the structural adaptation of bones (Carter, 1984, Turner, 1998, Turner, et al., 1994). Experiments and computer models have supported the idea that dynamic loading is associated with increased bone remodeling, whereas continuous loading is not (Basso and Heersche, 2002). Several characteristics of dynamic strain have been identified as contributors to the magnitude of bone adaptation. Animal studies have shown that bone formation is proportional to the peak strain magnitude caused by a dynamic mechanical loading stimulus (Rosen, et al., 1997). Rubin and Lanyon (1984), studying isolated turkey wings, found that bone hypertrophy was proportional to strain magnitude (Rubin and Lanyon, 1985). In humans, the high bone densities, found in athletes who experience the greatest loads in their respective sports, suggest that magnitude is particularly osteogenic (Heinonen, et al., 1995). Dynamic resistance exercises that involve high peak forces should effectively load the skeleton.

Strain rate may also play a role in influencing the adaptive remodeling response (Hsieh and Turner, 2001). Mosley and Lanyon (1998) studied the influence of strain rate on adaptive modeling in response to dynamic loading of the ulna in growing male rats. Strain rate was determined to be a major contributor to the adaptive osteogenic response. Turner et al. (1995) found that the formation of new bone was directly proportional to the rate of strain on bone tissue; however, relatively high strains alone were not sufficient to activate bone cells in an adult rat model (Turner, et al., 1995). In a study of ulnar diaphysis of adult female rats, Hsieh and Turner (2003) determined that the mechanical load required to initiate new bone formation decreased as the loading frequency increased (Hsieh and Turner, 2001). Increasing loading frequency provides a more effective application of mechanical forces to promote osteogenesis.

Changes in strain distribution are also likely to be potent osteogenic stimuli. The more unusual the strain distribution, the more potent the osteogenic potential (Lanyon, 1996, Rubin and Lanyon, 1985). Finally, the duration of exercise affects the osteogenic response. Bone cells demonstrate a desensitization phenomenon in the presence of extended mechanical loading. Rubin and Lanyon (1984) demonstrated that osteogenic response to mechanical loading was not increased when the loading regimen was lengthened from 36 to 1800 consecutive cycles/d in a functionally isolated avian bone preparation. This suggests that bone tissue may become desensitized to prolonged exercise.

Bone cells may react strongly to transients in their environment, but eventually "accommodate" to steady state signals (Burr, et al., 2002, Lanyon, 1996). Consequently, a change in mechanical loading will cause an initial change in bone remodeling, but the

reaction will eventually cease as the bone cells adjust to the new environment. The mechanism of accommodation has not been defined, but it could involve receptor down regulation of cytoskeletal reorganization that changes the cell's sensitivity to external stimuli. Thus, cellular accommodation may have the property of "path dependence", meaning that final bone mass will be dependent upon the temporal sequence of preceding mechanical loading (Martin, 2000).

The ultimate mechanical strain magnitude, rate, directional movement, and duration needed to cause changes in bone cell metabolism and eventual bone composition alterations has remained elusive. Unusual strain distributions, high strains, and high strain rates seem to be particularly osteogenic. Adaptive bone remodeling may be sensitive to strain "errors" which are not repeated frequently, rather that the repetitious strain cycles produced during normal predominate activities (Lanyon, 1996). Exercise regimens designed to control bone architecture should capitalize on this feature of the adaptive remodeling response. Exercise sessions, need not be prolonged, but should include novel strain distributions.

The Effects of Muscular Strength Training of Bone Adaptation

The exact mechanisms by which physical activity increases bone mass are largely unknown. According to animal experiments, the training effect is most likely transmitted by the stimulation of osteoblast activity and new bone formation and, perhaps, not so intensely, by inhibition of osteoclast activity and bone resorption (Kannus, et al., 1996, Yeh, et al., 1993). Furthermore, the effects of training may be specific to the anatomic sites at which the mechanical strain occurs (Haapasalo, et al., 1998, Kannus, et al., 1996,

Kaufman and Einhorn, 1993, Vuori, et al., 1994). Frost (1997) examined strain magnitudes as a component of osteogenicity. Except for trauma and jumping down form a height, the largest mechanical loads on bones usually come from muscle contractions, which work against the dual resistances of body weight multiplied by lever arms used to move the body around in the earth's gravitational field (Frost, 1997). Muscle contractions during strength training also impose loads that are dynamic and irregularly distributed (Chilibeck, et al., 1995). Thus, it would seem likely that skeletal muscle contractions imposed by muscular strength training have the potential to cause bone adaptation. Whether the adaptation is directly related to the magnitude of bone loading from muscle pull or to some other aspect of muscle contraction or muscle mass is unclear (Lohman, et al., 1995).

Substantial research has focused on the relationships between muscular strength training, muscle mass, and bone. Several studies, involving resistance training in young and pre-menopausal women, have demonstrated significant regional increases in BMD and muscle strength (Friedlander, et al., 1995, Hawkins, et al., 1999, Lohman, et al., 1995, Snow-Harter, et al., 1992), while other studies have not (Alfredson, et al., 1999, Gleeson, et al., 1990, Vuori, et al., 1994). Cross-sectional studies have shown that young female strength-trained athletes have greater BMD than endurance–trained athletes and untrained subjects (Davee, et al., 1990, Heinonen, et al., 1995). Additionally, several studies have reported a positive correlation between muscle strength and muscle mass and BMD and bone mineral content (BMC) (Nichols, et al., 1994, Nichols, et al., 1995, Snow-Harter, et al., 1990).

Isokinetics refers to a muscular action performed at constant angular limb velocity. Isokinetic muscular strength training consists of coupled shortening or concentric movement and/or lengthening or eccentric muscle action. Isokinetic training makes it theoretically possible for a person's muscle to exert a continual maximal concentric or eccentric muscular contraction throughout a movement's full range of motion. Both eccentric and concentric isokinetic muscle training have been shown to increase muscular strength (Colliander and Tesch, 1990, Godard, 1998, Hawkins, et al., 1999, Hortobagyi, et al., 1996, Johnson, et al., 1976). In several studies, eccentric muscle training has demonstrated superior strength gains (Hortobagyi, et al., 2001, Hortobagyi, et al., 1996, Nichols, 1995) and in one study, greater osteogenic potential (Hawkins, et al., 1999).

Higbie et al. (1996) studied the effects of isokinetic concentric and eccentric training on quadriceps muscle strength. Forty-four women (mean $\pm$ SD, age 20.0 $\pm$ 0.5 yrs) were randomly assigned to either: a concentric training group (n = 16), an eccentric training group (n = 9), or a control group (n = 19). Muscle strength (average torque) was tested before and after 10 weeks of unilateral concentric or eccentric knee-extension training. Average torque was measured during concentric and eccentric maximal voluntary knee extension at 60^{o}/sec using a Kin-Com dynamometer. Average concentric and eccentric torque increased 18.4 and 12.8 % for the concentrically trained group, 6.8 and 36.2 % for the eccentric group, and 4.7 and –1.7 % for the control group, respectively. The increase in the eccentric average torque was greater in the eccentric training group than the increase in the concentric average torque in the concentric group (Higbie, et al., 1996). This study demonstrated that gains in strength consequent to concentric and eccentric training are highly dependent on the muscle action used for training and testing.

Maximum voluntary force and force relative to motor unit activation have been found to be greater for eccentric muscular action than concentric action (Dudley, et al., 1990, Komi and Buskirk, 1972, Tesch, et al., 1990, Tesch, et al., 1990). Tesch et al. (1990) measured the surface electromyography (EMG) of the vastus lateralis and rectus femoris muscles of 14 healthy males (mean $\pm$ SE, 33 $\pm$ 2 yrs, n = 14). Electromyography readings were taken on two separate days and concentric exercise measurements were performed two days before eccentric measurements. Three bouts of 32 maximal voluntary unilateral concentric or eccentric quadriceps muscle actions were performed at a constant angular velocity (180^{o}/sec). Eccentric muscular contraction was associated with greater peak force production and lower EMG signals than concentric. These findings suggest that eccentric loading should generate greater forces on bone than concentric loading (Tesch, et al., 1990).

If the stimulus from muscle contraction to which bone responds is peak load, eccentric muscle action should produce greater bone adaptation than concentric muscle action. Hawkins et al. (1999) conducted a study where twelve women (mean $\pm$ SD, age 27.8 $\pm$ 1.7 yrs, n = 8) underwent 18 wks of isokinetic concentric and eccentric training. Eight women served as controls (mean age 21.9 $\pm$ 1.13, n = 8). Subjects trained one leg concentrically and the other eccentrically. Dominant vs. non-dominant limbs were not considered relative to exercise training. Training was performed on a KinCom dynamometer and consisted of three sets of four maximal concentric repetitions and three sets of three eccentric maximal repetitions per training session. Bone mineral density was measured by Dual-energy x-ray absorptiometry (DXA). Isokinetic knee flexion and extension strengths were determined eccentrically and concentrically. Concentric

training group strength improved 30 % in concentric extension, 21 % in eccentric extension, and 15% in eccentric flexion. Eccentric training group strength improved 27 % in eccentric extension, 21 % in concentric extension, 16 % in eccentric flexion, and 15% in concentric flexion. The control group demonstrated insignificant reductions in strength. There were no differences between exercise or control subjects in pre- or post-training TBBMD or total hip BMD. However, eccentric exercise significantly increased mid-femur segment BMD by 3.9 % ($p < 0.05$). Concentric exercise caused an insignificant increase in mid-femur BMD of 1.1 % (Hawkins, et al., 1999). This study demonstrated that isokinetic eccentric training was more osteogenic than isokinetic concentric training. One possible reason for greater bone adaptation with eccentric muscular training was that it provided a unique stimulus to the bone. Strains of abnormal distribution are very effective in stimulating bone formation. Alternatively, Evans and Cannon (1991), in a review on the metabolic effects of exercise-induced muscle damage, determined that muscle damage causes the generation of systemic and local factors known to be osteogenic. These factors, which are not generated during concentric exercise, might influence bone adaptation (Evans and Cannon, 1991).

Mechanotransduction

In order to adapt to mechanical loading, bone tissue adjusts through a feedback system in which changes in the local mechanical environment signal bone cells to modify bone structure to meet new strain requirements. Mechanotransduction involves the transmission of load induced biophysical signals to a cell, and ultimately throughout the cellular network, eventually effecting bone remodeling (Donahue, 1998). The specific mechanical signals detected by bone cells, and the ways in which the signals are

converted into cellular activity that results in bone remodeling are still under investigation. However, the majority of researchers hypothesize that osteocytes are involved in mechanotransduction. Osteocytes are osteoblasts, which have been trapped in the bone matrix after matrix formation and mineralization. Osteocytes have numerous long processes rich in microfilament, which are in contact with cell processes on other osteocytes, and with processes from inactive cells lining the bone surface, which also originate from senescent osteoblasts. Thus, osteoblasts, osteocytes and lining cells form a network, which is well–equipped for signal tranduction (Masi and Brandi, 2001). It is possible that increased strain in the local bone matrix signals osteocytes to transmit stimuli to the surface, where bone is formed until the strains are normalized (Huiskes, et al., 2000)

Deformation or strain on bone tissue causes flow of interstitial fluid through the lacunar-canalicular network of bone. Deformation causes extracellular fluid to move away from surfaces of greater concavity toward surfaces of greater convexity (Burr, et al., 2002). This creates hydrostatic pressure gradients within the bone's matrix that drive extracellular fluid flow (Basso and Heersche, 2002). Pressure gradients must be equilibrated via movement of fluid from regions of high pressure to regions of low pressure. Thus, fluid movement is created which places shear stresses on the plasma membranes of resident osteocytes (Owan, et al., 1997, Weinbaum, et al., 1994). Osteocytes have been shown to be sensitive to relatively small extracellular fluid shear stresses acting on membranes of their osteocyte processes within the network (Weinbaum, et al., 1994). Additionally, shear stresses are proportional to the rate of fluid flow. As bone is loaded more quickly, at a higher strain rate, fluid velocity and

consequent shear stresses increase (Burr, et al., 2002). It is most likely that fluid movement exerts shear stresses on the osteocyte processes, rather than a direct deformation of the osteocytic cell membrane by the mechanical stimuli itself (Owan, et al., 1997).

Osteocytes may produce biochemical messengers that cause signals to dissipate through the osteocytic network to the bone surface, where an osteoblast recruitment stimulus or an osteoclast inhibitory signal is created (Burger and Klein-Nulend, 1999). Osteocytes have been shown to respond to fluid shear stresses by elevating the production of nitric oxide and prostaglandins. Nitric oxide is an inhibitor of osteoclastic resorption (Klein-Nulend, et al., 1995) and prostaglandins stimulate osteoblast formation (Ajubi, et al., 1999, Cowin, et al., 1991). Osteocytes may also send signals to the bone surface by electric current and ion transport (Burger and Klein-Nulend, 1999, Cowin, et al., 1991, Huiskes, et al., 2000). The mechanism by which ionic signals are integrated and amplified within the osteocyte-bone lining cell-osteoblastic network is unclear; however, gap junctions may communicate the load-induced signals (Donahue, 1998). Gap junctions are membrane-spanning channels that allow passage of small molecules such as calcium ions (Ca^{2+}), inositol phosphates, and cyclic nucleotides, from one cell to another (Donahue, 1998). Both osteoblastic and osteocytic cells possess functional gap junctions enabling them to communicate with one another and each other (Donahue, 1998). Mikuni-Takagaki (1999) observed a Ca^{2+} influx in stretched primary rate osteocytic cell processes, which may support a specific role of stretch-activated calcium entry pathways (Mikuni-Takagaki, 1999).

Markers of Bone Cell Metabolism

Biochemical markers of bone turnover can be assessed in serum to provide a minimally invasive measure, which reflects the cellular events in bone. One of the principle advantages of bone biomarkers is that they can readily detect acute changes in bone cell metabolism (Lee, et al., 2000). Bone biomarker measurements may be vulnerable to many sources of biological variability. Seasonal variations, circadian variations, use of oral contraceptives, and menstrual influences have all been identified as possible sources of biological variation; however, their impact remains controversial (Watts, 1999). Serum concentrations of bone biomarkers are also affected by acute changes in circulatory dilution, transient plasma fluid shifts, and circulatory clearance factors that modify blood concentration. Additionally, day-to-day variability may occur in serum concentrations of biomarkers (Hannon and Eastell, 2000, Swaminathan, 2001). In summary, serum biomarkers of bone metabolism can only provide inference of events at the bone tissue level. Caution must be exercised when drawing conclusions and making recommendations based on changes that occur in serum biomarkers during interventional studies.

Receptor Activator of Nuclear Factor Kappa-beta Ligand and Osteoprotegerin

Receptor activator for nuclear factor kappa-beta (NF-kB) Ligand (RANKL), receptor activator of (NF-kB) (RANK), and osteoprotegerin (OPG) comprise a cytokine network, which regulates osteoclast metabolism. Receptor activator for nuclear factor kappa-beta activation by RANKL stimulates bone resorption (Schoppet, et al., 2002). The binding of RANKL by OPG interferes with bone resorption. Receptor activator for nuclear factor

kappa-beta is the receptor activator of (NF-kB) and is a type I transmembrane receptor with four cysteine-rich pseudorepeats and 616 amino acids (Hsu, et al., 1999, Nakagawa, et al., 1998). Receptor activator for nuclear factor kappa-beta mRNA is expressed by both osteoclast progenitors and mature osteoclasts (Nakagawa, et al., 1998). The activation of RANK results in the activation of intracellular signaling cascades that involve NF-kB (Anderson, et al., 1997) and c-Jun N-terminal kinase (JNK). Nuclear factor kappa-beta (NF-kB) and JNK are signaling receptors essential for RANKL-mediated osteoclastogenesis. Following RANK binding by RANKL, adaptor molecules including tumor necrosis factor receptor-associated factors (TRAFs), such as TRAF-6, and c-src are recruited. TRAF-6 recruitment is followed by signaling pathways down-stream which include the NF-kB pathway and the c-Jun N-terminal kinase/c-fos/c-jun pathway (Darnay, et al., 1998, Darnay, et al., 1999, Galibert, et al., 1998), both of which are essential for osteoclast formation and activation. In a third pathway, TRAF-6 and c-src cooperate to activate the serine/threonine kinase protein kinase-B (PKB/Akt) pathway, which mediates antiapoptotic signals and cytoskeletal reorganization. Interestingly, it appears that there are multiple cross-talks and redundancies between the three signaling pathways (Hofbauer and Heufelder, 2001). The precise function of the individual components of the RANK signaling pathway, their relative contribution to osteoclast function, and their interaction with other cytokines and growth factors has not been completely elucidated (Hofbauer and Heufelder, 2001).

