Changes in Bone Mineral Density and Biomarkers of Bone Turnover with Calcium Supplementation During Initial Military Cadet Exercise Training

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ABSTRACT

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Changes in Bone Mineral Density and Biomarkers of Bone Turnover with Calcium
Supplementation During Initial Military Cadet Exercise Training
(Under the direction of SHARON M. NICKOLS-RICHARDSON)

Osteoporosis is a condition involving decreased bone mineral density (BMD) and increased fragility of the skeletal system. Osteoporosis affects ~75 million individuals in the United States, Europe, and Japan. In the United States alone, hip fractures affect 500,000 individuals per year, and annual healthcare costs for osteoporotic fractures are approximately $14 billion. A high peak BMD can prevent or delay the onset of osteoporosis and its complications. Exercise and diet may affect peak BMD by as much as 20 to 40% each and have been identified as the two most important controllable factors determining BMD. The current study investigated the effect of a calcium, vitamin D, and vitamin K supplement combination during initial military cadet exercise training on: BMD, stress fracture occurrence, hormones associated with BMD, and biochemical markers of bone turnover. Significant changes in BMD, either between the supplemented group or the unsupplemented group or across time for both groups were not found. The majority of participants (n = 22) had unexpectedly high levels of physical activity prior to enrollment, and the initial military exercise training program included only moderate levels of activity. Therefore, the exercise stimulus to bone was likely insufficient to promote gains in BMD, regardless of the nutrient supplement status. Serum insulin-like growth factor-1 and osteocalcin significantly increased over time ($p < 0.05$ and $p < 0.001$, respectively), irrespective of treatment group. Significant decreases were found in dietary intake of calories ($p < 0.01$), carbohydrate ($p < 0.05$), protein ($p < 0.0001$), and fat ($p < 0.01$) over time. Decreases in reported dietary intake were likely due to less variety of foods eaten, and diminished compliance with food records. Significant differences were not found between groups or across time in dietary intakes of calcium, vitamin D, or vitamin K. Low dose supplementation with a calcium, vitamin D, vitamin K supplement during initial military training in young-adult cadets did not change BMD or alter stress fracture occurrence.

DEDICATION

This work is dedicated to my husband and my parents—all of whom have supported and encouraged me through the years, and to my daughter Katherine—who I hope to support and encourage as well.

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While my name appears on the cover of this work, it is the culmination of many efforts by many people. My endeavors alone would have had diminished results. I would like to express my heartfelt appreciation to the following people, who made completion of this thesis possible:

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CHAPTER 1

INTRODUCTION

Osteoporosis is a condition involving decreased bone mineral density (BMD) and increased fragility of the skeletal system. Osteoporosis affects ~75 million individuals in the United States, Europe, and Japan (Branca 1999). In the United States alone, 500,000 individuals sustain hip fractures per year, and healthcare costs for all osteoporotic fractures are approximately $14 billion, annually (Ray et al. 1997). Hip fractures due to osteoporosis increase mortality risks by 20%, and 25% of individuals sustaining hip fractures require long-term care following the injury (Branca 1999).

Low BMD is the hallmark feature of osteoporosis. The World Health Organization (WHO 2000) defines osteoporosis as a BMD of 2.5 standard deviations below the young-adult gender-matched mean. Losses in BMD typically occur with aging and result in increased risk of fractures. High BMD in young life can prevent or delay the onset of osteoporosis and its complications later in life (Teegarden et al. 1995).

In addition to a role in preventing osteoporosis, high BMD may assist in decreasing occurrence of stress fractures. Stress fractures involve damage to bone tissues and can occur during times of intense exercise training (Johnson et al. 1999). Stress fractures are a concern for athletes and have been a documented problem in military trainees for more than 100 years (Johnson et al. 1999). Financial and manpower costs to the military due to stress fractures in trainees are large; therefore, any factor that may decrease the incidence of stress fracture occurrence, and prevent subsequent losses in military personnel and finances, is of value.

Bone density is affected by many factors. Genetics, hormonal status, and medications, for example, all play roles in defining the mineral content and strength of bones. Habits or behaviors, such as diet, exercise, and tobacco use, also impact bone density.

Diet and exercise affect BMD by as much as 20 (Branca 1999) to 40% (Specker 1996) per factor. Diet and exercise have been identified as the two most important controllable factors determining BMD (Cooper and Eastell 1993), especially vital in women as their risks for bone loss are much greater compared to men (Toss 1992). Proper dietary intake ensures the presence of necessary vitamins and minerals to facilitate bone growth. Exercise increases

the rate of bone remodeling—the resorption, formation, and development of new bone (Price et al. 1994).

While some studies have investigated combined effects of physical activity and calcium supplementation on BMD, findings from these studies have been largely inconclusive. There is a scarcity of published data regarding effects of exercise in conjunction with vitamin and mineral supplementation specifically formulated to improve bone health. The current study was designed to investigate the effect of intake of a calcium, vitamin D, and vitamin K supplement combination during a 13- week exercise training period on BMD, selected hormones, and biochemical markers of bone turnover in young-adult males and females. Participants were students enrolled at Virginia Polytechnic Instituteand State University (VPI and SU) and were new recruits in the Corps of Cadets program.

Research Hypothesis

It was hypothesized that exercise training involved in the Corps of Cadets curriculum would result in significant increases in BMD at the lumbar spine, total proximal femur, total forearm, and whole body. These increases were expected for both the nutrient supplemented group and the control (unsupplemented) group. It was further hypothesized that the group supplemented with calcium, vitamin D, and vitamin K would have significantly greater increases in BMD at all four body sites compared to the control group. Stress fracture incidence was not expected to increase in either group across time.

CHAPTER 2

REVIEW OF LITERATURE

A great deal of research has been conducted regarding various aspects of bone density. This chapter provides an overview of some factors pertinent to the current study. Bone density, effects of dietary and exercise modifications, and BMD responses to hormonal alterations are addressed.

Bone density

Peak bone mass is the highest level of bone accumulation achieved in a lifetime. In females, 99% of final BMD (grams per squared centimeter or g/cm^2) is achieved by approximately age 22 years, but gains in BMD continue until the late second or early third decade of life (Teegarden et al. 1995). Bone mineral density peaks by age 29 in males, with a total gain of only ~ 0.1 g/cm^2 occuring between the ages of 19 and 24 years in the entire body (Rico 1993). Beyond peak BMD, men and women have similar femoral and lumbar spinal BMD until age 50 years, after which women typically lose BMD at an increased rate compared to males (Pumarino et al.1993).

Bone density is affected by many factors, but heredity may account for 50 to 80% of the variation in peak bone mass (Cooper and Eastell 1993, Pollitzer and Andersen 1989, Suleiman et al. 1997). Persons of African descent have higher levels of cortical bone than Caucasians, who in turn have higher BMD than Japanese (Pollitzer and Andersen 1989). Some ethnic differences in BMD begin *in-utero*, possibly due to metabolic differences during growth (Pollitzer and Andersen 1989). For example, persons of African descent have been found to have significantly higher levels of active vitamin D or 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$ or calcitriol) and parathyroid hormone (PTH) compared to their Caucasian counterparts (Pollitzer and Andersen 1989).

Total body weight and lean muscle mass play important roles in BMD attainment and maintenance (Douchi et al. 1998, Henderson et al. 1995, Lindsay et al. 1992). Hormonal status plays a large role in BMD development in females. Estrogen related menstrual disturbances, such as menopause and amenorrhea, are associated with a profound loss of bone mass (Kirchner 1995, Suleiman et al. 1997, Toss 1992, Young et al. 1994). Smoking is associated with a decrease in bone density and a corresponding increase in osteoporosis risk

(Law and Hackshaw 1997, Valimaki et al. 1994). Of the controllable risk factors, however, diet and physical activity levels are considered the two most important to BMD (Cooper and Eastell 1993, Toss 1992).

Bone density and osteoporosis

Low BMD is an indicator of osteoporosis, since the latter is defined as a BMD value that is greater than or equal to 2.5 standard deviations (SD) below the young adult, gender-matched mean (WHO 2000). With age, BMD declines due to decreases in physical activity, alterations in hormone status, and losses in muscle mass. Attainment of high peak bone mass during youth helps to decrease risks of osteoporosis (Teegarden et al. 1995). Furthermore, a diagnosis of osteoporosis is associated with increased risk of skeletal fractures. In fact, if BMD can be maximized during youth so that osteoporosis is avoided in later life, the risk of bone fracture is also lessened (Teegarden et al. 1995). Although De Laet and colleagues (1997) found that the risk of hip fracture increases with age at a rate beyond that associated with decreases in BMD in elderly individuals, achievement of peak BMD and attenuation of losses in BMD are critical components of osteoporosis prevention.