Receptor activator for nuclear factor kappa-beta ligand (RANKL) is produced by osteoblastic lineage cells and activated T cells. Receptor activator for nuclear factor kappa-beta Ligand stimulates its specific receptor RANK. Receptor activator for nuclear

factor kappa-beta Ligand and is a type II transmembrane protein and is expressed in three forms. There is a cell-bound peptide consisting of 317 amino acids with a molecular mass of 38 kd, a truncated ectodomain form created from the cell-bound form by enzymatic cleavage by TNF-alpha converting enzyme-like protease at positions 140 or 145 respectively, and a primary form which is secreted by activated T cells (Hofbauer and Heufelder, 2001). Although all forms are bioactive, the membrane bound protein appears to be the homeostatic form, while the production of soluble RANKL may signal pathological conditions (Hofbauer, et al., 2001). Osteoclast precursors derived from bone marrow cells and macrophages or spleen cells require macrophage colony-stimulating factor (M-CSF) to differentiate into RANKL-responsive osteoclasts. In the presence of M-CSF, RANKL promotes osteoclast differentiation (Matsuzaki, et al., 1998, Quinn, et al., 1998, Shalhoub, et al., 1999), osteoclast activation (Burgess, et al., 1999, Fuller, et al., 1998, Kong and Penninger, 2000), survival (Lacey, et al., 2000), and adherence to bone surface (O'Brien, et al., 2000). The manner in which RANKL is metabolized *in vivo* has not yet been fully elucidated.

Receptor activator for nuclear factor kappa-beta ligand expression is modulated by several cytokines. Stimulators of osteoclastogenesis such as, interleukin (IL)-6, IL-11, IL-17, IL-1-β, and tumor necrosis factor-alpha (TNF-α) increase the expression of RANKL. Interleuken-1 stimulates the activation of osteoclasts and TNF-α appears to activate the generation of osteoclasts (Jimi, et al., 1999, Kobayashi, et al., 2000). Both IL-1 and TNF-α are strong inducers of RANKL production (Brandstrom, et al., 1998, Hofbauer, et al., 1999, Vidal, et al., 1998). Ostoeclastogenic cytokines such as prostaglandin E2 may facilitate and cooperate with RANKL-induced osteoclast formation

and activation (Hofbauer and Heufelder, 2001, Wani, et al., 1999). Transforming growth factor-beta (TGF-β) super family members, such as bone morphogenetic proteins (BMPs), strikingly enhance osteoclast differentiation from their progenitors and survival of mature osteoclasts. Bone morphogenetic proteins are a family of multifunctional cytokines that stimulate or inhibit cell function and replication in numerous cell types. Bone morphogenetic protein-mediated signals cross-communicate with RANKL mediated signals in inducing osteoclast differentiation and function (Udagawa, 2002). Cytokines that inhibit osteoclastogenesis, such as TGF-β-1 and IL-13, suppress the expression of RANKL and stimulate OPG expression.

Several hormones may also affect RANKL expression and activity. Analysis of the RANKL gene promoter structure has revealed response elements for glucocorticoids and vitamin D (Kitazawa and Kitazawa, 2002, Kodaira, et al., 1999). Parathyroid hormone (PTH) triggers osteoclast formation by binding to osteoblasts and causing them to increase RANKL output and decrease OPG production. Parathyroid hormone promotes bone loss when elevated over long periods (Kong and Penninger, 2000). Estrogen may regulate RANKL and OPG to block osteoclast formation and preserve bone (Rosen, 2003).

Osteoprotegerin acts as a decoy receptor for RANKL (Lacey, et al., 1998), thus preventing activation of RANK (Simonet, et al., 1997). Osteoprotegerin, also known as osteoclastogenesis inhibitory factor (OIF), is a glycoprotein molecule composed of 401 amino acid residues with a signal peptide of 21 amino acids required for homodimerization (Yasuda, et al., 1998) and 4 characteristic cysteine-rich pseudorepeats (Tsuda, et al., 1997, Yasuda, et al., 1998). Osteoprotegerin is a member of the tumor

necrosis factor receptor (TNFR) superfamily, and exists in a 60-kd monomeric form and a disulfide-linked homodimeric form of 120 kd (Yamaguchi, et al., 1998). Monomeric and dimeric OPG have been shown to be indistinguishable in their specific activity to inhibit osteoclastogenesis (Tomoyasu, et al., 1998). The half-lives of OPG molecules *in vitro* are (mean $\pm$ SD) 15.0 $\pm$ 1.7 min for the monomeric form and 20.0 $\pm$ 1.2 min for the homodimeric forms. The metabolism and break down of OPG *in vivo* has not yet been clarified.

The effects of OPG *in vitro* are opposite to those of RANKL. Osteoprotegerin actions include the inhibition of differentiation, survival, and fusion of osteoclastic precursor cells, suppression of osteoclast activation, and promotion of osteoclast apoptosis (Burgess, et al., 1999, Fuller, et al., 1998, Hakeda, et al., 1998, Jimi, et al., 1999, Lacey, et al., 1998, Udagawa, et al., 2000, Yasuda, et al., 1998). Osteoprotegerin is produced by a variety of tissues including the cardiovascular system (heart, arteries, veins), kidney, lung, intestine and bone as well as hematopoietic and immune cells (Schoppet, et al., 2002). Skeletal versus non-skeletal contributions to circulatory OPG levels are unclear (Yano, et al., 1999).

Various cytokines, peptides, and hormones modulate the expression and production of OPG. Cytokines, including IL-1-α, IL-1-β, IL-13, and transforming growth factor-beta-1 (TGF-β-1), have been shown to up-regulate OPG mRNA levels (Brandstrom, et al., 2001, Collin-Osdoby, et al., 2001, Hofbauer, et al., 1999, Makiishi-Shimobayashi, et al., 2001, Saika, et al., 2001, Schoppet, et al., 2002, Vidal, et al., 1998, Wan, et al., 2001). Prostaglandin E2 and basic fibroblast growth factors suppress the expression of OPG

(Brandstrom, et al., 2001, Hofbauer, et al., 1999, Nakagawa, et al., 1999, Onyia, et al., 2000).

Hormones that may affect OPG levels include PTH, Vitamin D, and 17 B-estradiol. The active vitamin D metabolite $1,25(OH)_2D3$ has been shown to downregulate OPG mRNA levels in a variety of osteoblast and stromal cells (Kostenuik and Shalhoub, 2001). In contrast, $1,25(OH)_2D3$ increased OPG mRNA and protein levels in immortalized fetal osteoblasts. Due to the pro-resorptive effect of $1,25(OH)_2D3$ in coculture and organ culture models, this upregulation of OPG may represent a negative feedback signal to limit the extent of resorption (Quinn, et al., 2000). The steroid hormone 17 B-estradiol has been shown to up-regulate OPG mRNA levels (Hofbauer, et al., 1999, Saika, et al., 2001). Parathyroid hormone regulates serum calcium levels by exerting control over osteoclast activity, renal calcium reabsorption, and intestinal calcium absorption. Binding of PTH 1 receptor (PTH1R) by both PTH and PTH-related protein (PTHrP), causes a decrease in OPG production and increase in RANKL production (Quinn, et al., 2000). Several studies have shown a role for the cyclic adenosine monophosphate (cAMP) and cAMP dependent protein kinase (cAMP/PKA) pathway in the PTH-mediated downregulation (Brandstrom, et al., 1998, Halladay, et al., 2001, Kanzawa, et al., 2000). Glucocorticoids, known to promote bone resorption, suppress OPG messenger ribonucleic acid (mRNA) expression (Brandstrom, et al., 2001). OPG levels may also be regulated during the remodeling cycle. Bone resorption releases calcium, which causes increased concentrations of calcium in the osteoclast microenvironment. Treatment in MC3T3-E1 osteoblasts and ST2 stromal cells with calcium *in vitro* has been shown to increase OPG mRNA levels, suggesting that calcium

released locally during bone resorption may inhibit further bone resorption via a negative feedback mechanism (Takai, et al., 1998, Yasuda, et al., 1998).

Osteoprotegerin and RANKL function together to exert control over osteoclast activity and bone metabolism. Most receptors for growth factors, cytokines and hormones are present on osteoblasts rather than osteoclasts, regardless of whether the predominant action of these factors is bone formation or bone resorption (Kostenuik and Shalhoub, 2001). Furthermore, most factors which stimulate osteoclastogenesis do so through a pathway, which can ultimately be blocked by OPG (Tsuda, et al., 1997). In fact, RANKL induced osteoclast resorption is completely blocked by the addition of OPG *in vitro* (Burgess, et al., 1999, Lacey, et al., 1998).

Several mechanisms for OPG inhibition of RANKL have been investigated. Receptor activator of nuclear factor kappa-beta (RANKL) promotes the survival of osteoclasts by a mechanism, which may involve the inhibition of apoptosis. The mechanism of OPG action may involve the induction of osteoclast apoptosis through the antagonism of an essential RANKL induced survival signal (Akatsu, et al., 1998, Lacey, et al., 2000, Lacey, et al., 1998). Another possible mechanism for OPG inhibition of RANKL and osteoclast activity may include the disruption of the actin ring structure formed between the osteoclast and the bone surface during resorption. Osteoprotegerin may block RANKL induced cytoskeletal reorganization into actin rings (Burgess, et al., 1999, Hakeda, et al., 1998, Nakamura, et al., 1996). Additionally, the stage of osteoblast differentiation may affect expression of RANKL and OPG. Undifferentiated (pre-osteoblastic) bone marrow stromal cells express RANKL strongly, but secret low levels of OPG. These immature osteoblasts may recruit osteoclasts by increasing RANKL

expression and decreasing OPG expression (Mbalaviele, et al., 2000, Nagai and Sato, 1999). An increased osteoblastic RANKL-to-OPG ratio has also been correlated with the capacity of mature osteoblasts to support osteoclast formation and activation (Gori, et al., 2000, Nagai and Sato, 1999, Quinn, et al., 2000, Sakaguchi, et al., 2000). As osteoblasts approach senescence, they appear to produce increased amounts of OPG. Finally, analysis of both the RANKL and OPG gene promoter structures has revealed binding sites for the osteoblast-specific transcription factor cbfa-1. Cbfa-1 is essential for osteoblast differentiation, function and the generation of normal bone. Interestingly, Cbfa-1 deficient mice not only lack mature osteoblasts but lack osteoclasts as well (Ducy, et al., 2000). This may suggest that Cbfa-1 is an essential factor for the osteoblast and osteoclast lineages, respectively, and may represent a molecular link for osteoblastic regulation of osteoclast biology and bone resorption through the production of OPG and RANKL (Hofbauer and Heufelder, 2001, Thirunavukkarasu, et al., 2001).

The majority of studies measuring serum OPG have focused on relationships between circulating serum concentrations and disease states (Alvarez, et al., 1999, Coen, et al., 2002, Hegedus, et al., 2002, Sasaki, et al., 2001, Sezer, et al., 2003, Szalay, et al., 2003, Ueland, et al., 2001, Yano, et al., 1999). Other studies have focused on bone status (Fahrleitner-Pammer, et al., 2003, Rogers, et al., 2002) and age (Jung, et al., 2002, Kudlacek, et al., 2003, Szulc, et al., 2001, Yano, et al., 1999). Serum levels of RANKL have also been extensively monitored in diseased populations (Alvarez, et al., 1999, Hegedus, et al., 2002, Sezer, et al., 2003, Szalay, et al., 2003, Terpos, et al., 2003). Interestingly, both serum OPG (Sasaki, et al., 2002, Valleala, et al., 2003, Ziolkowska, et al., 2002) and RANKL (Szalay, et al., 2003) have been utilized as markers of bone cell

metabolism during pharmacological interventional studies, but not in exercise interventions. Neither serum OPG nor RANKL have been validated as bone biochemical markers, but their potential role as biomarkers has been discussed (Buckley and Fraser, 2002, Hofbauer and Schoppet, 2001).

Changes in serum RANKL during exercise have not been explored; however, mechanical strain down-regulates RANKL messenger ribonucleic acid (mRNA) and inhibits osteoclast formation *in vitro*. Rubin et al. (2002) measured changes in RANKL mRNA occurring in bone stromal cells subjected to equibiaxial strain - uniform stretching that entails both bending and compressive forces. Total RANKL messenger mRNA was measured utilizing semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RANKL mRNA was measured before the initiation of strain and after 6 and 24 hrs of strain. Six hours of applied strain caused similarly significant reductions in RANKL mRNA expression as 24 hrs of strain (approximately 40%). The fact that RANKL mRNA has been shown to respond to mechanical strain *in vitro* suggests that serum RANKL may respond to changes in bone cell metabolism during exercise interventions *in vivo*.

Changes in OPG mRNA have been monitored during the application of force in an experimental model of tooth movement in rats. Kobayashi et al. (2000) applied tensional force to the surface of maxillary bone in 28 male Wistar rats (aged 5 wk). Tensional force was applied to the bone surface through an orthodontic appliance. Osteoprotegerin mRNA levels were analyzed utilizing *in situ* hybridization. Expression of OPG mRNA in bone lining cells was enhanced during days one and two of force application. Furthermore, the mean pre-existing osteoclasts at day zero 14.4 $\pm$ 1.2 decreased to 11.2 $\pm$

0.7 on day one and 9.6 $\pm$ 0.8 on day two. Osteoclasts disappeared from the affected bone surface through apoptosis. Osteoprotegerin is known to have a negative effect on osteoclast recruitment and survival. Thus, there may be a sequential link in tensional force applied on bone lining cells, up-regulation of OPG, and the disappearance of osteoclasts (Kobayashi, et al., 2000). Since changes in OPG mRNA have been observed *in vitro* during application of tensional force, serum OPG may prove to be an excellent biomarker for the determination of changes that occur in bone cell activity with exercise interventions *in vivo*.

Osteocalcin

Osteocalcin is a 49-residue (5-8 kDa) polypeptide. In humans, the osteocalcin gene is located on chromosome 1 (1q25-q31) and is regulated at the transcriptional level by $1,25(OH)_2D3$ (Lee, et al., 2000). Osteocalcin is synthesized as an 11-kDa preproosteocalcin of 98 residues. Vitamin K or phylloquinone is an essential co-factor for the post-translational gamma-carboxylation of osteocalcin. During carboxylation, a second carboxyl group is added to specific glutamyl residues (Glu) at positions 17, 21, and 24 forming gamma-carboxyglutamyl residues (Gla). This modification leads to a conformational change, stabilizing the alpha-helical portion of the protein and conferring a greater affinity for calcium and hydroxyapatite (Hauschka and Carr, 1982). The actual function of osteocalcin is not known; however, it is thought to have a role in the organization of the extracellular matrix (Kraenzlin, et al., 1990) and the regulation of osteoblast function during the mineralization phase of bone formation (Woitge and Seibel, 2001).