Stress fractures

Stress fractures may be categorized as overuse injuries (Beck 1998). If bone is subjected to repeated cycles of stress through exercise, fractures can occur. These stress fractures are sustained at a lower threshold of load than would be required for bone fracture by a single application of stress (Johnson et al. 1999).

Stress fractures affect 1 to 30% of military trainees (Kelly et al. 2000, Cline et al. 1998). Discrepancies in the incidence rate of stress fractures are primarily due to differences in the criteria and diagnostic technologies used to define stress fracture occurrence. Nonetheless, the majority of stress fractures occur in female recruits (Cline et al. 1998).

Bone density is often considered a key factor that may predetermine stress fracture rates in military trainees (Kelly et al. 2000), but this is not a certainty. For example, in a study involving female army personnel, neither BMD nor calcium intake correlated with stress fracture risk at the tibia, metatarsal, pubic ramus, or femur (Cline et al. 1998). Conversely, a low femoral neck BMD was significantly correlated with a high incidence of stress fractures in female military members in a separate study (Lauder et al. 2000). A previous study of

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active-duty military women by Lauder and colleagues (1999) showed little evidence of low BMD at the lumbar spine or femoral neck, but the population reviewed in the study was a group with very high physical activity levels—a trait associated with high BMD. Other research has shown that low BMD, low calcium intake, or low dairy food intake was associated with high risk of stress fractures (Clarkson and Haymes 1995).

Two studies investigated the association between pre-enlistment physical fitness and stress fracture occurrence. In the first of these studies, Kelly and associates (2000) examined self-reported activity level and stress fracture incidence among female Navy recruits. A significant relationship between fitness and occurrence of pelvic stress fractures was not observed in Kelly's et al. (2000) study. In the second study, pre-enlistment physical fitness level, based on performance from a 2000 meter run, did not have an association with incidence of stress fractures of the tibia, metatarsals, or femur (Gofrit and Livneh 1994). However, it is questionable whether a single performance run provided a true indicator of usual physical activity level. Without an adequate measure of pre-enlistment fitness, associations between BMD and stress fractures were tenuous.

While relationships between BMD and stress fractures may be equivacable, it is clear that high intensity exercise is associated with high stress fracture rates (Lauder et al. 2000). While both intensity and duration of physical activity likely affect stress fracture rates (Kelly et al. 2000), intensity of activity appears to have more impact on stress fracture occurrence compared to duration of exercise (Branca 1999). High intensity exercise may not only increase development of stress fractures by physical insult, but may also add to fracture risk by decreasing BMD. High intensity exercise is any activity that places a greater strain on the skeletal system, in contrast to low strain effects associated with weight-bearing exercise, such as a brisk walk. For example, Etherington and colleagues (1999) measured BMD using calcaneus ultrasounds in Army recruits undergoing a 10-week basic training regimen. These researchers found that heel BMD was reduced during the study, and they attributed the decrease in bone density to an overly intense exercise regimen.

Exercise and bone density

Bone strength is largely determined by the bone size, internal architecture, bone mineral composition, and mineral distribution (Sievanan 2000). Bone is in a constant flux; a

continual process of resorption and formation called remodeling in the adult (Price et al. 1994). Exercise affects bone strength through accelerating the process of remodeling (Heaney 1994, Johnson 1999, Price et al. 1994). Bone remodeling begins about five days after the start of a new exercise pattern, with bone resorption leading the remodeling process; bone replacement by subsequent bone formation continues for the following six months (Beck 1998).

The period during which remodeling rates change is called the bone-remodeling transient (Heaney 1994). During this time, bone is fairly porous rendering it susceptible to injury (Beck 1998). Bone remodeling can be detrimental to bone strength or result in lower BMD if the resorption process takes place more rapidly than formation (Heaney 1994). Long-duration high intensity exercise has been associated with decreases in bone weight in animals and lowered BMD in humans (Forwood and Burr 1993). Kiiskinen found that increasing the duration of training from 30 minutes per day to three hours per day caused decreased femoral bone volume in mice, as reported by Woo(Woo et al. 1981).

Bone density is greatly affected by weight bearing exercise, especially during youth (Ulrich et al. 1999). The magnitude of the effect of exercise on BMD is not certain, as research shows varying results. Among prepubescent boys, every hour of weight bearing activity per day was associated with a 2% increase in total body BMD (Branca 1999). Physical activity, as assessed by self-reported questionnaire, accounted for a 3.6 to 5.8% increase in total skeletal BMD in adolescent girls (Vladimarsson et al. 1999). Vuori (1996) reported that exercise and physical activity were linked to an increased peak BMD of 7 to 8% in young adults, children and adolescents. Valimaki and associates (1994) studied activity levels in adolescents and young adults over an 11-year time period. Weight-bearing exercise was one of the most significant lifestyle factors influencing BMD of the lumbar spine and femoral neck (Valimaki et al. 1994).

After peak BMD is attained, weight-bearing activity can halt or slow bone losses associated with the aging process (Forwood and Burr 1993, Kelley et al. 2000). A study by Davee and associates (1990) found that women aged 20 to 30 years who participated in resistance training regimens had significantly higher BMD at the lumbar spine. Ulrich and colleagues (1999) found that self-reported weight-bearing physical activity levels were

significantly correlated with total body BMD among women aged 28 to 50 years. Resistance training for postmenopausal women was shown to increase BMD at the hip compared to aerobic training (Kerr et al. 2001).

Some evidence exists to support site-specific effects on BMD due to exercise training. Among adult male subjects, it was shown that men who engaged in upper body resistance-training activities had significantly greater BMD of the upper limbs than did runners or cross-trainers (Hamdy et al. 1994). A meta-analysis of eight studies showed BMD increases of 2.6% in site-specific measurements, but a lower gain was found in studies that did not assess BMD sites specific to the exercise loading program (Kelley et al. 2000).

The effects of exercise during military training has been the focus of many studies. The high- intensity of basic training is associated with high rates of stress fracture (Kelly et al. 2000, Cline et al. 1998). Army recruits aged 18 to 21 years performed 48 hours of physical training per week for 14 weeks. These subjects showed increased BMC of the leg, but also incurred a stress fracture rate of approximately 40% (Forwood and Burr 1993). In a separate study, weight-bearing activity increased tibial BMD in Swiss military trainees aged 20 to 22 years by 1.1 to 2.5%, while lumbar BMD decreased with the strenuous exercise (Casez et al. 1995). The losses in lumbar bone were recouped within two years, and were considered transitory. Wide variations in exercise levels were of concern in this study (Casez et al. 1995).

Even under conditions of inadequate calcium intake, exercise has been shown to increase BMD in female athletes (Anderson 2000, Weaver 2000). It is generally concluded that non-weight bearing exercise is of little benefit in improving or maintaining bone health (Ulrich et al. 1999), while either weight-bearing aerobic or resistance training is able to provide benefits to bone (Davee et al. 1990).

Diet and bone health

Dietary factors play key roles in achieving or maintaining peak bone mass. For example, fruit and vegetable intake have both been associated with higher BMD at the femoral neck (Muhlbauer and Li 1999, New et al. 2000). Chronic dieting, weight loss after 18 years of age, and low body mass index (BMI) have all been associated with low total hip, lumbar spine, radial shaft, and ultradistal wrist BMD (Holbrook and Barrett-Connor 1993).

These effects may be due to lower intake of nutrients, as well as to decreased loading of bone associated with reductions in body mass. There are many nutrients that play roles in bone health: calcium, phosphorous, vitamin D, sodium, copper, zinc, protein, and others. Discussion is limited to the three nutrients contained in the nutrient supplement used in the present study—calcium, vitamin D, and vitamin K. The mechanisms of action and human nutrient requirements for these micronutrients are reviewed here.

Calcium

Calcium is the single most abundant mineral in the human body, constituting 1.5 to 2% of body weight (Clarkson and Haymes 1995). The average adult has 1000 to 1200 grams of calcium in the body at any given time (Swaminathan 1999). Approximately 99% of calcium is used to form bone structures with most of the remainder found in the circulatory system (Clarkson and Haymes 1995). Calcium has been described as "the only nutrient whose storage form serves a functional role" (p 579S, Weaver 2000).

Calcium stores in bone and tissues, as well as the serum levels are constantly regulated by a system involving PTH, calcitriol, and calcitonin (Clarkson and Haymes 1995). A low level of serum calcium causes release of PTH. Parathyroid hormone stimulates calcium reabsorption by the distal tubule of the kidney, calcium release from bones, and production of calcitriol—the active form of vitamin D (Favus et al. 1999, Greenspan and Gardner 2001). Calcitriol increases intestinal absorption of calcium, acting to improve absorption across the brush border of the intestine, flow through cellular cytosol, and transport across the basolateral membrane into the bloodstream (Favus et al. 1999, Greenspan and Gardner 2001). The actions of PTH and vitamin D combine to increase the serum calcium level. Figure 1 diagrams the mechanism of regulation for hypocalcemia.

Figure 1. Parathyroid hormone, vitamin D, and calcium homeostasis

↓ Serum Calcium

↑ PTH

↑ Vitamin D activation   ↑ Calcium reabsorption by kidney   ↑ Calcium release from bone

↑ Intestinal Calcium absorption

↑ Serum Calcium
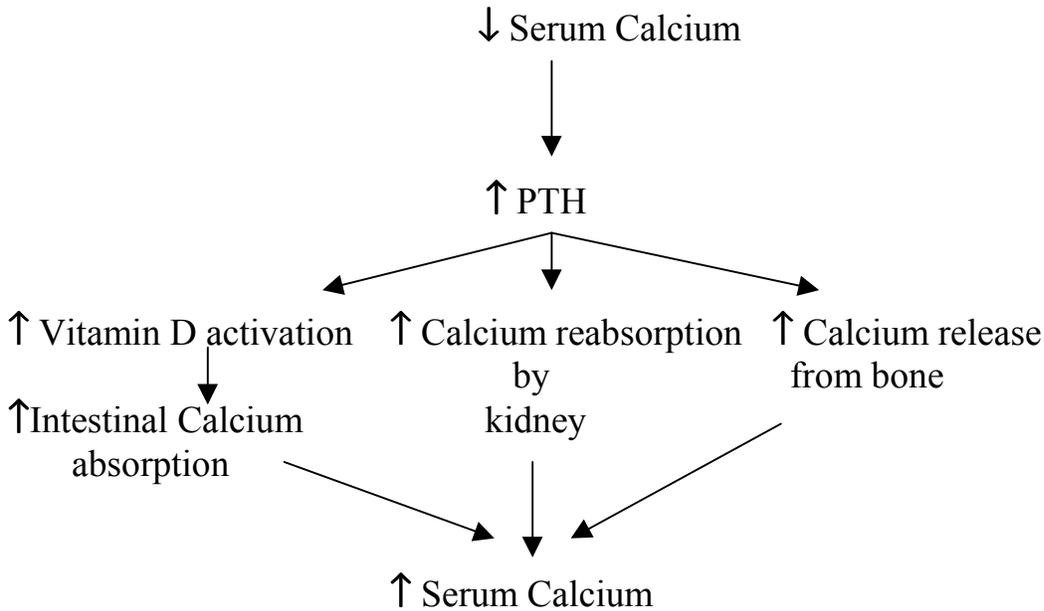
Most Americans consume inadequate amounts of dietary calcium, especially adolescent females (Clarkson and Haymes 1995). The average calcium intake in the United States was found to be 743 mg/day (National Research Council 1990). Women engaging in military field training consume similar nutrient profiles compared to nutrient profiles of average civilians, including the typical inadequate calcium intake (King et al. 1993). In situations involving intense exercise training, this deficiency may be even more marked, as Dietary Reference Intakes (DRIs) for nutrients do not account for varying activity levels (Clarkson and Haymes 1995).

Studies investigating calcium and BMD generally show improvements with high dietary calcium intakes or with calcium supplementation. Additionally, the effects of dietary calcium intake on BMD may be site-specific, as evidenced by one study in which usual dietary calcium intake did not correlate with forearm density measurements in young females aged 11 to 15 years (Maggiolini et al. 1999). Bone density was not measured at sites other than the forearm in this study. Calcium intake data obtained from one 24-hour dietary recall

was able to predict BMD 18 years later in 360 men and women (Holbrook and Barrett-Connor 1995).

Studies may be categorized by age group, which translates to bone formation rates of the subjects: rapid bone formation (generally considered <18 years of age), bone maintenance (18 years of age to menopausal), and bone degeneration (postmenopausal ages). Generally, more impressive results have been shown with dietary calcium supplementation during the years of peak bone formation. A study that involved 8- to 12-year-old identical twins in which one child from each twin set was supplemented with ~700 milligrams (mg) of calcium daily, as calcium citrate malate, reported that, on average, the supplemented child possessed 3 to 4% more BMD at the radius, spine, and Ward's triangle, compared to the unsupplemented twin after three years (Johnston et al. 1992). A group of 12-year-old girls consuming 500 mg of calcium per day, in the form of calcium citrate malate had a 1.3% increase in total body BMD compared to unsupplemented counterparts (Lloyd et al. 1993).

Women aged 18 to 30 years who consumed supplements of ~ 500 mg of calcium per day showed attenuation in total body BMC losses over 6 months (Peterson et al. 2000). Subjects were allowed to select their own calcium supplements; thus, the form of calcium consumed varied (Peterson et al. 2000). A meta-analysis of 33 studies revealed that supplementation with 1000 mg of calcium per day decreased BMD loss at the lumbar spine, femoral neck, Ward's triangle, and trochanteric region by 1% per year in 30- to 55-year-old women (Welten et al. 1995). A study by Baran and colleagues (1989) showed that the addition of ~600 mg of calcium per day attenuated vertebral bone losses in 30- to 42-year-old women. During this three-year study, women in the control group experienced a mean decline in BMD of 2.9% while the supplemented women experienced no bone loss (Baran et al. 1989).

A meta-analysis conducted by Cumming (1990) showed that dietary calcium intake decreased the rate of post-menopausal bone loss in all studies reviewed. The effect of calcium was most beneficial for subjects whose baseline dietary calcium intakes were low. A non-interventional study by Suleiman and colleagues (1997) showed that dietary calcium intake significantly correlated with bone mass at the spine and hip, and with total body BMC in healthy postmenopausal women aged 52 to 62 years.

Vitamin D

Vitamin D plays a crucial role in maintaining proper calcium balance in the body, thereby affecting bone health. Calcitriol triggers an increase in intestinal absorption of calcium (Clarkson and Haymes 1995); thus, inadequate vitamin D intake may result in insufficient absorption of dietary calcium. Plasma calcitriol level has a positive correlation with BMD and an inverse correlation with serum PTH (Swaminathan 1999). Dietary vitamin D and vitamin D synthesized by the body through sun exposure is converted to 25-hydroxy vitamin D—25(OH)D—in the liver. Plasma 25(OH)D was significantly lower in elderly individuals who sustained bone fractures compared to plasma 25(OH)D of uninjured counterparts (Swaminathan 1999). The average vitamin D intake in this country was found to be 1.5-2.1 micrograms/day, with fortified dairy products as the major source (National Research Council 1990).

Hunter and colleagues (2000) studied the effects of 800 International Units (IU) per day of vitamin D supplementation on BMD in postmenopausal women aged 47 to 70 years. Researchers found that while serum levels of vitamin D were elevated at 6 months in the supplemented group, other significant changes in vitamin D levels or in BMD were not found during the two-year study (Hunter et al. 2000). A recent study conducted in Finland evaluated effects of vitamin D supplementation on BMD, PTH level, and urinary calcium excretion (Sairanen et al. 2000). Subjects maintained a consistent calcium intake of 800 mg per day, and half were supplemented with 0.05 μg of calcitriol per day. Vitamin D supplementation resulted in significant increases in hip BMD, and positive changes in biomarkers of bone formation. Supplementation had to be decreased after two years, however, due to hypercalcuria (Sairanen et al. 2000).