The majority of osteocalcin secreted by the osteoblast is deposited in extracellular bone matrix. Serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite (Lee, et al., 2000). Thus, osteocalcin is considered a marker of bone formation (Rosenquist, et al., 1995). Garnero et al. (1994) reported that the intact osteocalcin molecule represents one-third of the circulating osteocalcin – one-third is comprised of a large n-terminal mid-molecule fragment, and another third by several other smaller fragments (Garnero, et al., 1994). Serum osteocalcin has a short half-life, approximately 5 min (Garnero, et al., 1994), and is hydrolysed mainly in the kidney (Price, et al., 1981) and, to a lesser extent, the liver (Farrugia and Melick, 1986).

Quantitative bone histomorphometry and combined calcium balance/calcium kinetics studies have validated the use of osteocalcin as a marker of bone formation (Calvo, et al., 1996, Delmas, 1995, Lee, et al., 2000, Swaminathan, 2001). Osteocalcin has been utilized extensively in both therapeutic (Fenkci, et al., 2003, Sasaki, et al., 2002, van Everdingen, et al., 2003, Zacharieva, et al., 2003) and exercise interventional studies (Bemben, et al., 2000, Lohman, et al., 1995, Rockwell, et al., 1990). Studies monitoring changes in serum osteocalcin during exercise interventions in women have produced equivocal results. In a study conducted by Lohman et al. (1995), 22 previously inactive pre-menopausal women (mean $\pm$ SD, 34.2 $\pm$ 2.6 yrs) were randomly assigned to an exercise intervention and 24 women (34.4 $\pm$ 2.7 yrs) to a control group. Osteocalcin was measured at 5,12 and 18 months. In addition to osteocalcin, TBBMD and regional BMD were measured by DXA. The exercise intervention consisted of three one-hour strength-training sessions per week for 18 months. Training consisted of three sets of 8-12 repetitions for twelve weight lifting exercises (free weights) and stressed all major muscle

groups. Additionally, absolute training load was progressively increased throughout the training period. Intact serum osteocalcin was measured by dual antibody radioimmunoassay. The intra-assay coefficient of variation (CV) was 6.8 % and the inter-assay CV was 9.5 %. Serum osteocalcin concentrations in the exercise group significantly increased (20 %, $p < 0.05$) from baseline to five months as compared to controls, and this difference between groups was sustained throughout the study. Though the change in TBBMD was not significant, there was a trend toward decreased TBBMD from a mean baseline value of 1.129 ± 0.082 g/cm^2 to 1.121 ± 0.077 g/cm^2 at 5 months, 1.116 ± 0.075 g/cm^2 at 12 months, and 1.115 ± 0.076 g/cm^2 at 18 months. Lumbar spine BMD significantly increased from a mean baseline value of 1.183 ± 0.152 g/cm^2 to 1.207 $\pm$ 0.146 g/cm^2 at 5 months (2.0% change), and 1.202 g/cm^2 at 12 months (1.6% change). Femur trochanter BMD increased significantly from a mean baseline value of 0.750 $\pm$ 0.097 g/cm^2 to 0.765 ± 0.099 g/cm^2 at 5 months (0.8% change), 0.768 ± 0.109 g/cm^2 at 12 months (2.4 % change), and 0.761 + 0.099 g/cm^2 at 18 months (1.6 % change) (Lohman, et al., 1995). This study demonstrated that regional changes in BMD occurred with concomitant changes in osteoblast activity. The study also demonstrated that regional BMD could increase without an elevation in TBBMD. This may support the hypothesis that strength training leads to a redistribution of bone mineral rather than an overall increase.

Rockwell et al. (1990) evaluated the effect of an eight-week training program on lumbar spine and femoral neck bone mass and serum osteocalcin concentrations in ten pre-menopausal women (mean $\pm$ SEM, 36.2 ± 1.3 yrs) and compared the results with seven sedentary women (40.4 ± 1.6 yrs). Weight training consisted of an eight-station

resistance-training machine circuit (Eagle-Cybex) performed twice a week for nine months. Subjects performed between one to two sets of 12 repetitions for each exercise throughout the training period. Weights were increased on an individual basis as strength improved. Strength was tested utilizing Eagle-Cybex circuit training machinery. Bone density was measured using quantitative digital radiography at baseline, four and a half, and nine months. Osteocalcin was measured using immunoradiometric assay. Individual strength increased 57 $\pm$ 0.8 % in the trained women over nine months. Femoral neck BMD did not significantly change during the study. Lumbar spine BMD decreased in the exercising women by 2.9 % at four and a half months and 4.0 % at nine months. There was no change in lumbar spine BMD in the controls. Osteocalcin concentrations were significantly higher in strength training subjects than controls (3.12 $\pm$ 0.31 ng/ml vs. 2.05 $\pm$ 0.28 ng ml^{-1}) at five months and continued to be significantly higher at nine months (2.87 $\pm$ 0.23 ng/ml vs. 2.21 $\pm$ 0.27 ng/ml) (Rockwell, et al., 1990). This study demonstrated that a significant change in osteocalcin might occur following only four and a half months of weight training in pre-menopausal women. Interestingly, the increase in serum osteocalcin concentrations was higher at four and a half months than at nine month of weight training. The bone remodeling cycle consist of approximately two to three weeks of bone resorption followed by two to three months of formation. Bone metabolism may increase more dramatically following the onset of exercise, and then subside as the exercise stimulus continues. Surprisingly, there was a net decrease in lumbar spine BMD (- 3.96 %) following nine months of weight training. One criticism of this study was that subjects were not randomly assigned to the exercise group or

sedentary control group. Subjects were allowed to choose enrollment as a weight trainer or a control.

Bamben et al. (2000) studied the musculoskeletal response to bilateral high- and low-intensity resistance training in 25 estrogen deficient post-menopausal women (mean $\pm$ SD, 51.4 $\pm$ 5.5 yrs). Subjects served as controls, or performed either a high-load [80%, one repetition maximum (1-RM), eight repetitions] or a high-repetition (40%, 1-RM, 16 repetition) training protocal, three d/wk for six months. The control group abstained from any resistance training. Subjects performed one to three sets of all exercises, which included; quadriceps extension, hamstring flexion, leg press, shoulder press, biceps curl, triceps extension, seated row, and the latissimus pull. Only one set was performed for each right and left hip exercise. Training was conducted using Cybex isotonic resistance training equipment. The weight lifted was progressively increased throughout the study based on subject performance. Muscle strength was assessed by a 1-RM test for each training lift at baseline, three months, and six months. Dual energy x-ray absorptiometry (Lunar DPX-IQ) was used to measure TBBMD and regional BMD areas including the proximal femur (femoral neck, Ward's triangle, trochanter, total hip) both pre- and post-training. An immunoradiometric assay kit (Diagnostic Systems Laboratories) was utilized to determine serum osteocalcin concentrations. Both training groups showed significant strength gain in the lower body (30%) and in hip strength (37-40%). There were no group differences in absolute change or in percent change from pre- to post-training for any BMD site. However, there was a trend for decreased TBBMD in the high-load group (-1.1% $\pm$ 0.4) after six months of training. There was a trend for serum osteocalcin concentrations to increase after the training period for all three groups.

Serum osteocalcin increased from approximately 7.0 ± 0.2 ng/ml to 9.0 ± 0.2 ng/ml in the high-load group (n = 10); 5.0 ± 3.0 ng/ml to 6.0 ± 0.1 ng/ml in the high repetition group (n = 7); and from 13 ± 5.0 ng/ml to 16.5 ± 4.0 ng/ml in the control group (n = 8). The overall percent change in osteocalcin was positively related to percent changes in the total hip (r = 0.41) and the trochanter (r = 0.42) sites (Bemben, et al., 2000). This study demonstrated that both high-load and high repetition training protocols significantly improved strength in sedentary post-menopausal women. Serum osteocalcin concentrations increased in both trained and untrained subject groups. Day-to-day intra-subject fluctuations and/or biological factors may have contributed to observed changes in serum osteocalcin in untrained individuals. Bone mineral density results of this study may have been limited by a short training duration of six months.

The short-term effects of exercise on osteocalcin concentrations have also been studied in young women. In a study conducted by Rudberg et al. (2000), seven young women near the age of peak bone mass (mean ± SD, 23 ± 2 yrs) jogged at a moderate tempo for 40 minutes. Serum was drawn from the arm both before exercise, immediately after, and 20 minutes following the cessation of exercise. Hemoconcentration, which can occur during exercise and cause reduced plasma volume, was measured and osteocalcin measurements were adjusted accordingly. Serum osteocalcin was measured using a radioimmunoassay with double antibody technique (INCSTAR, Stillwater, MN). Serum osteocalcin concentration from pre- to post-exercise did not change (Rudberg, et al., 2000).

Brahm et al. (1997) studied osteocalcin changes in ten women (mean ± SD, age 29 ± 11 yrs) who performed a standardized running exercise test on a motor-driven treadmill

with loads corresponding to 47 and 76 % of VO_2 max, followed by a maximal effort until exhaustion. Venous samples were drawn at rest, after each of three loads, and after 30 min and 24 hrs of recovery. Serum osteocalcin was measured by radioimmunoassay (CIS Bio International, Gif-Sur-Yvette Cedex, France) and had intra- and inter-assay coefficients of variation (CVs) of 2.7 and 5.5 %, respectively. Osteocalcin decreased from a baseline value of 12.5 $\pm$ 3.4 ng/ml to 12.3 $\pm$ 3.3 ng/ml (15.4%) at 30 min of recovery, but returned to baseline levels following 24 hrs of recovery (Brahm, et al., 1997). This study demonstrated that serum concentrations of osteocalcin might fluctuate immediately following exercise; however, concentrations return to baseline following 24 hrs of recovery. Though findings on acute changes in serum osteocalcin are equivocal, recent exercise should be controlled for at the time of serum biomarker sample collection in order to avoid erroneous conclusions caused by acute fluctuations.

The measurement of serum concentrations of osteocalcin may be affected by several analytical factors. Osteocalcin is sensitive to *in vitro* degradation, freeze-thaw cycles, and haemolysis. Garnero et al. (1994) have reported a 17 % *in vitro* degradation of osteocalcin over 2 hrs of incubation at room temperature (Garnero, et al., 1994). The C-terminal fragment of osteocalcin is easily cleaved leaving the stable N-terminal mid-fragment. Assays that measure both intact and the N-terminal mid fragment of osteocalcin have been shown to be less susceptible to changes during storage (Blumsohn, et al., 1995, Swaminathan, 2001) and appear to be more stable and reproducible (Delmas, et al., 2000). Swaminathan et al. (2001) have recommended that serum osteocalcin samples only be thawed once in order to prevent degradation, concomitant generation of fragments, and corresponding erroneous results. Finally, haemolysis may decrease intact

osteocalcin possibly owing to proteolysis by enzymes released from lysed red blood cells (Lee, et al., 2000).

Several biological factors may affect serum concentrations of osteocalcin. Osteocalcin has a diurnal rhythm. Concentrations of osteocalcin tend to be highest from 0200 to 0400 hrs and nadir from 1200-1600 hrs (Eastell, et al., 1992, Garnero and Delmas, 1998, Markowitz, et al., 1987). The amplitude of the rhythm is 5-20 % of the 24 hour mean concentration (Hannon and Eastell, 2000). Thus, serum samples must be collected within a consistent and well-defined time frame. The effect of the menstrual cycle on osteocalcin is controversial. Several studies have shown small but significant changes in osteocalcin concentrations during the menstrual cycle (Gorai, et al., 1995, Martin, 2000, Nielsen, et al., 1990, Watts, 1999, Woitge, et al., 1999, Zittermann, et al., 2000). Other studies have not demonstrated changes in osteocalcin concentrations during the luteal phase and follicular phases of menstrual cycle (Chiu, et al., 2000, Chiu, et al., 1999, Schlemmer, et al., 1993, Tarallo, et al., 1990). Delmas and colleages (2000) have concluded that menstrual cycle effects on markers of bone turnover are small and may be regarded as insignificant (Delmas, et al., 2000).

There may be seasonal changes in osteocalcin. Douglas et al. (1996), who measured intact osteocalcin with a radioimmunoassay, found osteocalcin concentrations to be significantly higher in the spring than in the autumn (Douglas, et al., 1996). Thomsen et al. (1989), who also utilized an intact osteocalcin radioimmunoassay, found a significant seasonal variation of 23 % around the yearly mean, with the zenith in February and nadir in July (Thomsen, et al., 1989). Woitge et al. (1998), utilizing a two-site immunoradiometric assay, found osteocalcin concentrations in winter (October through

April) to be significantly higher (2%) than in summer (May through September) (Woitge, et al., 1998). Seasonal variations in levels of bone markers of bone turnover may be explained by vitamin D deficiency in winter and may be less marked at low latitudes (Hannon and Eastell, 2000).

Research on the effects of oral contraceptives on osteocalcin has also produced conflicting results. Karlsson et al. (1992) reported a 50 % decrease in osteocalcin after two-three months of contraceptive use, whereas Tarallo et al. (1990) found osteocalcin concentrations to be unaffected by oral contraceptives (Karlsson, et al., 1992, Tarallo, et al., 1990). In a cross-sectional study of older women (age range 35-49 yrs) there were significant decreases in serum osteocalcin concentrations in older women taking oral contraceptives, suggesting the effect of oral contraceptives may be age-dependent (Hannon and Eastell, 2000). The effect of oral contraceptives is small and therefore of little significance in interpreting concentrations of markers on bone turnover, particularly in younger women.

Serum osteocalcin concentrations have been shown to be vulnerable to within-subject biological variability. In a cohort of 259 healthy post-menopausal women (age range 51-89 yrs) who had four sequential serum osteocalcin measurements over three yrs, the within-subject coefficient of variation (CV) was 12 % for serum osteocalcin (Garnero and Delmas, 1999). Delmas et al. (2000) reduced the long-term precision error of serum osteocalcin, measured seven times over 18 months, from 28 % to 12 % by using an assay that measured both the intact and N-mid fragments of osteocalcin instead of using just the intact molecule (Delmas, et al., 2000).

Sources of analytical and biological variability contribute to uncertainty about changes in osteocalcin that occur during interventional studies. To make a determination that a treatment has a real biological effect, the change induced by the treatments must be greater than the change that might occur by chance alone. Regardless, patterns of change in serum biomarker concentrations can be used as an indication of change in bone cell activity during interventional studies.

N telopeptide

During the resorption phase of remodeling, osteoclasts acidify and dissolve the inorganic phase of bone and subsequently degrade the organic component of bone with either activated collagenase or cathepsins. The majority of the organic component of the bone matrix is type-I collagen. Type-I collagen has two cross-link forming sites, one in the amino-terminal and the other in the carboxyterminal region of the molecule. During the process of protease degradation, amino- and carboxy-terminal fragments of collagen are released with cross-links attached. These fragments are called telopeptides. Antibodies have been raised against both telopeptides, and immunoassays have been created to measure both the C-terminal telopeptide (CTx) and the N-terminal telopeptide (NTx). These assays have been shown to be the preferred markers to assess bone resorption (Watts, 1999)

Serum NTx has been verified as a indicator of collagen type-I degradation and bone resorption (Clemens, et al., 1997); although, there is some debate as to its specificity to bone (Robins, 1994). Clemens et al. (1997) demonstrated that serum NTx originates directly during to proteolytic cleavage of bone collagen by osteoclast rather than by degradative processes occurring in the liver and kidney (Clemens, et al., 1997). Both

urinary (Chesnut, et al., 1997, Greenspan, et al., 2002) and serum (Chesnut, et al., 1997, Garnero, et al., 1994, Gertz, et al., 1998, Greenspan, et al., 1998, Prestwood, et al., 1999, Rubin, et al., 2003, Valleala, et al., 2003) NTx have been utilized as resorption biomarkers in therapeutic interventions. Urinary NTx has been used as a marker of resorption during long-term exercise interventions (Eliakim, et al., 1997, Ryan, et al., 1998).

Eliakim et al. (1997) monitored urinary NTx changes in 38 healthy males (mean $\pm$ SD, 16 $\pm$ 0.7 yrs). Twenty males were randomly assigned to an intervention group and 18 to a control group. The intervention group underwent a five week training program (2 hrs/d, 5 d/wk) of endurance exercise consisting of running, aerobic dance, competitive sports (e.g. basketball), and occasional weight lifting. Urinary NTx decreased significantly from a baseline mean value of 323.3 $\pm$ 58.5 nM/mM creatinine (Cr) to 267.8 $\pm$ 53.9 nM/mM Cr (- 21 $\pm$ 3%,) in the trained group at 5 wks. Urinary NTx change was not significant in controls who had a mean baseline value of 203.9 $\pm$ 45.0 nM/mM Cr that decreased to 182.5 $\pm$ 36.7 nM/mM Cr (– 6 $\pm$ 4 %) at 5 wks (Eliakim, et al., 1997). An enzyme-linked immunosorbent assay (ELISA) [Osteomark kit (Ostex International) was utilized to measure urinary NTx concentrations. The intra-assay CV for the assay was 5-8%, and the inter-assay CV was 3-5 %. In this study, a relatively brief training intervention, lead to a robust response in circulating NTx in adolescent males. Elevated rates of bone remodeling occur during puberty and may have contributed to the magnitude of NTx depression observed in this study.

Urinary NTx was also utilized to monitor changes in bone metabolism in post-menopausal women undergoing a total body resistance-training program. Twenty-seven

healthy post-menopausal women (mean ± SD, age 62 ± 7 yrs) participated in a strength-training program three times/wk for 16 wks. Though both upper and lower body strength significantly increased by 36-65% and 32-98%, respectively after training, urinary NTx did not change significantly (Ryan, et al., 1998). Though serum NTx has not been utilized as a marker of resorption during exercise intervention, it has been utilized extensively as a biomarker of bone turnover in diseased populations. (Akin, et al., 2003, Jakob, et al., 2002, Mikosch, et al., 2003, Montagnani, et al., 2003, Scariano, et al., 2002). Serum NTx may offer more long-term clinical precision than urinary NTx and may be a more precise indicator of overall skeletal metabolism (Chandani, et al., 2000). Thus, serum NTx has excellent potential for use a marker of bone resorption during exercise interventions.

Serum and urinary NTx concentrations might be affected by several biological factors. Gertz et al. (1998) found a significant diurnal variation of serum NTx in 13 elderly osteopenic women (mean ± SD, age 69 ± 3 yrs). Mean peak levels occurred at 0400 hrs and a mean nadir occurred at 1300 hrs (Gertz, et al., 1998). The effects of the menstrual cycle on NTx have also been explored. Gorai et al. (1995) found a significant decrease in urinary NTx during the luteal phase of the menstrual cycle (Gorai, et al., 1995). Menstrual cycle fluctuations have not been assessed for serum NTx. In one study, urinary NTx levels where found to be 28 % lower in women using oral contraceptives compared to controls (Garnero, et al., 1995). However, the effect of oral contraceptives on serum NTx has not yet been elucidated. N-telopeptides may also undergo seasonal variation. Woitge et al. (2000) studied urinary NTx levels in 15 pre-menopausal women (mean ± SD, 25 ± 10 yrs) over an 18-month period. Urine samples

were obtained every 4 wks. NTx showed a difference in amplitude of 15.4 ± 15.8 nM/mM Cr over the 18-month period and was highest in November. Seasonal variation of urinary NTx did not reach statistical significance; however, due to high inter-assay variability (Woitge, et al., 2000).

Serum NTx intra-subject variability has also been considered. Eastell et al. (2000) studied short- and long-term intra-subject variability in 277 post-menopausal women, (mean + SD) aged 63.6 ± 10.2 yrs. Short-term intra-subject variability (over three days) for serum NTx was 6.3 %. Long-term biological variability (over three months) was 7.5 % (Eastell, et al., 2000). Scariano et al. (2001) collected seven blood samples over four to six months from 12 pre-menopausal women (age range 25-52 yrs). The intra-subject CV was (mean + SD) 10.7 ± 4.0 %. Thus, intra-subject variation may contribute to changes in serum measurements of NTx over the course of short and long-term interventional studies. In addition to analytical and biological variation, and intra-subject variability, biomarker assays have intra- and inter-assay variation. Technique may differ between duplicated measures and values quantified by different assay kits may have inherent variability. Thus, CVs must be calculated in order to assess the accuracy of serum measurements.

Summary

The adult skeleton is a dynamic organ that undergoes a constant process of remodeling. One function of bone remodeling is to provide a mechanism for the skeleton to adapt to its mechanical environment. Wolf's law states that bone accommodates to loads imposed on it by altering its mass and distribution of mass (Hortobagyi and Katch,

1990). Therefore, bone strain arising from mechanical loading can stimulate the remodeling process, which results in bone better suited to withstand applied loads (Friedlander, et al., 1995). Remodeling commences 5 days after stimulation (the initiation of altered strain pattern or magnitude) and consists of a two to three week period of osteoclastic tunneling (resorption) followed by two to three months of osteoblastic formation (Rosen, 2003).

Mechanical loading during physical activity results in internal forces (stress) and deformation (strain) (Bennell, et al., 1999). These stresses and strains are thought to provide the stimulus for the structural adaptations of bone (Carter, 1984, Turner, 1998, Turner, et al., 1994). Experiments and computer models have supported the idea that dynamic loading is associated with increased bone remodeling, whereas continuous loading is not (Basso and Heersche, 2002). Several characteristics of dynamic strain have been identified as essential contributors to the magnitude of bone adaptation. Peak strain magnitude, strain rate, and unusual strain distribution are thought to be particularly osteogenic. In order to adapt to mechanical loading, bone tissue must sense changes in the local mechanical environment and signal bone cells to modify their activity. Mechanotransduction involves the transmission of load induced biophysical signals to a cell, and ultimately throughout the cellular network, eventually effecting bone remodeling (Donahue, 1998). The specific mechanical signals detected by bone cells, and the ways in which the signals are converted into cellular activity that results in bone remodeling are still under investigation. However, the majority of researchers hypothesize that osteocytes are involved in mechanotransduction. Osteocytes are osteoblasts, which have been trapped in the bone matrix after matrix formation and

mineralization. Osteocytes have numerous long processes rich in microfilament, which are in contact with cell processes on other osteocytes, and with processes from inactive cells lining the bone surface, which also originate from senescent osteoblasts. Thus, osteoblast, osteocytes and lining cells form a network, which is well–equipped for signal tranduction (Masi and Brandi, 2001). It is possible that increased strain in the local bone matrix signals osteocytes to transmit stimuli to the surface, where bone is formed until the strains are normalized (Huiskes, et al., 2000).

The ultimate mechanical strain magnitude, rate, directional movement, and duration needed to mediate changes in bone cell metabolism and thus lead to favorable modifications of architecture and mass has remained elusive. However, isokinetic muscular strength training has excellent osteogenic potential. Isokinetics refers to a muscular action performed at constant angular limb velocity. Isokinetic training makes it theoretically possible for a person's muscle to exert a continual maximal concentric or eccentric muscular contraction throughout a movement's full range of motion. Isokinetic muscular training imposes loads on bone that are dynamic, large in strain magnitude, and irregularly distributed. Both eccentric and concentric isokinetic muscle training have been shown to increase muscular strength. However, eccentric muscle training may provide superior strength gains and greater osteogenic potential. Long-term isokinetic muscular training has the potential to cause alterations in the rate of bone turnover that result in increased bone formation.

In order to assess the efficacy of long-term exercise training interventions, changes in bone tissue and bone cell activity must be estimated. Biochemical markers of bone turnover provide minimally invasive measures, which reflect the cellular events in bone.

Biomarker assays rely on the measurement, in serum or urine, of matrix protein enzymes synthesized by osteoblasts or osteoclasts that spill over into the body fluids, or on osteoclast-generated degradation of products of the bone matrix itself. One of the principle advantages of the biomarkers is that, they can readily detect acute changes in bone cell activity. Serum biomarker concentrations have only a remote relationship with local metabolically active bone tissue. Thus, blood concentrations of biomarkers can only allow inference of events occurring at the bone tissue level.

Receptor activator for nuclear factor kappa-beta Ligand (RANKL), receptor activator of nuclear factor kappa-beta (RANK) and osteoprotegerin (OPG) comprise a cytokine network, which acts as a key controller of bone cell metabolism. Receptor activator for nuclear factor kappa-beta activation by RANKL stimulates osteoclast differentiation, activation, survival, and adherence to bone surface. Osteoprotegerin acts as a soluble decoy receptor for RANKL. Thus, OPG inhibits osteoclast differentiation and activity. Serum assays for OPG and RANKL have recently been developed. Serum OPG and RANKL levels have been monitored in both healthy and diseased individuals and have been utilized as indices of change in bone cell metabolism during therapeutic interventions. Their potential use as biomarkers of bone during exercise interventional studies remains to be elucidated. Neither serum OPG nor RANKL have been validated as biochemical markers of bone turnover; however, their potential as markers has been discussed (Buckley and Fraser, 2002, Hofbauer and Schoppet, 2001, Jung, et al., 2002).

Serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite crystals during the formation phase of remodeling. Thus, osteocalcin is considered a marker of bone formation. Bone histomorphometry and combined calcium

balance/calcium kinetics studies have validated the use of osteocalcin as a marker of bone formation. Osteocalcin has been utilized extensively as a biomarker of bone turnover in both therapeutic and exercise intervention studies. During the resorption phase of remodeling, osteoclasts acidify and dissolve the inorganic phase of bone and subsequently degrade the organic component of bone. Protease degradation releases amino- and carboxy-terminal fragments of collagen with cross links attached. These fragments are called telopeptides. The N-terminal telopeptide (NTx) has been verified as an indicator of bone resorption (Clemens, et al., 1997). Both urinary and serum NTx have been utilized as resorption biomarkers in therapeutic interventions. Conversely, only urinary NTx has been used as a marker of resorption during long-term exercise interventions (Eliakim, et al., 1997, Ryan, et al., 1998). Serum NTx has excellent potential for estimating changes in bone resorption that occur during exercise interventional studies. The evaluation of serum levels of OPG and RANKL, with concomitant evaluation of markers of bone remodeling, osteocalcin and NTx, may provide a new and more complete perspective of regulatory and metabolic changes that occur in bone cells as result of mechanical loading during high load eccentric and concentric muscular strength training.

CHAPTER III – JOURNAL MANUSCRIPT

Muscular Strength Training Modifies Regulation of Bone Remodeling: Inferences From Serum Biomarkers in Young Women

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ABSTRACT

Biochemical markers of bone turnover allow inference of the events occurring at the bone tissue level and may detect changes in bone cell activity earlier than densitometric technologies. Serum concentrations of receptor activator for nuclear factor kappa-beta ligand (RANKL), osteoprotegerin (OPG), osteocalcin, and N-telopeptide (NTx) were measured in women aged 20 $\pm$ 1.5 years (mean $\pm$ SD) who underwent 32 weeks of unilateral isokinetic concentric or eccentric muscular strength training. Changes in serum biomarkers were compared with changes in arm and leg flexor and extensor muscle strength. Dual X-ray absorptiometry (DXA) measures of bone mineral density (BMD) and bone mineral content (BMC) of the total forearm, total tibia, and total body also were assessed. The mean serum OPG concentration increased from 4.6 $\pm$ 1.9 pmol/L to 5.2 $\pm$ 2.1 pmol/L (↑14.9 %, mean $\pm$ SD; $p = 0.05$, $n = 20$) following long-term isokinetic exercise training that also increased elbow extensor and knee flexor muscular strength ($p \leq 0.05$) and total forearm BMD ($p = 0.04$). The ratio of OPG/RANKL also increased over the course of the study ($p = 0.045$). Serum concentrations of other measured bone biomarkers did not change during training. Serum concentrations of OPG, a suppressor of osteoclastogenesis, increased with high-load muscular strength training that led to local increases in muscle strength and BMD. These adaptations may represent an exercise-mediated suppression of osteoclast differentiation and activity. The central role of the RANKL-OPG cytokine system in the regulation of bone cell biology is well established. Further research is needed to confirm the efficacy of using serum OPG and RANKL as biomarkers of bone cell metabolism in healthy populations undergoing long-term exercise interventions.

Keywords: Receptor Activator for Nuclear Factor Kappa-Beta Ligand (RANKL), Osteoprotegerin, Osteocalcin, Serum N-telopeptide (NTx), Unilateral Isokinetic Eccentric and Concentric Muscular Strength Training

Introduction

Isokinetic training imposes loads on bone that are dynamic, large in strain magnitude, and irregularly distributed. Both eccentric and concentric isokinetic muscle training have been shown to increase muscular strength. [11, 20, 25, 32, 34] In several studies, eccentric muscle training has demonstrated superior strength gains [31, 32, 49] and in one study, greater osteogenic potential. [25] In order to assess the efficacy of exercise training-modalities, changes in bone tissue and bone cell activity must be estimated. Utilization of multiple biomarkers of bone turnover is one methodology for the assessment of changes in bone cell metabolism. One of the principle advantages of biomarkers is that they can readily detect acute changes in bone cell activity. [43]

Receptor activator for nuclear factor kappa-beta (RANK), it's activation ligand (RANKL) and osteoprotegerin (OPG) comprise a cytokine network which regulates osteoclast metabolism.[29] Receptor activator for nuclear factor kappa-beta (RANK) activation by RANKL stimulates osteoclast differentiation, [45, 52, 66] osteoclast activation, [6, 17, 38] survival, [42] and adherence to bone surface. [50] Osteoprotegerin acts as a decoy receptor for RANKL. Thus, OPG inhibits the differentiation, survival, and fusion of osteoclast precursors and the activation and survival of osteoclasts.[28, 30, 39, 64, 73] The majority of studies measuring serum OPG have focused on relationships between circulating serum concentrations and bone status, [15, 55] age, [35, 41, 69, 79] and disease states. [2, 10, 26, 62, 65, 68, 74, 79] Serum RANKL has been extensively monitored in diseased populations. [2, 26, 65, 68, 70] Interestingly, serum OPG [61, 76, 82] and RANKL [68] have been utilized as markers of bone cell metabolism during pharmacological interventional studies but not in exercise interventions. Neither serum OPG nor RANKL have been

validated as bone biochemical markers but their potential roles as diagnostic bone biomarkers for disease states have been discussed. [5, 29, 35] For these reasons, we were interested in examining changes that occur in serum OPG and RANKL during isokinetic muscular strength training.

Osteocalcin is a polypeptide produced by mature osteoblasts. The majority of osteocalcin secreted by the osteoblast is deposited in extracellular bone matrix. Serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite. [43] Thus, osteocalcin is considered a marker of bone formation. [57] The actual function of osteocalcin is not known; however, it is thought to have a role in the organization of the extracellular matrix [40] and the regulation of osteoblast function during the mineralization phase of bone formation.[78] Quantitative bone histomorphometry and combined calcium balance/calcium kinetics studies have validated the use of osteocalcin as a marker of bone formation. [7, 12, 43, 67] Osteocalcin has been utilized extensively as a marker of bone formation in both therapeutic [16, 61, 77, 81] and exercise intervention studies. [4, 44, 54]

During the resorption phase of remodeling, osteoclasts acidify and degrade the organic component of bone with either activated collagenase or cathepsins. Cross linked N-terminal telopeptides (NTx) are one product of protease degradation.[3, 9] Serum NTx has also been extensively utilized as a biomarker of bone turnover in diseased populations. [1, 33, 46, 47, 63] Both urinary [8, 22] and serum [8, 18, 19, 23, 51, 58, 75] NTx have been utilized as resorption biomarkers in therapeutic interventions and during long-term exercise interventions. [14, 59]

The aim of this study was to evaluate changes that occurred in the osteoclast regulatory cytokines, OPG and RANKL, in response to 32 weeks of muscular strength training in young women that involved either intense eccentric or concentric isokinetic loading. A secondary objective was to examine the cytokine response in the context of concomitant changes in blood markers of bone remodeling, osteocalcin and NTx.