Calcium and vitamin D combination

Several studies have examined effects of concurrent supplementation with calcium and vitamin D, some utilizing fortified dairy products and others using vitamin and mineral supplements. A group of 9- to 13-year-old girls supplemented with 700 mg of calcium per day via dairy products experienced an ~6% increase in total BMC compared to a group of unsupplemented 11-year-old girls (Chan et al. 1995). Lumbar spinal BMD increased by ~22.8% in the supplemented group, compared to 12.9% in the control group during this 12-

month study. A study involving 12-year-old girls who received supplementation with ~400 mg of calcium per day via milk products showed a 1.3% improvement in total body BMD versus a control group (Cadogan et al. 1997). Pre-adolescents with diseases such as milk allergies frequently have limited intakes of calcium and vitamin D due to restricted consumption of dairy products. Infante and Tormo (2000) found that among 30 pre-adolescents with diseases requiring restricted dairy intake, half had either osteopenia or osteoporosis.

In the years following peak bone mass formation, calcium with vitamin D may prove beneficial to bone maintenance. This vitamin and mineral combination showed protective effects for vertebral BMD in a group of 30- to 42-year-old women. A 2.5% attenuation in bone loss occurred for women who consumed ~600 mg of additional calcium per day via dairy products compared to women who did not consume additional dairy food products (Baran et al. 1989).

A vitamin D plus calcium combination also appears beneficial for humans in the age groups susceptible to bone loss. In a double-blind placebo-controlled study, elderly individuals in the experimental group received 500 mg of calcium plus 700 IU of vitamin D daily. The supplemented subjects had a 50% less incidence of hip fractures compared to an unsupplemented group of elderly individuals (Tanne 1997). Withdrawal of calcium and vitamin D supplements in a group of women and men aged 68 years or greater has also been examined (Dawson-Hughes et al. 2000). Subjects who participated in a three-year study of supplement use were monitored for an additional two years after supplements were withdrawn. Male subjects retained slight improvements in total body BMD, but they lost the BMD gains that they had accomplished at the hip and spine. Female subjects returned to their pre-study BMD levels and did not sustain any lasting benefits from having received supplementation (Dawson-Hughes et al. 2000).

Vitamin K

Vitamin K is required for the carboxylation of glutamic acid (Swaminathan 1999). Several bone tissues are dependent on vitamin K, including matrix gla protein. Gla proteins contain dicarboxylic glutamyl residues which enhance calcium-binding activity (Favus et al. 1999). Matrix gla protein is thought to be involved in the control of mineral deposition

(Favus et al. 1999). Osteocalcin, which comprises ~15% of non-collagenous bone protein, is vitamin K dependent (Swaminathan 1999). Osteocalcin in the blood of osteoporotic subjects was shown to be undercarboxylated, and responded favorably to vitamin K supplementation Favus et al. 1999). Undercarboxylated osteocalcin has also been associated with increased fracture risk (Swaminathan 1999). Vitamin K supplementation has been associated with some increases in BMD levels (Swaminathan 1999), but few studies have been conducted assessing these effects. The average intake of vitamin K in the United States is 300-500 micrograms/day, but additional vitamin K is synthesized by intestinal bacteria (National Research Council 1990).

Effects of diet plus exercise

It is plausible that an exercise program, combined with vitamin and/or mineral supplementation, would have a greater effect on BMD than either exercise or nutrient intake alone. Indeed, the increased level of calcium required during bone remodeling makes this a likelihood. Holmes-Walker and colleagues (1995) stated that premenopausal women require exogenous calcium sources during exercise to optimize the bone-building effects of exercise. However, studies regarding interactions between dietary intakes of nutrients and exercise have been inconclusive.

Results from a correlational study of over 1600 European females aged 11 to 23 years showed that neither calcium intake nor exercise affected bone mineral status (Kardinaal et al. 2000). In a two-year study, women aged 20 to 35 years were supplementated with calcium to provide a total daily intake of 1500 mg (Friedlander et al. 1995). Exercise resulted in a 1.3 to 2.6% increase in femoral trochanter region BMD, while calcium did not have an effect on BMD (Friedlander et al. 1995). In a cross-sectional study of women aged 25 to 34 years, vertebral BMD was associated with exercise level, but not with dietary calcium intake (Kanders et al. 1988). If effects of exercise were eliminated, a correlation between calcium intake and bone mass was observed. Both males and females were divided into age groups in a 15-year longitudinal study (Welten et al. 1994). Weight-bearing exercise among males and females correlated with high BMD, but dietary calcium intake was not a significant predictor of bone health in any age group (Welten et al. 1994). However, subjects in Welten's et al. (1994) study had high dietary protein intakes that may have had a negative interaction with

calcium. Similar results were shown in a separate study with 18-year-old women in whom past activity levels were associated with current BMD at the lumbar spine, proximal femur, distal tibia, and distal fibula—but past calcium intakes were not (Henderson et al. 1995). However, Henderson's et al. (1995) study did not include an intervention, but rather relied on self-reported activity and diet information. In fact, none of the aforementioned studies were originally designed to investigate an interaction between dietary calcium intake and exercise in relation to BMD.

A meta-analysis of 17 studies showed that calcium intakes totaling 1000 mg per day, combined with exercise, resulted in significantly greater improvements in lumbar spine BMD but not in distal radius BMD, than exercise alone (Specker 1996). Effects of calcium supplementation were greatest in the lumbar spine region (Specker 1996). Additionally, both calcium intake and exercise were shown to have significant and positive correlations with spinal, hip, and total body BMD in women aged 52 to 62 years (Suleiman et al. 1997). Yet again, this meta-analysis did not include studies that were originally or exclusively designed to investigate the interactive effect of dietary calcium intake and exercise on bone.

Hormones and bone mineral density

Hormones play vital roles in all phases of bone metabolism (i.e., growth, maintenance, and degeneration) throughout the lifecycle. Estrogen, perhaps, is the hormone most frequently associated with bone changes due to the bone loss associated with estrogen decreases after menopause (Kohrt et al. 1998). While estrogen and PTH are vital to maintenance of bone health and calcium balance, several other hormones also have important functions. Testosterone, insulin-like growth factor-1 (IGF-1), and growth hormone (GH) all play roles in bone growth and maintenance. Additionally, levels of these hormones may be affected by physical activity.

Testosterone

Testosterone is a steroid hormone produced primarily by the testicular Leydig cells and is controlled partially by luteinizing hormone (Amin et al. 2000, Greenspan and Gardner 2001). In males, only ~5% of testosterone is secreted by sources other than the Leydig cells. In females the ovaries produce ~25% of testosterone found, while the remainder is converted in peripheral tissues from precursor molecules (Greenspan and Gardner 2001). Testosterone

plays important roles in sexual maturation especially in males and has been shown to affect both muscle formation and bone development (Greenspan and Gardner 2001, Hakkinen et al. 2000).

The role of testosterone in bone development and maintenance has been debated. It has been demonstrated that young hypogonadic males have low BMD and that testosterone replacement can partially reverse this trend (Amin et al. 2000, Greenspan and Gardner 2001). Osteoporotic women also show improvements in bone mass with androgen supplementation (Greenspan and Gardner 2001). In older males, however, hypogonadism may play a much smaller role in the control of bone status (Amin et al. 2000).

Much of testosterone's actions are thought to be through its influences on GH secretion, but some research indicates that testosterone may also raise IGF-1 levels through non-GH-dependent mechanisms (Ohlsson et al. 1993). Androgens inhibit osteoclast activity and the production of interleukin-6 (a pro-bone resorptive cytokine), thereby potentially decreasing bone resorption rates. Additionally, testosterone has been shown in *in vitro* studies to have anti-apoptotic actions that serve to protect osteoblasts from cell death (Falahati-Nini et al. 2000). Receptors for testosterone have been found in cartilage, which can convert this hormone to dihydrotestosterone, a more active metabolite (Ohlsson et al. 1993). Overall, it has been postulated that while estrogen plays a more important role in stimulating bone formation in young females and preventing bone resorption in older females, testosterone has a strong impact on bone formation rates, especially in young males (Falahati-Nini et al. 2000).

Testosterone is affected by exercise, but the degree to which it is affected is unclear. Hakkinen and colleagues (2000) showed acute elevations in testosterone levels in response to resistance training, but did not show a prolonged systemic change in the hormone. These findings were corroborated by a study by Kraemer and associates (1998). Conversely, a more recent study did not demonstrate acute or chronic changes in serum testosterone in response to strength or endurance training (Bell et al. 2000). Alen and associates (1988) have suggested that while total serum testosterone may show little long-term change with exercise, testosterone turnover rates may be affected. In support of Alen's et al. (1988) hypothesis, Hakkinen and colleagues (2000) also postulate that exercise may cause alterations in the testosterone receptor.

In young males, testosterone has an important role in bone formation. Additionally, exercise plays a larger role in controlling testosterone levels in this age group compared to any other age group (Kraemer et al. 1998).

Insulin-like growth factor-1

Insulin-like growth factor-1 has been described as "perhaps the most abundant growth factor secreted by bone" (p B83, Thompson et al. 1996). Not only is IGF-1 abundant in the bone, but it appears to affect bone health through a myriad of pathways (Canalis and Agnusdei 1996). *In vitro* studies have shown that IGF-1 stimulated proliferation of bone cells and collagen synthesis and contributed to up to 50% of the proliferation in bone cell cultures (Hayden et al. 1995). Insulin-like growth factor-1 also stimulated bone growth in *in vivo* studies, and increased osteoclast differentiation (Hayden et al. 1995). Insulin-like growth factor-1 increases both longitudinal bone growth (Ohlsson et al. 1993) and circumferential bone growth (Thompson et al. 1996). Previous research has documented decreased levels of IGF-1 in osteoporotic postmenopausal women compared to healthy counterparts indicating a possible relationship with bone resorption through an impact on osteoclasts (Thompson et al. 1996).

The roles of exercise and diet as modulators of IGF-1 are controversial because of conflicts in study results. Several studies have shown little change in IGF-1 levels associated with exercise programs, but other investigations have found large changes (Kraemer et al. 1998). Exercise may result in short-term alterations in IGF-1, which could impact bone health despite the transient nature. One recent study found significant transient differences in IGF-1 concentrations following heavy and moderate bouts of resistance training (Raastad et al. 2000). Cadogan and associates (1997) showed a significant increase in IGF-1in adolescent girls who were supplemented with one pint of milk daily. These findings were also associated with a significantly higher increase in total body BMD among the experimental group compared to control group.

Growth hormone

Growth hormone also influences bone health throughout the life cycle. During youth and adolescence, GH is crucial for longitudinal bone growth and for maximizing skeletal development (Favus et al. 1999, Ohlsson et al. 1993, Saggese et al. 1993). Growth hormone

has also been shown to be vital to maintaining bone health in adults. Johansson and colleagues (1994) found that indices of GH secretion strongly correlated with total body, lumbar spine, and hip BMD in healthy males aged 25 to 59 years. In female rats with GH releasing hormone (GHRH) deficiencies, deterioration in bone structure similar to that associated with aging was found (Thompson et al. 1996). Much of the action of GH on bone metabolism may occur through stimulation of IGF-1 production (Favus et al. 1999, Thompson et al. 1996).

Serum GH levels typically rise in response to exercise (Hakkinen et al. 2000, Kraemer et al. 1998, Raastad et al. 2000). However, changes in GH appear to vary widely, depending on the type and frequency of exercise (Raastad et al. 2000). Hakkinen and colleagues (2000) found that exercise stimulated a significant elevation in GH in men aged 40 to 75 years and in women aged 36 to 42 years. It is plausible that exercise may be able to counteract decreases in GH that are typically observed during aging (Greenspan and Gardner 2001).

In summary, diet and exercise both play major roles in bone health. Young-adults in military training situations are susceptible to changes in bone density and to stress fractures. Use of a dietary supplement containing a combination of calcium, vitamin D, and vitamin K during military cadet training may positively influence BMD and stress fracture rate and therefore should be investigated further.

Review of methods

There are a wide variety of methods for measuring bone mineral density, hormones, and biochemical markers of bone change. These methods vary in accuracy, specificity, and convenience of use. Selecting valid and appropriate means of assessment is of paramount importance. Current methods of evaluating these parameters are discussed below.

<u>Measurement of bone mineral density</u>

Bone density can be measured by many methods, including single-photon absorptiometry, dual-energy photon absorptiometry, and quantitative computed tomography (Johnston et al. 1991). An established tool for deriving BMD and predicting osteoporosis (Sievanen 2000), dual energy X-ray absorptiometry (DXA) scans are required for precise measurement of BMD at the spine and hip (Johnston et al. 1991). In addition to measuring BMD, DXA is able to measure bone mineral content (BMC) and provides data regarding soft tissue composition (Madsen et al. 1997). Dual energy X-ray absorptiometry has been

validated as a tool for precise estimation of three body composition components and for accurately measuring changes in total body composition (Madsen et al. 1997).

<u>Measurement of testosterone, insulin-like growth factor-1, and growth hormone</u>

Radioimmunoassays are widely utilized to measure hormone concentrations. Monoclonal antibodies are chosen which are specific to the hormone to be measured (Greenspan and Gardner 2001). The radioimmunoassay utilized for testosterone measurement can detect hormone levels as low as 4 ng/dL (Diagnostic Products Corporation 1999). Insulin-like growth factor was measured by radioimmunoassay as described in the study by Weber et al. (1998). The GH radioimmunoassay used in the current study utilizes a double antibody technique with crossreactivity < 1%, to improve specificity (Diagnostic Products Corporation 1997).

<u>Measurement of biomarkers of bone turnover</u>

Osteocalcin is a hormone that acts as a clinically significant marker of bone formation and turnover (Adachi 1996). It is manufactured by osteoblasts and chondrocytes (Calvo et al.1996). Osteocalcin production is dependent on both vitamin D and vitamin K (Calvo et al. 1996) and forms about 20% of the non-collagenous proteins found in bone (Adachi 1996). In the bone, osteocalcin is thought to interact with hydroxyapatite, and may be involved in the differentiation of osteoblasts (Calvo et al. 1996). Osteocalcin is produced during bone formation, and some of the hormone is released into the bloodstream during this phase, indicating formation (Adachi 1996, Calvo et al. 1996). Osteocalcin is also released from the bone matrix during resorption, thus may be thought of as an indicator of bone turnover (Favus et al. 1999). Radioimmunoassay is a validated method, widely accepted for measuring circulating osteocalcin levels (Calvo et al. 1996). The Biomedical Technology radioimunoassay kit has been shown to provide results consistent with commercial research laboratories (Biomedical Technologies Inc. 1999).

Bone collagen is found in the form of fibrils, which are connected by covalent cross-links called telopeptides (Favus et al. 1996). N-telopeptide cross-links (NTx) are amino-terminal telopeptides, and serve as markers of bone resorption (Adachi 1996, Favus et al. 1999). N-telopeptides are released into circulation during bone resorption and are eventually excreted in the urine (Favus et al. 1999). Enzyme-linked immunosorbent assays (ELISA)

provide an efficient and convenient measure of urinary N-telopeptides (Osteomark NTx, 2001), can be as sensitive a measure as radioimmunoassays, and provides a working range of 1.0 to 50.0 nanograms per milliliter (Greenspan and Gardner 2001). N-telopeptide levels are reported adjusted to urine creatinine levels to standardize for urine concentration.

Diet records

Diet records are established tools for assessing nutrient intakes. Diet records are considered the "gold standard" for evaluating dietary intake in experimental studies (Taitano et al. 1995). Studies designed to validate other forms of dietary-intake assessment use multiple-day food records as the standard for verifying the accuracy of alternative methods (Margetts et al. 1989, Musgrave et al. 1989).

Summary

Osteoporosis is a chronic, destructive condition affecting millions worldwide. It has high physical and financial costs. High BMD during youth can prevent or delay the onset of osteoporosis (Teegarden et al. 1995) and may assist in decreasing occurrence of stress fractures. Stress fractures are a concern for athletes and military personnel. These injuries have been a documented problem in military trainees for more than 100 years (Johnson et al. 1999).

Bone density is affected by many factors. Diet and exercise have been identified as the two most important controllable factors determining BMD (Cooper and Eastell 1993). Proper dietary intake ensures the presence of necessary vitamins and minerals, such as calcium, vitamin D, and vitamin K. Exercise increases the rate of bone remodeling—the resorption, formation, and development of new bone (Price et al. 1994). Numerous studies have confirmed the vital importance of proper nutrition and weight-bearing exercise in the maintenance of bone health.

Findings from previous studies on the combined effects of exercise and calcium supplementation have been largely inconclusive. There is a scarcity of published data regarding effects of exercise in conjunction with a vitamin and mineral supplement specifically formulated for bone health on bone health. The current study investigated the effect of a calcium, vitamin D, vitamin K supplement combination during exercise-training on

BMD, selected hormones, and biochemical markers of bone turnover in young-adult Corps of Cadets recruits at VPI and SU.