Materials and Methods

Study Sample

Twenty women (mean $\pm$ SD) aged 20 $\pm$ 1.5 yrs were chosen from 102 subjects who participated in the Trial in Bone Injury Abatement for Ladies (TiBIAL) study conducted at Virginia Polytechnic Institute and State University from the years 2000 through 2002. Subjects were recruited from the campus and the surrounding community of Blacksburg, Virginia. All subjects had a body mass index (BMI) $\geq$ 18 and $\leq$ 25 and had not participated in any structured exercise program or athletic training during the 12 months prior to participation in the study. Subjects did not evidence any of the following exclusion criteria: 1) previous injuries such as fractures or surgeries to the dominant limb; 2) inadequate daily calcium consumption of < 1200 mg/day; 3) any bone or metabolic disorder; 4) any use of medications that may change normal bone metabolism; and 5) menstrual irregularities. Subjects were randomly assigned to 32 wks of unilateral isokinetic eccentric or concentric training of their non-dominant limbs using a Biodex® System 3 dynamometer. The subject subset was selected from among

all subjects in the larger study sample who met the following criteria: completion of 32 wks of either eccentric (n = 10) or concentric (n = 10) unilateral isokinetic training; training attendance > 71 %; serum samples for weeks 0 and 32, and intra-subject coefficients of variation (CVs) < 20% for all assayed biomarkers.

Isokinetic Muscular Strength Training

The unilateral isokinetic resistance training consisted of 32 wks of non-dominant limb elbow flexion/extension and knee extension/flexion resistance training on a Biodex® System 3 dynamometer. Initially, subjects completed two sets of six repetitions for knee extension/flexion and elbow flexion/extension at an angular velocity of 60°/sec with 2 min of recovery between sets. An additional set of six repetitions per exercise was added weekly until subjects were performing five sets of six repetitions for each exercise, resulting in 30 total repetitions/exercise/training per session. Thereafter, the training volume was held constant. Three training session were conducted each week on non-consecutive days. Technicians encouraged subjects to perform to the maximum of their ability.

Muscular Strength Testing

Measurements of peak concentric and eccentric torque in flexion and extension for both the exercise-trained and untrained limbs were obtained using the Biodex® System 3 dynamometer at 0 and 32 wks. These tests were performed at a controlled angular velocity of 60°/sec for the knee and the elbow and included concentric extension and flexion, as well as eccentric extension and flexion. Prior to testing days, subjects

completed three preliminary sub-maximal (~50% of perceived maximal effort) strength-training sessions with the Biodex® system to familiarize them with the equipment and the performance expectations for the power tests. Strength testing was administered during two sessions separated by $\geq$ 3 days. Subjects were tested in the concentric mode on the first day of strength testing, followed by the eccentric mode on the second day of testing. The isokinetic strength testing protocol began with a 3-min warm-up of low intensity stationary leg cycling on a Monark® cycle ergometer followed by 3-to-5 min of static stretching exercises. Six practice trials were administered, followed by the maximal strength tests. For knee extension/flexion testing, the subjects were positioned on the Biodex® system with the dynamometer axis of rotation aligned with the knee and with 85° hip-flexion. The testing began with the knee at ~90° flexion, with the initial movement being knee extension to ~0°, and the second movement being knee flexion to ~90° to complete the first repetition. The highest values obtained from any of the trials for peak torque were recorded. For the elbow flexion/extension tests, subjects were positioned with the dynamometer axis of rotation aligned with the elbow and with 40° shoulder-abduction. The elbow extension/flexion testing was followed by the procedures described above for the knee.

Dual Energy X-ray Absorptiometry

A QDR 4500 A Elite Fan Beam X-Ray Bone Densitometer (DXA) (Hologic, Bedford, MA) was utilized for total body (TB) bone mineral density (BMD), TB bone mineral content (BMC), and total forearm and total tibial regional BMD and BMC measures.

Subjects lay supine on the DXA table for one TB scan to determine TB BMD and TB BMC. For the current study, each subject had the non-dominant (trained) forearm and lower legs scanned to determine BMD and BMC of the radius and ulna and proximal tibia and tibial shaft, respectively. Standard TB and forearm protocols were used during scans. A modified forearm protocol was used for measurement of the tibia. All scans were conducted on the campus of Virginia Polytechnic Institute and State University and were analyzed by the same technician to eliminate inter-tester variation. Quality control for BMD and BMC were ensured by daily scans of an anthropomorphic phantom lumbar spine prior to any subject testing. Reliability testing with DXA in the BONE laboratory has resulted in CVs of 0.73% and 1.09% for TB and total forearm BMD, respectively.

Biomarker Assays

Blood samples were collected after a 48-hr abstention from exercise and a 12-hr abstention from any food or liquid intake other than water. Blood samples were collected in a serum vaccutainer, allowed to stand at room temperature for 30 min, and the serum was separated by then centrifuge for 12 min at 2500 rpm. The serum was then pippetted into vials and stored at -80°C until assayed. For the current study, serum samples obtained at 0 and 32 wks were analyzed for OPG, RANKL, osteocalcin and NTx. Additionally, serum alkaline phosphatase and calcium were measured to allow evaluation of markers relevant to bone metabolism and to rule-out relevant bone and

systemic pathology. In order to limit inter-assay variability within subjects, the same assay kit was utilized to measure subject samples from 0 and 32 wks.

Serum concentrations of soluble, uncomplexed human RANKL were measured by enzyme immunoassay (EIA) [sRANKL EIA (Biomedica), Windham, NH] and were quantified by a standard ELISA reader at 450 nm. The intra-assay CV was 7.8%. Serum concentrations of OPG were also measured by EIA [Osteoprotegerin (Biomedica), Windham, NH] and quantified at 450 nm. The intra-assay CV for serum OPG was 5.3%. Both intact osteocalcin and N-terminal mid-molecule fragments of osteocalcin were measured by EIA [Mid-Tact Human Osteocalcin EIA Kit (Biomedical Technologies Inc.), Stoughton, MA] and read at 450 nm. Osteocalcin assays had an intra-assay CV of 6.6 % and an inter-assay CV of 22.3 %. Serum NTx was measured by a competitive enzyme-linked immunosorbent assay [Osteomark NTx Serum (Osteomark), Seattle, WA] and read at 450 nm. Assay values were reported in nanomoles Bone Collagen Equivalents per liter (nM BCE/L). The NTx intra-assay CV was 9.6 % and the inter-assay CV was 19.6%.

Serum alkaline phosphatase was measured by a modified colorimetric assay [Stanbio Alkaline Phosphatase LiquiColor Procedure No. 2900 (Stanbio), Boerne, TX]. In the assay, alkaline phosphatase hydrolyzed 4-nitrophenol phosphate to form 4-nitrophenol and phosphates. The assay was then modified by the addition of sodium hydroxide 0.25 moles (M), which ended the reaction. The amount of 4-nitrophenol formed was directly proportional to the activity of alkaline phosphatase, which was detected by a spectrophotometer at an absorbance reading of 405 nm. The intra-assay CV was 3.5 %. Serum calcium was measured by a quantitative colorimetric assay

[Stanbio Total Calcium LiquiColor (Arsenazo III) Procedure No. 0155 (Stanbio), Boerne, TX]. In this assay, Arsenazo III formed a violet Arsenazo III: calcium complex, which was detected by a spectrophotometer at an absorbance reading of 650 nm. The intra-assay CV for this assay was 4.3 %.

Statistical Analysis

Prior to performing statistical analyses, composite variables were created from peak torque strength measurements. Variables created included: knee extensor; knee flexor; elbow extensor; elbow flexor; a sum of elbow extensor and flexor measurements (total arm), and a sum of knee extensor and flexor variables (total leg). Repeated measures analysis of variance (ANOVA), with training group as the between subjects factor, and paired sample *t*-tests were utilized to compare baseline measurements with post-training values. Correlation matrixes were created to determine if relationships existed between percentage change in biomarkers, bone densitometry measures, and composite measures of peak torque strength. Finally, multiple regression analyses were conducted to ascertain if changes in muscular strength and bone densitometry variables were systematically related to changes in bone biomarkers. All analyses were performed using SPSS.

Results

Study Sample

The means and SD for baseline age, weight, height, BMI, TB and regional BMD and BMC, and peak torque muscle strengths for eccentric and concentric trained subjects are provided in **Table 1** and **Table 2**. The eccentric trained group had higher baseline knee extensor strength ($p = 0.04$) than the concentric group. There were no other differences in baseline parameters between groups.

Strength Testing

Peak torque measurements quantified the training effects of the study and provided an estimate of peak power for muscle groups that operate across the knee and elbow joints, within both the concentric and eccentric training modes. Strength changes in the muscle groups that performed the high load unilateral muscular strength training were compared to strength changes that occurred in untrained muscle groups. There were no significant differences between eccentric and concentric training groups for percentage change in any peak muscular strength measure (**Table 4**). In the combined isokinetic training group ($n = 20$), elbow extensor muscular strength substantially increased 13.8 % ($p = 0.004$) and knee flexor strength increased 22.3 % ($p = 0.009$), while no changes occurred in the untrained limbs (**Table 4**). Elbow flexor, knee extensor, total leg and total arm peak muscular strength substantially increased in both the trained and untrained limbs (**Table 4**). There was a trend for a higher percentage change in total arm strength ($p = 0.64$) for the trained limb in the eccentric trained group than in concentric trained individuals, 37.4 % vs. 19.0 %, respectively (**Table 4**).

Bone Densitometry

Table 4 depicts percentage changes that occurred in TB and regional BMD and BMC in concentric (n = 10), eccentric (n = 10), and isokinetic (n = 20) training groups over the course of the study. Isokinetic training increased total forearm BMD 0.8 % ($p = 0.04$, $n = 20$). Total forearm BMC and total tibial BMD increased in both the trained and untrained limbs. There were no differences in BMD or BMC change between eccentric and concentric trained groups. However, there was a trend ($p = 0.07$) for the concentric trained group to have higher gains in forearm BMD than eccentric trained individuals, 1.5 % vs. 0.2%, respectively. Total body BMC and BMC did not change in any training group.

Bone-related Serum Parameters

Osteoprotegerin and RANKL. As depicted in **Figure 1**, the mean serum OPG concentration significantly increased (14.9 %) from 4.6 $\pm$ 1.9 pmol/L to 5.2 $\pm$ 2.1 pmol/L ($p < 0.05$, $n = 20$), in the collective isokinetic trained group. There was no change in serum RANKL concentration ($p = 0.41$, $n = 20$). Alterations in serum levels of OPG and RANKL were not different between training groups (**Table 3**). The ratio of OPG/RANKL significantly increased, from 13:1 to 20:1, over the course of the study ($p = 0.04$, $n = 20$).

Osteocalcin and Serum NTx. As illustrated in **Figure 2**, serum osteocalcin and serum NTx did not change with long-term high load unilateral isokinetic exercise. There were no substantial differences between eccentric and concentric training groups (**Table 3**).

Changes in Biomarkers and Changes in other Relevant Variables. In the collective isokinetic trained group, percentage change in biomarkers of bone metabolism where not related to one another, nor to percentage changes in BMD, BMC, or strength. In the eccentric trained group, the percentage change in serum OPG concentration was negatively correlated with the percentage change in RANKL concentration ($r = -0.68$, $p = 0.03$, $n = 10$). Additionally, the ratio of OPG/RANKL was positively correlated with change in knee extension strength in the trained limb($r = 0.69$, $p = 0.03$, $n = 10$) (**Figure 3**). Linear regression revealed that change in the ratio of OPG/RANKL explained 47 % of the change in knee extensor strength.

DISCUSSION

In the current study, both unilateral concentric and eccentric isokinetic training significantly increased the strength of muscle groups that operate across the knee and elbow joints. Elbow extensor and knee flexor strength substantially increased in the trained limbs, but not in the untrained limbs. Interestingly, elbow flexor, knee extensor, and total arm and leg strength increased in both trained and untrained limbs. The muscular strength changes that occurred in untrained extremities may have been indicative of a cross training effect of high load muscular strength training. Though there were no substantial differences between eccentric and concentric training groups, there was a trend for a superior elbow extensors/flexor (total arm) strength gain in the eccentrically trained group than the concentrically trained group. Eccentric muscular contraction has been shown to generate maximal forces 25-30 % higher than those

produced by concentric muscular contraction [71]. Differential strength changes in eccentric vs. concentric strength training modalities have been previously examined. Higbie et al. [27] studied the effects of 10 wks of isokinetic concentric and eccentric training on quadriceps muscle strength in 44 women (mean $\pm$ SD), age 20.0 $\pm$ 0.5 yrs who were randomly assigned to either: a concentric training group (n = 16), an eccentric training group (n = 9), or a control group (n = 19). The average torque was measured during concentric and eccentric maximal voluntary knee extension at 60^{o}/sec using a Kin-Com dynamometer. Average concentric and eccentric torque increased 18.4 and 12.8 % for the concentrically trained group, 6.8 and 36.2 % for the eccentrically trained group, and 4.7 and –1.7 % for the control group, respectively. The increase in average torque for the concentrically trained group was greater when measured with concentric average torque testing than with eccentric testing. The increase in the eccentric average torque was greater in the eccentrically trained group than the increase in the concentric average torque by the concentrically trained group [27]. The authors concluded that the gains in strength consequent to concentric and eccentric training were highly dependent on the muscle action used for training and testing.

In the current study, isokinetic training resulted a small but significant increase in total forearm BMD without concomitant changes in TB BMD. This study supports the contention that isokinetic resistance training causes a localized rather than systemic effect on BMD. Total forearm BMC and total tibia BMD increased in both the trained and untrained limbs. One explanation of these bilateral changes, is that the mean age ($\pm$ SD) of subjects was 19.8 $\pm$ 1.5 yrs, which is still considered an age for peak bone mass

accrual. [24] Therefore, changes in BMC and BMD that occurred in both trained and untrained limbs were most likely attributable to skeletal growth.

Maximum voluntary force and force relative to motor unit activation have been found to be greater for eccentric muscular action than concentric action [13, 37, 71, 72]. Thus, eccentric muscular contraction should generate greater forces on bone than concentric loading.[71] Additionally, eccentric exercise has been shown to more osteogenic previously. Hawkins et al. [25] studied 12 women (mean $\pm$ SD, age 22.8 $\pm$ 1.17 yrs, n = 8) who underwent 18 wks of isokinetic concentric and eccentric training on a KinCom dynamometer. Subjects trained one leg concentrically and the other eccentrically. Eight women served as controls (mean age 21.9 $\pm$ 1.13, n = 8). Bone mineral density was measured by DXA (Hologic QDR-1500, Waltham, MA). Eccentric exercise significantly increased mid-femur segment BMD by 3.9 % ($p < 0.05$), whereas concentric exercise caused a nonsignificant increase in mid-femur BMD of 1.1 % [25]. Surprisingly, in the current study there was an unexpected trend for concentric isokinetic training to cause a larger increase in total forearm BMD than eccentric training. The superior increase in forearm BMD with concentric exercise training may have been attributable to superior training attendance. The mean training adherence for the concentric group was 85.4 % while the mean attendance for the eccentric group was 76.5 %.

The exact mechanisms by which physical activity increases bone mass are largely unknown. According to animal experiments, the training effect is most likely transmitted by the stimulation of osteoblast activity and new bone formation and, perhaps, not so intensely, by inhibition of osteoclast activity and bone resorption [36, 80].