CHAPTER 3

MATERIALS AND METHODS

The experimental design for this research proposal was approved by the Institutional Review Board for research involving human subjects at VPI and SU. Additionally, the Commandant of the Corps of Cadets at VPI and SU approved the project prior to its initiation.

Participants

Male (n = 22) and female (n = 8) adults, aged 18 to 22 years, regardless of ethnicity, participated in this longitudinal study. All incoming VPI and SU Corps of Cadets freshmen (N=272) were invited to participate in the screening process. Potential participants were provided with a packet containing an introductory letter describing the study, a Medical History/Screening Form, and a 3-Day Dietary Record. Interested potential participants returned their Medical History Forms and 3-Day Dietary Records in enclosed, self-addressed, stamped envelopes to the investigators for screening. Recruits were also invited to participate during their initial training week at VPI and SU.

Volunteers meeting the inclusion criteria were invited to participate in the full study. Inclusion guidelines included proper age range, general good health status, and absence of exclusion criteria. Exclusion criteria included history of bone disease or recent fractures (n = 1), use of medications affecting bone metabolism (n = 0), endocrine, renal, and/or metabolic diseases including kidney stones and blood clotting disorders (n = 0), and cigarette smoking (n = 0).

Few studies have been conducted regarding changes in BMD with calcium supplementation and the initiation of military exercise training in young adult individuals. However, for purposes of establishing an appropriate sample size based on smallest changes expected over time, changes in lumbar spine BMD for active-duty Army women as reported by Lauder and colleagues (2000) were used. Necessary data included sample size (n = 185), mean lumbar spine BMD with usual physical activity patterns (1.240 g/cm^2), estimated lumbar spine BMD with increased military training (1.283 g/cm^2), and estimated standard deviation of measurements (0.040). The correlation between lumbar spine BMD was greater than 0.80. The effect size (d = 1.04) for the change in BMD was calculated. To examine the main effect of exercise training on BMD measures over time (main effect for 2 x 2 ANOVA

with repeated measures over time), where d = 1.04, alpha = 0.05, r = 0.80, and n = 30, a statistical power of 0.98 was found. Inclusion of thirty participants was established to allow sufficient power to detect differences in BMD measures, the dependent variables of primary interest. Forty-three cadets volunteered, but several were ineligible due to age (n = 6) or to medical concerns (n = 1). Thirty-six cadets were originally enrolled in the study, but several dropped from the Corps of Cadets program (n = 4) and two quit the study due to time constraints, leaving 30 cadets to complete the study.

Study protocol

Each participant meeting the selection criteria for the study was provided with an Informed Consent Form at least one week in advance of the baseline testing session. Testing sessions were conducted at two timepoints: baseline at the beginning of the Fall, 2000 (August) semester and in December, 2000, after ~4 months of Corps regulated exercise training. Testing sessions were completed in the Bone metabolism, Osteoporosis, and Nutrition Evaluation (BONE) Laboratory, Room 229 Wallace Hall, on the VPI and SU campus. Testing sessions involved measurement of body height and weight and completion of dual energy X-ray absorptiometry (DXA) scans. Body height was measured using a wall-mounted stadiometer (Heightronic™, Measurement Concepts, North Bend, WA), and body weight was measured using a digital scale (Scaletronix, Wheaton, IL). Participants were lightly clothed and shoeless during body height and weight measurement.

Fasting blood and second void urine samples were collected at baseline and after 4-months of military training, either during the bone density testing appointment, or another scheduled time for the convenience of the participant, but within seven days of corresponding DXA scans. One participant was unable to provide baseline blood and urine samples, so DXA results were included in the study, but evaluation of hormonal changes and biomarkers of bone change were not. For remaining participants, whole blood (10 mL) was drawn following an overnight fast (nothing to eat or drink except for water) of at least 10 hours. Blood was centrifuged, and serum was pipetted into 2 mL cryovials and stored at -80° C until analyzed. Participants also provided a second void urine sample (~ 60 mL; instructed to urinate once in the morning before the sample was collected). Urine samples were pipetted into 3, 2 mL cryovials and stored at -80° C until analyzed.

For both testing sessions, each participant was allowed to complete all procedures in an unhurried fashion. At the follow-up testing session in December, 2000, a Medical/Physical Activity Update was completed. Participants also completed another set of 3-Day Dietary Records, or provided 3-Day Food Recalls.

Experimental treatment

Cadets began physical training in the week prior to the beginning of the Fall semester. Cadets were assigned to one of the four Reserve Officer Training Corps (ROTC) detachments located at VPI and SU and performed Physical Training (PT) activities with these ROTC groups. The Air Force and Navy ROTC programs performed PT 2 times per week, while Army and Marine Corps ROTC exercised 3 times weekly. Cadets with difficulty maintaining physical standards were identified for remedial PT programs. Cadets were also free to participate in extra-curricular exercise activities, such as sports or weight lifting, and were encouraged to perform extra PT sessions with other ROTC groups.

After the first testing session (baseline), participants were randomly stratified based on gender and initial dietary calcium intake, to either a calcium supplementation or control group with equal numbers of males and females in each group. Each calcium supplement "chew" (soft, chewy supplement with texture of a Tootsie Roll) contained 500 mg of calcium carbonate, 100 I.U. of vitamin D, and 40 ug of vitamin K. The supplement chews were commercially marketed products and were available in pharmacies and grocery stores in Blacksburg, Virginia. Participants receiving supplements were instructed to consume 1 chew per day for the duration of the study. Due to unavailability of an appropriate placebo, control subjects did not receive any supplementation during the study, but completed ROTC-based PT training programs as did the supplemented group.

Monitoring compliance

The Medical/Physical Activity Update and 3-Day Dietary Records or 3-Day Food Recall were also collected in mid-October. Supplement compliance was monitored via e-mails twice monthly throughout the study. Supplies of supplements were provided monthly, and unconsumed supplements were collected at these times to corroborate reported compliance.

Bone mineral density

Dual-energy X-ray absorptiometry scans adhered to the following protocol: the participant was appropriately positioned on or next to the DXA table for completion of one lumbar spine, one nondominant total proximal femur, one nondominant forearm, and one whole body scan (QDR 4500A, Hologic Inc., Bedford, MA). For consistency, one investigator administered and analyzed all DXA scans, using the Whole Body Analysis (version 8.25a, Hologic Inc., Bedford, MA) and standard spine, hip, and forearm protocols and analyses software. Quality control procedures were performed throughout the study to ensure internal quality control of DXA scans. Quality control scans were performed each day prior to subject testing, to ensure proper instrument calibration.

Measurement of hormones and biochemical markers of bone turnover

Serum and urine samples were thawed and returned to room temperature prior to measurement of target compounds. All assays were run in duplicate. Radioimmunoassay (RIA) was used to measure GH, IGF-1, and total testosterone. Growth hormone was measured using a double antibody RIA kit (Diagnostic Products Corporation, Los Angeles CA). The intra-assay coefficient of variation (CV) for GH was 13.6 %. The IGF-1 measurements were accomplished in accordance with protocol described by Weber et al. (1998). The intra-assay CV for IGF-1 was 7.7 %. Total testosterone was measured with a Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles CA). The intra-assay CV for testosterone was 9.0 %. Serum osteocalcin was measured by RIA (Biomedical Technologies Inc., Staughton, MA). The intra-assay CV for osteocalcin was 8.3 %. Urinary NTx was measured by enzyme-linked immunosorbent assay (Osteomark, Ostex Int., Seattle, WA). Urinary NTx was normalized to urinary creatinine, which was measured by quantitative spectrophotometry (#555-A, Sigma Diagnostics, St. Louis MO). The intra-assay and inter-assay CV for NTx were 7.7 % and 1.0 %, respectively.

Dietary intake

Three-day food diaries were collected at baseline, mid-study, and at the final data collection appointment. Participants were provided with instructions for home completion of these records. All 3-Day Dietary Records or Food Recalls were coded and analyzed for average daily dietary intake of energy, protein, carbohydrate, fat, calcium, phosphorous,

vitamin D, vitamin K, and caffeine, using the Food Processor® Dietary Analysis software (version 5.0, Esha Research, Salem, OR). Supplement compliance for each study period was calculated from participants' reports. Average daily calcium, vitamin D, and vitamin K consumed was computed for each member of the supplement group, and was included in their individual dietary analysis. Some participants were unable or unwilling to complete mid-study or final food diaries, so three-day food recalls were obtained via telephone for these subjects.