In the current study, OPG was significantly increased following 32 wks of high-load unilateral isokinetic exercise. This supports the contention that physical exercise causes a suppression of osteoclastogenesis and osteoclast activity. The argument of exercise-induced alterations in osteoclast regulatory cytokines was further supported by an increased ratio of OPG/RANKL following isokinetic training. In fact, percentage change in the ratio of OPG/RANKL was positively correlated with percentage change in knee extensor strength in the eccentric trained group. Alterations in the relative serum concentrations of OPG and RANKL may indicate exercise-generated adaptations in osteoblast signaling. Interestingly, change in OPG was negatively correlated with change in RANKL in the eccentric trained group only. This provides evidence for a superior osteogenic effect of eccentric isokinetic training, at a cellular level. Analytical and biological variability, long-term intra-subject variability, and other acute factors influencing measured serum concentrations of OGP and RANKL have not been well characterized. Consequently, changes that occurred in serum OPG and RANKL during this interventional study may have the potential to be affected by factors that cannot yet be quantified.

Osteocalcin, a marker of bone formation, did not change following long-term unilateral isokinetic training. Substantial increases in osteocalcin, without concomitant changes in BMD, have been previously observed during strength training interventions; [44, 54] however, bilateral high-load muscular training was utilized. In the current study, unilateral isokinetic training may have provided an inadequate stimulus for the detection of systemic changes in serum osteocalcin. Additionally, the bone remodeling cycle has been shown to consist of approximately 2-to-3 wks of bone resorption

followed by 2-to-3 months of formation. [56] Serum concentrations of osteocalcin may have risen and subsided prior to the collection of serum at 32 wks. Changes in serum concentrations of NTx were also unsubstantial in the current study. As with serum osteocalcin, unilateral isokinetic muscular strength training may have been insufficient to stimulate measurable changes in systemic concentrations of serum NTx. The timing of serum collection may have also contributed to the nonsignificant finding.

In summary, serum concentrations of OPG and the ratio of OPG/RANKL were elevated following 32 wks of high-load unilateral isokinetic exercise training in young women. The mean serum OPG level increased 14.9 % following long-term unilateral isokinetic exercise training that also significantly increased total forearm BMD. These alterations in osteoblast cytokines that control osteoclastogenesis may represent an exercise-mediated suppression of osteoclast differentiation and activity. Osteoprotegerin and RANKL were negatively related in women undergoing eccentric isokinetic muscle training. This is the first time that OPG and RANKL have been utilized as markers of bone cell activity during long-term high-load strength training. The central role of the RANKL/OPG cytokine system in the regulation of bone cell biology is well established. Though further research is needed to confirm the efficacy of using serum OPG and RANKL as markers of bone cell metabolism in healthy populations, this study has shown for the first time that high load isokinetic muscular training may alter osteoblastic regulation of osteoclastogenesis.

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Table 1. Mean (± SD) baseline characteristics for eccentric and concentric training groups

Parameter	Concentric Trained Group (n = 10)	Eccentric Trained Group (n = 10)
Age (yr)	20.1 ± 2.0	19.5 ± 0.9
Weight (kg)	58.9 ± 6.9	58.8 ± 5.3
Height (cm)	162.5 ± 6.3	163.0 ± 7.84
Body mass index (kg/m^2)	22.3 ± 2.4	22.2 ± 3.0

Table 2. **Mean (± SD) of bone densitometry and peak torque strength measurements at baseline for eccentric and concentric trained groups**

	Concentric Trained Group (n = 10)		Eccentric Trained Group (n = 10)	
Parameter	Trained Limbs	Untrained Limbs	Trained Limbs	Untrained Limbs
Bone Mineral Density (g/cm^2)				
Total body	1.097 ± 0.070		1.101 ± 0.091	
Total forearm	0.551 ± 0.023	0.567 ± 0.028	0.556 ± 0.039	0.555 ± 0.380
Total tibial	0.991 ± 0.123	1.001 ± 0.116	1.001 ± 0.117	.0975 ± 0.112
Bone Mineral Content (g)				
Total body	2061.00 ± 215.74		2133.23 ± 271.14	
Total forearm	11.59 ± 1.12	12.05 ± 1.31	11.86 ± 1.29	11.89 ± 1.46
Total Tibial	25.33 ± 5.14	26.26 ± 5.65	26.47 ± 3.54	26.62 ± 3.57
Peak Torque (Nm)				
Elbow Flexor	56.52 ± 7.68	59.52 ± 7.49	63.00 ± 12.16	67.33 ± 19.86
Elbow extensor	44.07 ± 7.26	42.51 ± 8.48	46.89± 10.37	49.02 ± 11.04
Knee flexor	116.51 ± 33.36	117.99 ± 35.19	120.62 ± 15.85	131.54 ± 21.17
Knee extensor	173.29 ± 33.78[a]	183.08 ± 42.69	208.83 ± 38.22[a]	218.93 ± 37.46
Total arm	100.59 ± 12.50	102.03 ± 14.42	109.89 ± 22.28	116.36 ± 9.83
Total leg	289.80 ± 58.55	301.07 ± 74.92	329.45 ± 49.58	350.47 ± 55.11

[a] Significant difference between concentric and eccentric training groups by paired *t*-test, $p < 0.05$.

Table 3. **Mean (± SD) of serum bone biomarkers at baseline and at 32 Weeks**

Biomarker	Concentric n = 10)		Eccentric (n = 10)		Isokinetic (n = 20)	
	0 Weeks	32 Weeks	0 Weeks	32 Weeks	0 Weeks	32 Weeks
RANKL (pmol/L)	0.32 ± 0.18	0.34 ± 0.16	0.46 ± 0.22	0.41 ± 0.22	0.39 ± 0.21	0.37 ± 0.19
OPG (pmol/L)	4.38 ± 0.94	4.94 ± 1.57	4.90 ± 2.52	5.47 ± 2.62	4.64 ± 1.87	5.21 ± 2.12[a]
Osteocalcin (ng/ml)	18.2 ± 9.3	17.7 ± 8.0	24.2 ± 13.8	26.9 ± 11.0	21.21± 11.9	22.3 ± 10.5
NTx (nM BCE)	14.5 ± 5.2	15.2 ± 5.1	17.0 ± 5.9	16.82± 6.6	15.8 ± 5.6	16.0± 5.8
Alkaline Phosphatase (U/L)	68.2 ± 16.3	73.5 ± 22.0	71.0 ± 6.7	73.2 ± 7.5[a]	69.6 ± 12.2	73.4 ± 16.0
Calcium (mg/dl)	9.7 ± 0.6	9.8 ± 0.7	9.63± 0.4	9.7 ± 0.6	9.7 ± 0.5	9.8 ± 0.6

Key: RANKL, receptor activator for nuclear factor kappa; OPG, osteoprotegerin; NTx, Cross-linked N-telopeptides of bone collagen; BCE, bone collagen equivalents
[a] Significantly different from baseline by repeated measures ANOVA, $p < 0.05$

Table 4. Percentage change in bone densitometry measures, bone biomarkers, and peak torque muscular strength measures for eccentric, concentric and isokinetic training groups

Parameter	Eccentric Trained Group (n = 10)		Concentric Trained Group (n = 10)		Isokinetic Trained Group (n = 20)	
	% Change Trained Limb	% Change Untrained Limb	% Change Trained Limb	%Change Untrained Limb	% Change Trained Limb	% Change Untrained Limb
Bone Mineral Density						
Total body	0.04		0.4		0.2	
Total forearm	0.2	0.3	1.5[a]	0.9	0.8[a]	0.6
Total tibial	1.3	2.6[a]	0.3	0.3	0.8[a]	1.5[a]
Bone Mineral Content						
Total body	-0.2		0.5		0.2	
Total forearm	1.7[b]	1.0[b]	1.7[a]	1.7[b]	1.7[c]	1.4[b]
Total Tibial	1.3	2.1	-0.2	-0.03	0.5	1.0
RANKL	-10.2		16.5		3.2	
OPG	16.0		13.7		14.9[a]	
Osteocalcin	38.6		5.0		21.8	
NTx	7.9		28.1		18.0	
Alkaline Phosphatase	3.4		6.8		5.0[a]	
Calcium	1.1		1.1		1.1	
Peak Torque						
Elbow Flexor	51.5[b]	28.2	27.5[a]	12.7	39.5[b]	20.4[b]
Elbow extensor	18.8[b]	14.9	8.8	11.9	13.8[b]	9.05
Knee flexor	27.5[a]	5.7[a]	17.1	13.4	22.3[b]	9.6
Knee extensor	35.4[b]	18.1	41.8[b]	21.3	38.6[c]	18.1[b]
Total arm	37.4[b]	18.7	19.0[a]	12.1	28.2[c]	15.4[b]
Total leg	32.2	11.1	30.1[c]	17.7[a]	31.4[c]	14.4[b]

Key: RANKL, receptor activator for nuclear factor kappa; OPG, osteoprotegerin; NTx, Cross-linked N-telopeptides of bone collagen; BCE, bone collagen equivalents

[a] Significantly different from baseline by paired *t*-test, $p < 0.05$

[b] Significantly different from baseline by paired *t*-test, $p < 0.01$

[c] Significantly different from baseline by paired *t*-test, $p < 0.001$

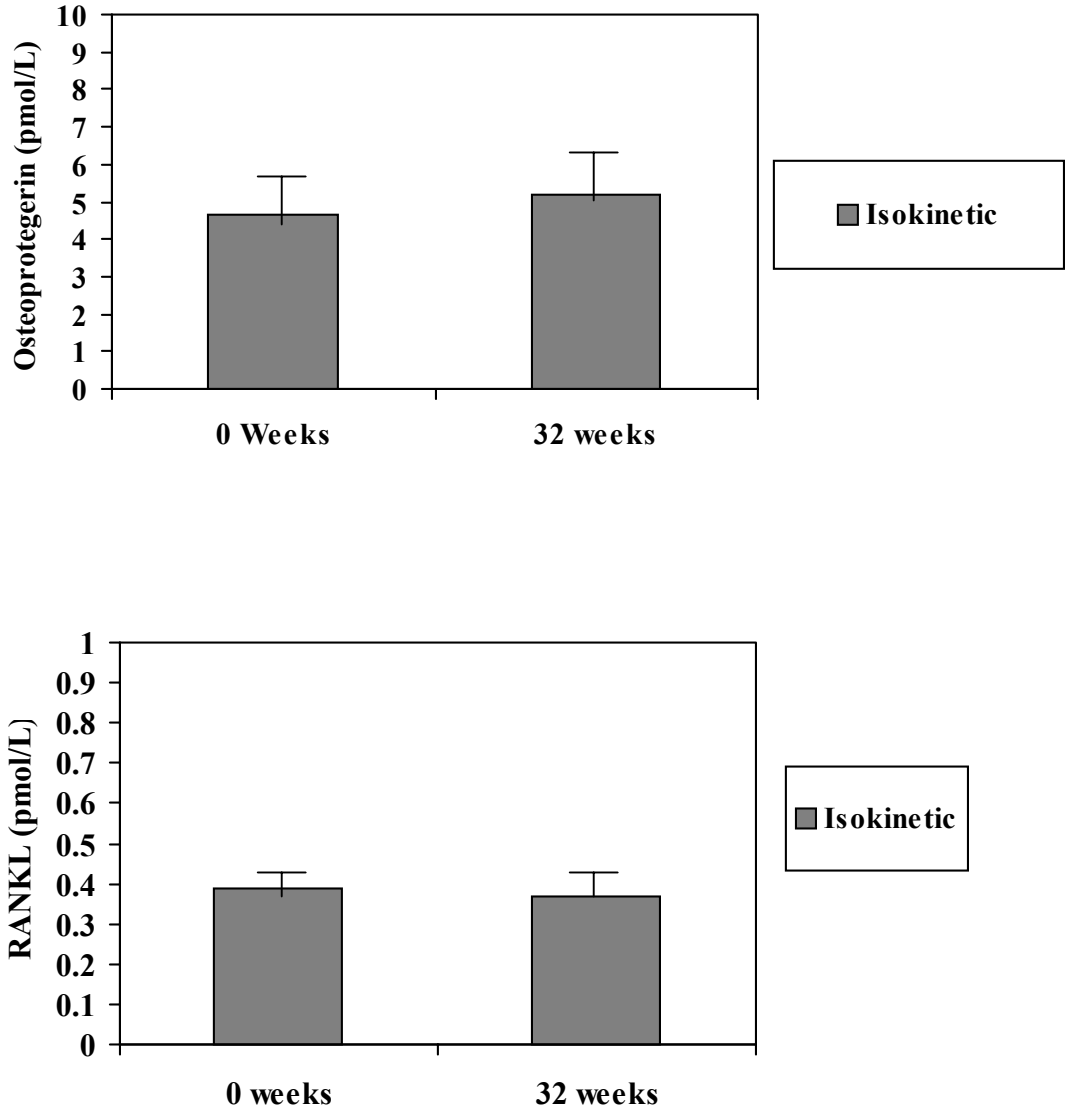

Figure 1. Regulatory cytokines at baseline and post-training in the isokinetic group (n = 20)

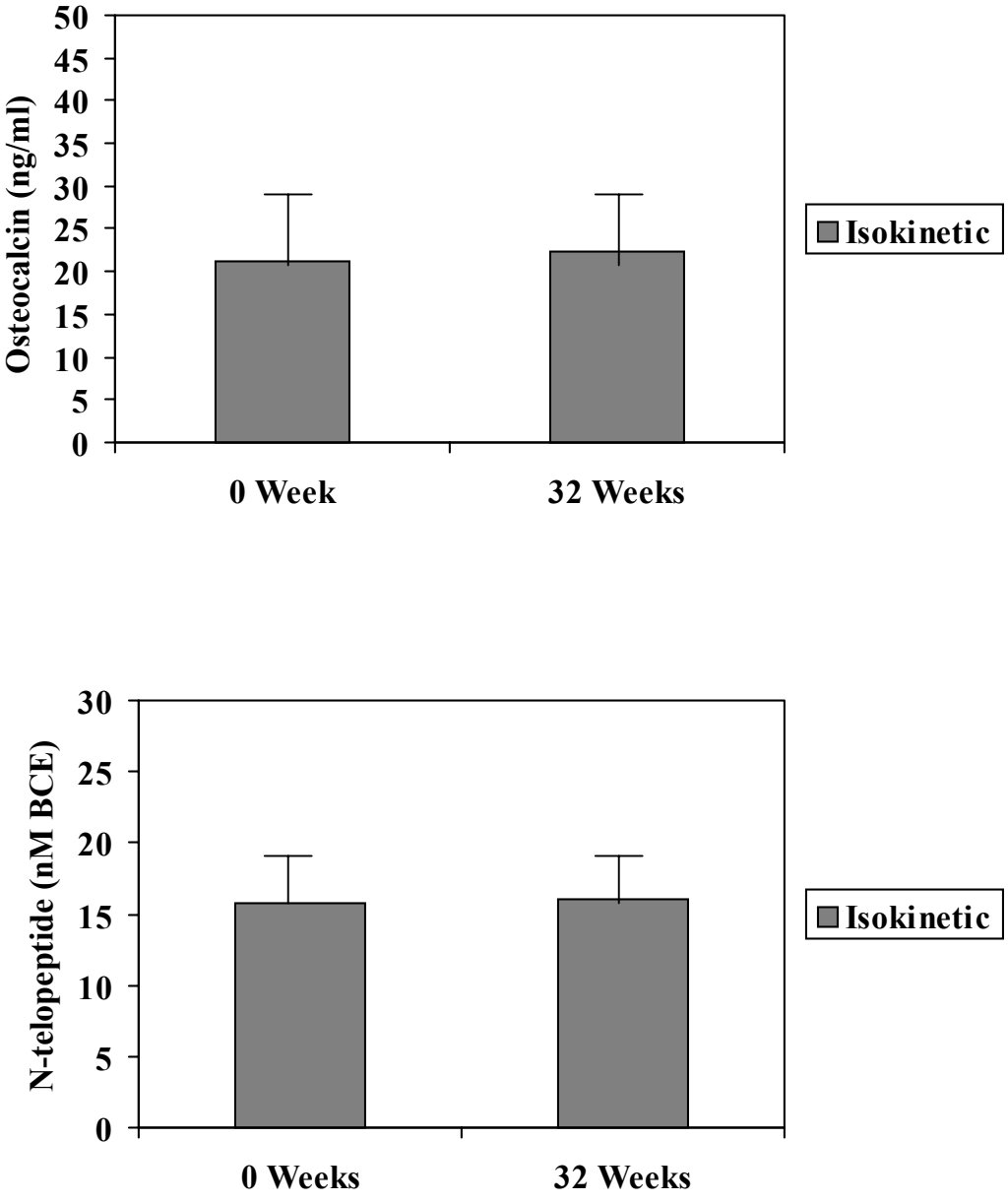

Figure 2. Bone remodeling biomarkers at baseline and post-training for the isokinetic group (n = 20)