Stress fractures

Information about potential stress fractures was obtained from participants' medical history updates. Subjects were queried about all injuries that affected their ability to perform exercise training. Stress fractures were defined as a diagnosis of stress fracture by the Student Health Clinic. Any subject ($n = 0$) reporting an insidious onset of atraumatic pain in an extremity, which affected their ability to exercise (Kelly et al. 2000, Johnson et al. 1999), was referred to the student health center for evaluation.

Statistical analyses

Statistical analyses were performed using the Statistical Analysis System (SAS) software. Descriptive statistics were completed to describe participants. *T*-tests were conducted to compare variables of interest between the supplemented group and the control group at baseline. A 2 x 2 (group x time) analysis of variance (ANOVA) with repeated measures on the time factor was used to measure changes within and between groups for variables of interest across time, from baseline to 13 weeks.

CHAPTER 4

RESULTS

Descriptive statistics

Thirty participants aged 18 to 20 years completed the study. The sample was comprised of 22 males and eight females, evenly divided between supplement and control groups. Selected baseline characteristics for the sample, for each study group, and for gender by group are presented in Tables 1 and 2. Two-tailed *t*-tests revealed that statistically significant differences at baseline between the control and supplement groups did not exist (p > 0.05) in any of the parameters assessed including age, height, weight, BMD, GH, testosterone, IGF-1, osteocalcin, NTx, or dietary intakes. Moreover, statistically significant differences in age, height, weight, BMD, GH, testosterone, IGF-1, osteocalcin, NTx, or dietary intakes did not exist at baseline when supplement and control groups were further divided by gender. Thus, data for males and females remained pooled in their respective supplement or control groups for all subsequent analyses.

Table 1--Selected Baseline Characteristics

CHARACTERISTIC	ALL SUBJECTS (N=30) [a]	CONTROL GROUP (N=15) [a]	SUPPLEMENT GROUP (N=15) [a]
Age (years)	18.5 ± 0.6	18.3 ± 0.6	18.6 ± 0.5
Height (cm)	175.1 ± 10.2	174.6 ± 9.4	175.6 ± 11.0
Weight (kg)	70.0 ± 10.5	67.9 ± 11.7	72.0 ± 9.3

[a] Mean ± standard deviation (SD)

Table 2--Selected Baseline Characteristics, by Gender

Characteristic	All subjects [a]		Control group [a]		Supplement Group [a]	
	Males (n = 22)	Females (n = 8)	Males (n = 11)	Females (n = 4)	Males (n = 11)	Females (n = 4)
Age (years)	18.4 ± 0.5	18.3 ± 0.5	18.2 ± 0.4	18.0 ± 0.3	18.7 ± 0.5	18.5 ± 0.7
Height (cm)	178.7 ± 7.8	164.1 ± 6.2	178.0 ± 5.7	163.2 ± 5.6	179.4 ± 9.7	165.0 ± 7.4
Weight (kg)	73.5 ± 9.2	60.2 ± 7.5	72.0 ± 10.1	56.7 ± 8.2	75.1 ± 8.5	63.6 ± 5.7

[a] Mean ± standard deviation (SD).

The greatest number of supplements missed was 20 out of 91, by two subjects over a 13-week period—an average of 1.67 supplements missed per week. In the subjects with the greatest number missed, one did not have access to his supplement for one week. Overall, subjects missed less than one supplement per week. Forgetfulness was the main reported cause of supplement non-compliance.

Bone mineral density and stress fractures

Bone density data were available for all 30 subjects. Significant changes in any measure of bone density, whether across time or between supplement and control groups were not found. Table 3 and Figure 2 present baseline and final BMD values for selected sites for all participants. Subjects did not report any loss of exercise or any medical attention required due to occurrence of stress fractures.

Table 3--Baseline and Final bone mineral density findings (g/cm^2)

Bone site (g/cm^2)[a]	Baseline— Supplement (n = 15)	Baseline— Control (n = 15)	Final— Supplement (n = 15)	Final— Control (n = 15)	P-Value[b]
Whole Body	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	NS[c]
Lumbar Spine 2-4	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.2 ± 0.1	NS
Lumbar Spine 1-4	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	NS
Femoral Neck	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	NS
Trochanter	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	NS
Ward's Triangle	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	NS
Total proximal femur	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	NS
Forearm—UD[d]	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	NS
Forearm—Mid	0.6 ± 0.1	0.6 ± 0.04	0.6 ± 0.1	0.6 ± 0.04	NS
Forearm—1/3[d]	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	NS
Forearm—Total	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	NS

[a] Mean ± standard deviation (SD)
[b] For Final vs. Baseline, combined groups (n = 30 vs. n = 30)
[c] NS = Not Significant
[d] UD = Ultra-distal, 1/3 = proximal 1/3

Figure 2--Initial versus final bone mineral density scores

Initial bone mineral density (BMD) versus final BMD scores (g/cm^2) for:
  a.   Whole body, b. Total forearm, c. Lumbar spine
  (n = 15 for supplement group and for control group in each graph)

a. Whole Body BMD

b. Total Forearm BMD

c. Lumbar spine (L1-L4) BMD
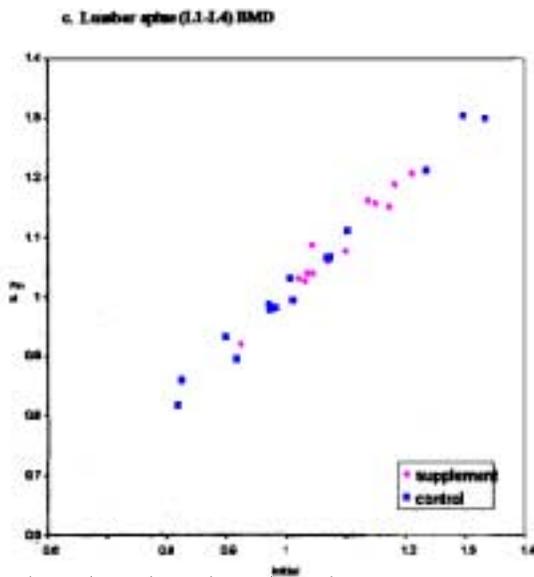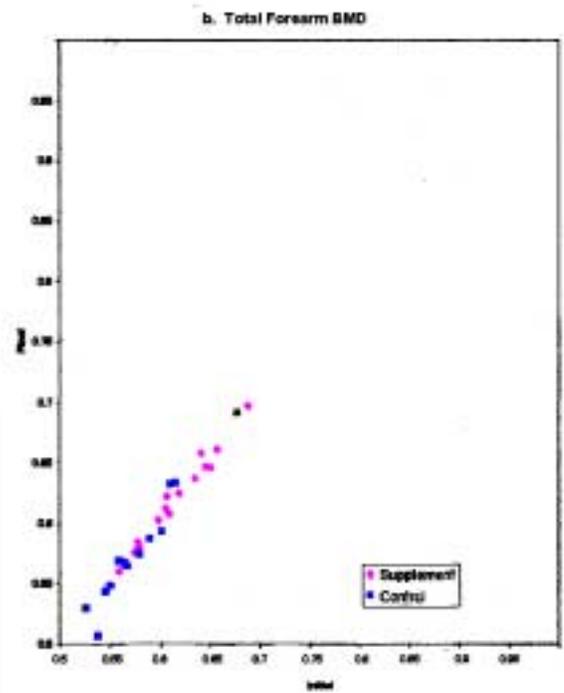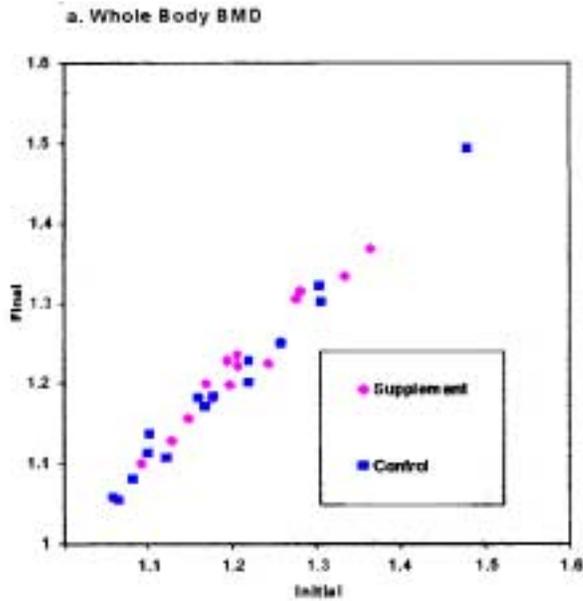
Growth hormone

Growth hormone data were available for 29 subjects. Data for four study participants (two control, two supplement group) were not utilized due to high levels of variation between duplicates, providing 25 samples for statistical analysis. In subjects with undetectably low levels of GH, a value of "1" was assigned.