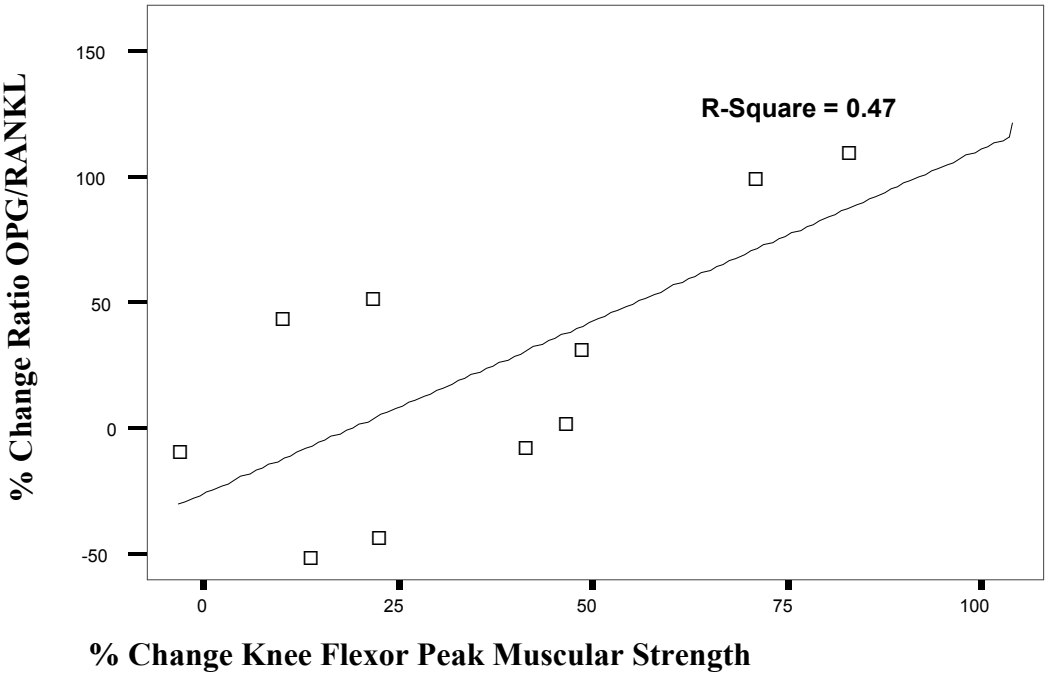

Figure 3. Relationship between percentage change ratio OPG/RANKL and knee extension peak torque strength in eccentric trained individuals

CHAPTER 4 – SUMMARY, CLINICAL IMPLICATIONS, AND FUTURE RESEARCH

Summary

The purpose of this study was to determine if 32 wks of high-load unilateral eccentric or concentric isokinetic resistance training caused alterations in muscular strength with concomitant modifications in bone cell activity and bone mineral density and content. Simultaneous evaluation of serum OPG, RANKL, osteocalcin and NTx were utilized to provide a more complete perspective of regulatory adaptations that occurred in bone cells as a result of mechanical loading from high-load muscular strength training. Only OPG demonstrated change.

The elevation of serum OPG during this isokinetic exercise intervention supports the contention that high-load muscular strength training causes a suppression of osteoclastogenesis and osteoclast activity. The argument of exercise-induced osteoclast suppression was further supported by an increased ratio of OPG-to-RANKL. The central role of the RANKL/OPG cytokine system in the regulation of bone cell biology is well established. In contrast, the validity and efficacy of OPG and RANKL as markers of bone cell metabolism have not been elucidated. Nevertheless, the present study shows a potential for OPG and RANKL use as biomarkers of bone metabolism in healthy populations undergoing long-term exercise training. Further research is needed.

CLINICAL IMPLICATIONS

The current study revealed that long-term high-load isokinetic muscular strength training might cause substantial changes in muscle strength with concomitant small but significant changes in local BMD and BMC in young women. The study also

demonstrated that high-load isokinetic training might cause a suppression of osteoclastogenesis. If this contention can be verified by additional research, isokinetic muscle training may become the quintessential mode of exercise for maintaining and improving skeletal strength and composition. Verification of the osteogenic potential of high-load isokinetic training may eventually lead to improved exercise prescription and exercise program design in clinical settings.

FUTURE RESEARCH

This study touches on several areas that are worthy of further research. Isokinetic muscular strength training has been shown to enhance strength and improve local levels of BMD and BMC. High-load isokinetic training may also cause alterations in osteoblastic control of osteoclastogenesis. The measurement of serum OPG and RANKL alone or in combination with other bone biomarkers may provide a more precise indication of adaptations that occur in bone cell metabolism during strength training. The following research initiatives could increase the knowledge base and understanding of the osteogenic impact of high-load muscular training interventions and the accuracy of bone biomarkers that evaluate their osteogenic effects.

1. Bone biomarkers measurements have been shown to be vulnerable to many sources of biological variability. Biomarkers may be vulnerable to seasonal variations, circadian variations, use of oral contraceptives, and menstrual influences. Biomarkers can also be affected by acute changes in circulatory dilution, transient plasma fluid shifts, and circulatory clearance factors that modify blood concentration. The factors that contribute to biological variability in serum OPG and RANKL have not been elucidated.

Several research studies could be undertaken to evaluate biological variability in OPG and RANKL. A long-term study could be conducted that measured OPG and RANKL over the course of a year. This study could provide information on long-term intra-subject variability and could estimate seasonal variability. A short-term study could be undertaken to evaluate fluctuations in OPG and RANKL throughout the menstrual cycle. This study could also study the effects of oral contraceptive use in women. A 24-hr study could be performed to determine diurnal variations in OPG and RANKL in a healthy population. Serum levels of RANKL, OPG and the RANKL-to-OPG ratio could also be correlated with other well-known markers of bone formation and resorption, to determine if they are accurate and relevant bone biomarkers. Osteoprotegerin and RANKL have the potential to be excellent markers of bone metabolism during therapeutic and exercise interventional studies. However, observed changes in serum OPG and RANKL during interventions will be suspect, until factors that cause variability have been identified and accounted for.

2. In the current study, unilateral isokinetic training caused substantial changes in strength and small but significant localized changes in BMD and BMC. Bilateral isokinetic eccentric training may provide a more robust osteogenic stimulus and a more substantial elevation in bone accrual. Young women or post-menopausal women could be randomly assigned to a high-load isokinetic training group or a control group. Training would consist of bilateral eccentric isokinetic training, three times per week for 32 wks. Serum levels of OPG, RANKL, osteocalcin, NTx and other relevant biomarkers could be measured. In order to assess changes in biomarkers throughout the period of training, serum samples could be collected at 0, 4, 12, 24, and 32 wks. Serum samples

would be collected at the same time of day and during the same phase of the menstrual cycle in an effort to control for biological variability. Changes in serum biomarkers would be compared with changes in flexor and extensor muscle strength and measures of BMD and BMC of the total forearm, total tibia, and total body as determined by DXA.

3. Previous research has looked at the impact of both high-load muscular strength training and high impact aerobic exercise interventions on BMD and BMC. It may be beneficial to explore changes in serum OPG and RANKL that occur during several different exercise training regimes. Women could be assigned to a high-load isokinetic strength-training group, a high impact aerobic group, a low-impact aerobic group, or a control group. Alterations in serum levels of OPG, RANKL and other relevant biomarkers would be compared to changes in strength and total body and regional BMD and BMC as determined by DXA. This study could determine if other modes of exercise cause alterations in osteoblast control of osteoclasts.

CHAPTER 5

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APPENDIX A

BIOMARKER ASSAYS

In accordance with methods delineated in the TiBIAL study protocol, blood samples (10 ml) were collected, and then centrifuged for 12 minutes at 2500 rpm to allow plasma separation. The plasma was stored at -80° C until final analysis. Serum samples attained at 0 and 32 weeks were analyzed for OPG, RANKL, osteocalcin, and NTx. Additionally, serum alkaline phosphatase and calcium were measured to allow evaluation of markers relevant to bone metabolism and to rule out relevant bone and systemic pathology. In order to limit inter-assay variability within subjects, the same assay kit was utilized to measure subject samples from 0 and 32 weeks.

The serum RANKL test kit [sRANKL EIA (Biomedica), Windham, NH] was an enzyme immunoassay designed to determine serum levels of soluble, uncomplexed human RANKL. In the assay, serum RANKL and biotinylated anti-RANKL (a detection antibody) were pipetted into wells pre-coated with recombinant OPG. Any RANKL present bound to the pre-coated OPG and formed a sandwich with the detection antibody. Serum levels of RANKL were then quantitated by an enzyme catalysed color change, which was read by a standard ELISA reader at 450 nm. The amount of color was proportional to the amount of sRANKL present in the sample. Each RANKL kit had 96 wells available and each well required 100 ul of serum or control. The intra-assay coefficient of variation (CV) was 7.8%.

In the OPG assay [Osteoprotegerin (Biomedica), Windham, NH], OPG bound to a pre-coated capture antibody. Serum OPG was quantitated by an enzyme catalysed color change that was detectable by a standard ELISA reader at 450 nm. The amount of color developed was directly proportional to the amount of OPG present in the

sample. Each assay kit had ninety-six wells available and each well required a sample or control volume of 50 ul. The intra-assay CV was 5.3%.

Serum samples collected at 0 and 32 weeks also underwent analysis for both intact osteocalcin and n-terminal mid-molecule fragments of osteocalcin [Mid-Tact Human Osteocalcin EIA Kit (Biomedical Technologies Inc.), Stoughton, MA]. The osteocalcin assay was an enzyme immunoassay utilizing two antibodies and had an intra-assay CV of 6.6 % and an inter-assay CV of 22.3 %. The concentration of osteocalcin in the sample was proportional to the absorbance and values were obtained by comparison to a standard curve prepared on the same assay plate. Absorbance was measured at 450 nm on an ELISA plate reader.

Serum NTx was measured by a competitive enzyme-linked immunosorbent assay [Osteomark NTx Serum (Osteomark), Seattle, WA]. NTx epitope was adsorbed onto a 96 well microplate. Diluted samples were added to the microplate wells, followed by a horseradish peroxidase labeled monoclonal antibody. The NTx in the sample competed with the NTx epitope in the microplate well for antibody binding sites. The amount of bound and labeled antibody was then measured by colorimetric generation of a peroxide substrate. Absorbance was determined spectrophotometrically at 450 nm on a standard ELISA microplate reader and NTx concentrations were calculated using a standard calibration curve. Assay values were reported in nanomoles Bone Collagen Equivalents per liter (nM BCE). The NTx assay intra-assay CV was 9.6 % and the inter-assay CV was 19.6 %.

Serum alkaline phosphatase was measured by a modified colorimetric assay [Stanbio Alkaline Phosphatase LiquiColor Procedure No. 2900 (Stanbio), Boerne, TX].

In the assay, alkaline phosphatase hydrolyzed 4-nitrophenol phosphate to form 4-nitrophenol and phosphates. The assay was then modified by the addition of sodium hydroxide 0.25 moles (M), which ended the reaction. The rate at which 4-nitrophenol was formed was directly proportional to alkaline phosphatase activity. The 4-nitrophenol product was yellow in color and was detected by a spectrophotometer at an absorbance reading of 405 nm. The intra-assay CV was 3.5 %.

Serum calcium was measured by a quantitative colorimetric assay [Stanbio Total Calcium LiquiColor (Arsenazo III) Procedure No. 0155 (Stanbio), Boerne, TX]. This assay provided a quantitative colorimetric determination of total calcium in serum. Arsenazo III formed a violet Arsenazo III:calcium complex, which was detected by a spectrophotometer at an absorbance reading of 650 nm. The concentrations of calcium were proportional to the absorbance of the violet color.

APPENDIX B

Raw Data

Table 1. Baseline Physical Characteristics

Subject	Age years	Height (cm)	Weight (kg)	BMI kg/m^2
13	19	160.7	49.6	19.2
16	19	161.8	54.7	21
36	19	160	68.1	26.2
52	20	168.3	70.7	25.2
64	18	160.3	52.5	20.2
91	19	159.4	60.6	24.2
330	20	154.9	55.7	23.2
347	20	172.1	59.6	19.9
352	22	172.1	63.8	21.3
361	25	154.9	54.5	22.7
11	19	172.1	61.5	20.5
25	21	173.4	68.3	22.8
27	21	165.1	54.5	20.2
37	19	163.2	61.9	22.9
38	19	166.4	54	19.3
40	19	152.4	56	24.3
62	19	165.1	54.2	20.1
63	20	158.8	52.4	20.1
72	19	148.6	64.8	29.5
314	19	165.1	60.5	22.4
Mean	19.8	162.7	58.9	22.3
SD	1.5	6.9	6.0	2.7
LL	18	148.6	49.6	19.2
UL	25	173.4	70.7	29.5

Table 2. Peak Torque Total Leg and Total Arm Data Trained Limbs

Subject	Total Leg (Nm) 0 Wk	Total Leg (Nm) 32 Wk	Total Arm Nm) 0 Wk	Total Arm (Nm) 32 Wk
13	241.3	329.4	88.4	121.5
16	231.9	285.0	96.4	103.8
36	297.1	406.2	104.7	110.0
52	321.0	411.8	92.2	110.3
64	242.0	457.3	91.6	143.8
91	381.8	488.8	122.9	168.8
330	207.6	269.2	90.4	95.7
347	343.4	369.8	118.3	108.2
352	350.4	359.1	100.4	110.4
361	281.5	351.2	*	*
11	340.40	584.4	112.4	206.9
25	285.1	474.5	107.1	135.2
27	355.1	434.1	123.0	159.0
37	429.5	419.7	155.3	178.2
38	298.7	355.2	95.6	108.4
40	254.2	382.3	92.0	132.3
62	311.2	408.3	*	*
63	349.9	369.8	100.7	157.5
72	366.4	553.3	123.5	186.4
314	304.0	323.0	79.4	93.4
Mean	309.6	401.6	106.1	133.9
SD	56.6	81.3	18.2	33.4
LL	207.6	269.2	79.4	155.3
UL	429.5	584.4	93.4	206.9
% Change		31.4		27.7

* Subjects with missing values were eliminated from relevant statistical analyses

Table 3. Peak Torque Total Leg and Total Arm Data Untrained Limbs

Subject	Total Leg (Nm) 0 Wk	Total Leg (Nm) 32 Wk	Total Arm Nm) 0 Wk	Total Arm (Nm) 32 Wk
13	264.6	314.2	82.6	106.9
16	241.8	252.5	103.6	94.3
36	278.4	387.3	98.4	101.7
52	295.5	331.7	103.8	109.5
64	255.2	426.3	92.7	131.0
91	461.6	454.1	126.7	156.4
330	206.1	242.8	84.4	92.5
347	347.7	345.8	116.0	117.5
352	376.8	395.6	110.1	114.1
361	283.0	325.6		
11	321.9	464.6	127.9	172.2
25	341.0	401.6	101.1	144.9
27	383.2	399.4	148.7	133.8
37	462.2	393.6	173.7	168.3
38	312.8	310.3	97.2	89.8
40	271.2	312.1	92.4	124.6
62	349.5	355.4		.
63	381.5	423.3	93.3	135.0
72	385.5	531.5	125.8	165.3
314	295.9	280.0	87.1	87.3
Mean	325.8	367.4	110.0	124.2
SD	68.8	73.9	23.5	27.2
LL	206.1	242.8	82.6	87.3
UL	462.2	531.5	173.7	172.2
% Change		14.4		15.4

* Subjects with missing values were eliminated from relevant statistical analyses

Table 4. Peak Torque Flexor and Extensor Data Trained Limbs

Subject	Elbow Flexor (Nm) 0 Wk	Elbow Flexor (Nm) 32 Wk	Elbow Extensor (Nm) 0 Wk	Elbow Extensor (Nm) 32 Wk	Knee Flexor (Nm) 0 Wk	Knee Flexor (Nm) 32 Wk	Knee Extensor (Nm) 0 Wk	Knee Extensor (Nm) 32 Wk
13	57.1	80.2	31.3	41.3	142.3	204.6	99	124.8
16	54.7	66.4	41.7	37.4	136.6	175	95.3	110
36	57	64.3	47.7	45.7	170.5	258.6	126.6	147.6
52	54.6	63	37.6	47.3	195.1	270.5	125.9	141.3
64	49.7	87.7	41.9	56.1	145.9	296.8	96.1	160.5
91	70.6	105.8	52.3	63	186	344.4	195.8	144.4
330	49.6	59	40.8	36.7	127.2	171.5	80.4	97.7
347	66.9	60.3	51.4	47.9	218	232.3	125.4	137.5
352	48.5	59.2	51.9	51.2	218.8	221.7	131.6	137.4
361	*	*	*	*	192.5	240.5	89	110.7
11	61.8	140.6	50.6	66.3	200.4	366.2	140	218.2
25	61.3	80.4	45.8	54.8	188.2	321.3	96.9	153.2
27	72.1	105.7	50.9	53.3	232.9	283.1	122.2	151
37	87.9	106	67.4	72.2	290.6	281.5	138.9	138.2
38	53.1	65.8	42.5	42.6	187.6	212.8	111.1	142.4
40	55.1	78.6	36.9	53.7	157.1	229.7	97.1	152.6
62	*	*	*	*	187.3	264.6	123.9	143.7
63	59.7	117.0	41	40.5	211.5	258.8	138.4	111
72	69.3	118.9	54.2	67.5	245.3	364.2	121.1	189.1
314	46.7	48.3	32.7	45.1	187.4	206.2	116.6	116.8
Mean	59.8	83.7	45.5	51.3	118.6	141.4	191.1	260.2
SD	10.4	26.1	8.8	10.6	25.5	27.8	39.6	57.3
LL	46.7	48.3	31.3	36.7	80.4	97.7	127.2	171.5
UL	87.9	140.6	67.4	72.2	195.8	218.2	290.6	366.2
% Change		39.5		13.8		22.3		

* Subjects with missing values were eliminated from relevant statistical analyses

Table 5. Peak Torque Flexor and Extensor Data Untrained Limbs

Subject	Elbow Flexor (Nm) 0 Wk	Elbow Flexor (Nm) 32 Wk	Elbow Extensor (Nm) 0 Wk	Elbow Extensor (Nm) 32 Wk	Knee Flexor (Nm) 0 Wk	Knee Flexor (Nm) 32 Wk	Knee Extensor (Nm) 0 Wk	Knee Extensor (Nm) 32 Wk
13	53.4	63.6	29.2	43.3	100.0	121.8	164.6	192.4
16	63.4	59.6	40.2	34.7	106.2	101.1	135.6	151.4
36	57.5	62.6	40.9	39.1	119.7	143.5	158.7	243.8
52	59.4	59.9	44.4	49.6	111.9	122.4	183.6	209.3
64	54.9	81.7	37.8	49.3	96.6	151.2	158.6	275.1
91	74.6	96.6	52.1	59.8	201.8	153.7	259.8	300.4
330	50.6	51.8	33.8	40.7	77.6	89.8	128.5	153.0
347	66.0	66.9	49.6	50.6	132.9	146.6	214.8	199.2
352	55.5	60.9	54.6	53.20	142.1	150.6	234.7	245.0
361	*	*	*	*	91.1	112.2	191.9	213.4
11	76.2	110.4	51.7	61.8	127.3	176.7	194.6	287.9
25	57.3	86.1	43.8	58.8	125.3	145.7	215.7	255.9
27	81.5	87.2	67.2	46.6	133.5	140.1	249.7	259.3
37	110.4	101.5	63.3	66.8	158.7	136.4	303.5	257.2
38	54.4	52.4	42.8	37.4	114.5	125.4	198.3	184.9
40	52.6	79.5	39.8	45.1	104.1	112.5	167.1	199.6
62	*	*	*	*	139.0	114.6	210.5	240.8
63	56.8	91.5	36.5	43.5	152.2	141.2	229.3	282.1
72	69.9	103.7	55.9	61.6	159.6	186.6	225.9	344.9
314	46.9	48.2	40.2	39.1	101.2	102.5	194.7	177.5
Mean	63.4	745.8	45.8	48.9	124.8	133.7	201.0	233.7
SD	15.1	19.7	10.1	9.6	29.1	24.8	43.2	51.1
LL	46.9	48.2	29.2	34.7	77.6	89.8	128.5	151.4
UL	110.4	110.4	67.2	66.8	201.8	186.6	303.5	344.9
% Change		20.4		9.1		9.6		18.1

* Subjects with missing values were eliminated from relevant statistical analyses

Table 6. Total Body Bone Mineral Density and Bone Mineral Content

Subject	Total BMD (g/cm^2) 0 Wk	Total BMD (g/cm^2) 32 Wk	Total BMC (g) 0 Wk	Total BMC (g) 32 Wk
13	1.038	1.051	1891.56	1845.75
16	1.09	1.099	2033.22	2046.29
36	1.074	1.055	1943.83	1959.6
52	1.022	1.054	2053.59	2107.45
64	1.146	1.138	2136.16	2157.5
91	1.194	1.183	2179.38	2146.45
330	1.055	1.07	1835.41	1890.76
347	1.038	1.037	2022.02	2027.86
352	1.226	1.231	2593.87	2587.35
361	1.084	1.092	1920.98	1934.25
11	1.261	1.267	2594.44	2570.85
25	1.072	1.064	2169.43	2173.49
27	1.157	1.149	2263.68	2234.6
37	1.149	1.131	2201.31	2222.7
38	0.938	0.945	1741.78	1705.00
40	1.122	1.132	1976.61	1964.47
62	1.107	1.102	2189.41	2192.3
63	1.039	1.043	1878.32	1879.4
72	1.158	1.169	2457.63	2441.8
Mean	1.098	1.101	2097.12	2099.80
SD	0.079	0.077	241.24	235.33
LL	0.938	0.945	1741.78	1705.00
UL	1.261	1.267	2594.44	2587.35
% Change		0.2		0.2

Table 7. Regional Bone Mineral Density Trained Limbs

Subject	Forearm BMD (g/cm^2) 0 Wk	Forearm BMD (g/cm^2) 32 Wk	Tibial BMD (g/cm^2) 0 Wk	Tibial BMD (g/cm^2) 32 Wk
13	0.558	0.572	1.249	1.232
16	0.537	0.555	0.865	.876
36	0.519	0.542	0.842	.831
52	0.598	0.595	1.137	1.160
64	0.575	0.586	1.019	1.013
91	0.558	0.562	1.058	1.065
330	0.603	0.608	0.940	0.944
347	0.607	0.609	1.087	1.101
352	0.568	0.549	1.117	1.107
361	0.569	0.568	0.800	0.820
11	0.530	0.535	0.999	0.997
25	0.502	0.512	0.897	0.901
27	0.494	0.500	1.200	1.204
37	0.588	0.574	0.985	1.047
38	0.537	0.545	0.930	0.949
40	0.558	0.572	1.249	1.232
62	0.537	0.555	0.865	0.876
63	0.519	0.542	0.842	0.831
72	0.598	0.595	1.137	1.160
314	0.575	0.586	1.019	1.013
Mean	0.553	0.558	0.996	1.004
SD	0.032	0.029	0.117	0.115
LL	0.494	0.500	0.800	0.820
UL	0.607	0.609	1.249	1.232
% Change		0.8		0.8

Table 8. Regional Bone Mineral Density Untrained Limbs

Subject	Forearm BMD (g/cm^2) 0 Wk	Forearm BMD (g/cm^2) 32 Wk	Tibial BMD (g/cm^2) 0 Wk	Tibial BMD (g/cm^2) 32 Wk
13	.591	.595	1.256	1.246
16	.565	.574	.907	.913
36	.514	.526	.876	.831
52	.622	.611	1.124	1.164
64	.567	.575	.973	1.004
91	.574	.588	1.026	1.057
330	.584	.593	.938	.972
347	.598	.598	1.078	1.092
352	.565	.553	1.137	1.149
361	.526	.531	.830	.815
11	.531	.532	.965	.961
25	.521	.518	.819	.894
27	.497	.505	1.108	1.113
37	.616	.598	.977	1.020
38	.541	.553	.881	.925
40	.591	.595	1.256	1.246
62	.565	.574	.907	.913
63	.514	.526	.876	.831
72	.622	.611	1.124	1.164
314	.567	.575	.973	1.004
Mean	.561	.564	.989	1.003
SD	0.033	0.030	0.112	0.113
LL	0.497	0.505	0.819	0.815
UL	0.622	0.611	1.256	1.246
% Change		0.6		1.5

Table 9. Regional Bone Mineral Content Trained Limbs

Subject	Forearm BMC (g) 0 Wk	Forearm BMC (g) 32 Wk	Tibial BMC (g) 0 Wk	Tibial BMC (g) 32 Wk
13	10.59	10.96	22.11	22.27
16	11.44	11.32	22.77	23.22
36	11.04	11.09	24.48	24.72
52	12.21	12.04	24.88	23.7
64	11.72	12	29.87	29.53
91	10.62	10.77	30.45	30.35
330	10.25	10.55	17.53	17.54
347	11.98	12.37	22.72	22.21
352	14.17	14.79	35.38	36.08
361	11.89	12.04	23.12	23.19
11	12.11	12.2	30.07	30.02
25	12.15	12.59	26.81	26.81
27	14.08	14.33	31.65	31.9
37	12.31	12.61	25.94	26.6
38	10.37	10.46	19.07	18.27
40	10.8	11.13	23.58	23.61
62	12.3	12.21	25.32	26.64
63	9.59	9.81	29.15	29.9
72	12.86	12.92	25.76	26.95
314	11.98	12.27	27.3	27.81
Mean	11.72	11.92	25.90	26.07
SD	1.19	1.23	4.34	4.54
LL	9.59	9.81	17.53	17.54
UL	14.17	14.79	35.38	36.08
% Change		1.6		0.8

Table 10. Regional Bone Mineral Content Untrained Limbs

Subject	Forearm BMC (g) 0 Wk	Forearm BMC (g) 32 Wk	Tibial BMC (g) 0 Wk	Tibial BMC (g) 32 Wk
13	11.33	11.85	31.07	30.84
16	10.67	10.97	18.87	18.71
36	12.33	12.63	22.81	21.88
52	15.39	15.59	36.96	37.57
64	11.76	11.80	23.09	23.36
91	12.49	12.52	28.93	29.33
330	12.25	12.64	30.20	30.63
347	13.73	13.54	31.25	31.45
352	12.16	12.12	26.05	26.48
361	9.95	10.15	18.84	18.18
11	11.10	11.14	24.01	23.56
25	12.68	12.82	27.81	30.99
27	9.39	9.59	28.07	29.26
37	13.81	13.67	24.73	24.96
38	11.38	11.80	26.28	27.58
40	11.33	11.85	31.07	30.84
62	10.67	10.97	18.87	18.71
63	12.33	12.63	22.81	21.88
72	15.39	15.59	36.96	37.57
314	11.76	11.80	23.09	23.36
Mean	11.97	12.13	26.44	26.76
SD	1.35	1.30	4.60	4.92
LL	9.39	9.59	18.84	36.96
UL	15.39	15.59	18.18	37.57
% Change		1.4		1.0

Table 11. Biomarker of Bone Turnover

subject	OPG (Pmol/L) 0 Wk	OPG (Pmol/L) 32 Wk	RANKL (pmol/L) 0 Wk	RANKL (pmol/L) 32 Wk	Osteocalcin (ng/ml) 0 Wk	Osteocalcin (ng/ml) 32 Wk	NTX (nM BCE) 0 Wk	NTx (nM BCE) 32 Wk
13	5.64	5.42	0.33	0.38	18.2	22.4	16.4	19.7
16	4.27	3.70	0.14	0.20	17.4	18.7	15.9	17.1
36	6.03	8.10	0.20	0.15	43	34.2	19	22.6
52	4.62	5.62	0.40	0.31	14	22.1	17.8	20.6
64	3.81	3.39	0.12	0.22	14.2	8.2	18.9	12.6
91	4.06	6.96	0.30	0.28	14.6	22.8	19.1	18.8
330	3.59	3.48	0.70	0.63	18.4	8.8	13.5	11.0
347	4.44	4.35	0.36	0.56	15.9	10.9	10.1	9.8
352	4.51	4.30	0.50	0.42	18.9	15.5	11.5	8.6
361	2.79	4.11	0.13	0.2	7.7	13.1	2.7	10.8
11	2.51	4.60	0.28	0.22	42	43.1	17.1	18.2
25	3.98	6.20	0.42	0.25	17.1	19.9	16	14.5
27	6.3	7.92	0.39	0.29	47.6	44.3	12.6	33.3
37	4.11	3.41	0.66	0.52	5.2	19.3	17.2	14.9
38	4.00	2.76	0.62	0.74	30	29.7	32.2	22.1
40	11.58	11.59	0.29	0.31	19.6	27.6	14.7	13.5
62	4.51	4.02	0.19	0.25	24.2	31.3	18.7	12.6
63	4.00	3.86	0.92	0.85	21.2	23.7	17.6	13.3
72	3.72	4.58	0.50	0.41	29.5	22.1	9.9	15.4
314	4.25	5.73	0.32	0.24	5.5	8.1	14.4	10.4
Mean	4.64	5.20	0.39	0.37	21.2	22.3	15.7	16.0
SD	1.87	2.12	0.21	0.19	11.9	10.5	5.6	5.8
UL	11.58	11.59	.92	0.85	47.6	44.3	32.2	33.3
LL	2.51	2.76	0.12	0.15	5.2	8.1	2.7	8.6
% Change		14.9		3.2		21.8		18.0

Table 12. Additional Biomarkers of Bone Turnover

Subject	Calcium (mg/dl) 0 Wk	Calcium (mg/dl) 32 Wk	Alkaline Phosphatase (U/L) 0 Wk	Alkaline Phosphatase (U/L) 32 Wk
13	10.4	9.7	72.8	74.2
16	9.7	10.4	42.0	46.9
36	10.2	10.5	96.6	111.8
52	8.9	9.3	84.4	95.1
64	10.1	8.7	68.5	70.0
91	9.1	8.7	63.9	76.1
330	8.8	9.9	72.1	73.0
347	10.2	10.4	76.6	89.9
352	9.7	10.1	5	38.2
361	10.1	10.4	54.6	59.8
11	10	10.5	74.2	69.9
25	9.5	8.8	81.2	73.4
27	9	10.4	75.7	72.7
37	10.1	9.5	80.3	89.4
38	10.1	9.6	65.5	64.3
40	9.3	9.8	70.4	76.4
62	10.1	8.7	68.5	70.0
63	9.5	10	62.4	66.0
72	9.5	9.8	62.5	68.9
314	9.2	10	69.5	81.4
Mean	9.7	9.8	69.6	73.4
SD	0.5	0.6	12.2	16.0
LL	8.8	8.7	42.0	38.2
UL	10.4	10.5	96.62	111.8
% Change		1.1		5.1

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Nutrition, Exercise and Fitness: New Directions, New Opportunities 1996
Lipid Clinic Preceptorship 1996
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Army Medical Department Officer Basic Course 1993
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