Significant differences in GH levels, whether analyzed across time or between groups, were not found. Baseline GH levels had a mean of 2.2 ± 1.7 ng/ml, while final levels averaged 2.2 ± 2.8 ng/ml. Table 4 contains further breakdown of GH levels and changes.

Testosterone

Samples were available from 29 study participants for assessment of testosterone. Mean total testosterone level did not significantly differ, either between groups or across time. At baseline, testosterone averaged 5.1 ± 3.3 ng/ml, while final levels were 5.0 ± 3.0 ng/ml. Table 4 contains testosterone data.

Insulin-like growth factor-1

Twenty-nine subjects provided serum samples for measurement of IGF-1. Serum levels of IGF-1 increased significantly across time, with baseline levels of 320.8 ± 70.5 ng/ml and final readings of 372.7 ± 92.3 ng/ml ($p < 0.05$). Significant differences in IGF-1 between the control and the supplement groups did not exist. Table 4 provides additional information on IGF-1 findings from this study.

Osteocalcin

Osteocalcin data were available for 29 subjects. Table 4 contains osteocalcin levels by study group. Serum osteocalcin levels, as measured by radioimmunoassay, significantly increased across time. Mean baseline osteocalcin levels for all participants were 21.5 ± 7.1 ng/ml, while final values had a mean of 24.4 ± 7.0 ng/ml ($p < 0.001$). Significant differences in osteocalcin levels between study groups were not found.

N-telopeptide crosslinks

Twenty-nine subjects provided urine samples for analysis of n-telopeptide crosslinks. All 29 samples were analyzed for n-telopeptides, but technical difficulties allowed for assessment of creatinine of only 10 participants. Mean baseline n-telopeptide crosslinks in

bone collagen equivalents (BCE) per mM creatinine were 196.3 ± 249.3. Mean final n-telopeptide crosslinks were 118.4 ± 50.4. Significant differences across time were not found in these 10 participants. Results between groups were not analyzed due to the small sample size available for assessment.

Table 4--Results of Hormone Assays, by group

Hormone	Baseline—Supplement [a] (n = 14)	Baseline—Control [a] (n = 15)	Final—Supplement [a] (n = 14)	Final—Control [a] (n = 15)	P-Values Time [b]	Group [c]
Osteocalcin (ng/ml)	21.6 ± 6.5	21.4 ± 7.8	24.7 ± 6.3	24.2 ± 7.8	0.0010	NS [d]
Growth Hormone (ng/ml)	2.0 ± 1.6 [e]	2.4 ± 1.9 [f]	1.4 ± 0.8 [e]	2.8 ± 3.8 [f]	NS	NS
Testosterone (ng/ml)	5.0 ± 3.4	5.2 ± 3.4	5.0 ± 3.1	5.1 ± 3.1	NS	NS
IGF −1 (ng/ml)	318.9 ± 70.7	322.5 ± 72.7	341.8 ± 53.7	401.5 ± 111.8	0.0146	NS

[a] Mean ± standard deviation
[b] Across time, control and supplement groups combined (n = 29)
[c] Between supplement and control groups
[d] NS = Not Significant
[e] n = 12
[f] n = 13

Dietary factors

Initial or mid-study food diary data were not available for four subjects. Statistical analysis of dietary factors was derived using the remaining 26 subjects. The mean caloric intake was 2550 ± 733 kcal/day, average calcium intake 1087.6 ± 640.5 mg/day, mean vitamin D intake 143.1 ± 106.5 IU/day, and the average vitamin K intake was 36.9 ± 56.7 μg daily. Caloric intake significantly decreased across time from 2550 ± 733 to 2121± 605 kcal/day ($p < 0.01$). Significant differences in caloric intake between supplement and control groups were not found. Carbohydrate, protein, and fat intake levels also decreased across time. Carbohydrate intake (grams/day) decreased from 340.5 ± 120.7 to 297.2 ± 88.1 ($p < 0.05$). Protein intake changed from 97.1 ± 29.5 to 72.9 ± 25.3 grams/day ($p < 0.001$). Fat

intake decreased from 92.1 ± 29.4 to 75.2 ± 24.8 grams/day ($p < 0.05$). Significant changes in caffeine or fiber intake were not found.

Significant differences in dietary calcium, vitamin D, or vitamin K intakes, whether between groups or across time, were not found. Tables 5 and 6 include specific values for dietary factors.

Table 5--Baseline and Final Values for Selected Dietary Factors (by Groups)

Nutrient/day	Baseline—Controls [a] (n = 14)	Baseline—Supplement [a] (n = 12)	Final—Controls [a] (n = 14)	Final—Supplement [a] (n = 12)	P-Value between groups[b]
Calories (Kcal)	2725 ± 825	2363 ± 592	2246 ± 702	1995 ± 480	NS[c]
Protein (g)	98.6 ± 27.8	95.6 ± 32.1	79.7 ± 30.8	66.2 ± 16.8	NS
Carbohydrate (g)	377.7 ± 136.5	300.9 ± 89.4	310.0 ± 99.8	284.4 ± 75.9	NS
Fat (g)	95.0 ± 32.6	89.0 ± 26.2	80.4 ± 28.4	69.9 ± 20.1	NS
Dietary Fiber (g)	17.1 ± 6.9	16.2 ± 6.0	13.9 ± 7.3	14.5 ± 3.3	NS
Calcium (mg)	1198.3 ± 624.0	969.9 ± 658.0	812.3 ± 329.8	1107.8 ± 320.7	NS
Phosphorus (mg)	1126.6 ± 358.9	995.5 ± 351.0	1084.9 ± 506.3	805.1 ± 300.2	NS
Vitamin D (IU)	144.9 ± 107.9	141.2 ± 109.1	164.9 ± 141.0	207.1 ± 154.5	NS
Vitamin K (mcg)	38.1 ± 40.5	35.8 ± 71.0	36.4 ± 33.1	82.9 ± 84.8	NS
Caffeine (mg)	77.5 ± 79.7	37.4 ± 36.2	44.2 ± 65.4	52.9 ± 104.4	NS

[a] Mean ± standard deviation
[b] Between control and supplement groups at any timepoint
[c] NS = Not Significant

Table 6--Baseline and Final Values for Selected Dietary Factors (Groups Combined)

Nutrient/day	Baseline [a] (n = 26)	Final [a]	P-Value across time[b]
Calories (Kcal)	2550 ± 733	2121 ± 604	0.0041
Protein (g)	97.1 ± 29.5	72.9 ± 25.3	0.0001
Carbohydrate (g)	340.5 ± 120.7	297.2 ± 88.1	0.0361
Fat (g)	92.1 ± 29.4	75.1 ±24.8	0.0035
Dietary Fiber (g)	16.7 ± 6.4	14.2 ± 5.6	NS[c]
Calcium (mg)	1087.6 ± 640.5	960.0 ± 353.2	NS
Phosphorus (mg)	1063.2 ± 355.4	945.0 ± 433.0	NS
Vitamin D (IU)	143.1 ± 106.5	164.9 ± 141.0	NS
Vitamin K (µg)	36.9 ± 56.7	34.0 ± 33.2	NS
Caffeine (mg)	58.1 ± 64.8	35.1 ± 52.1	NS

[a] Mean ± standard deviation
[b] Effect across time, combined groups (n = 26)
[c] NS = Not Significant

CHAPTER 5

DISCUSSION

During the 13-week period of this study, significant changes in BMD among study participants were not found. Subjects' exercise patterns both during the study and prior to participating in the project, likely had a large effect on BMD measurements. From the initial medical history, self-reported weight-bearing exercise averaged an estimated seven hours per week—fairly high activity levels. Restrictions or guidelines for pre-study exercise patterns were not employed, in order to obtain a study sample that would be more representative of the population entering basic training or military cadet programs. These groups tend to have a wide variety of fitness levels (Gofrit and Livneh 1994, Kelly et al. 2000). Unfortunately, it is possible that that subjects who volunteered to participate in the current study were those individuals with an interest in fitness and health and may have had better exercise patterns compared to an average cadet.

During the school year, subjects participated in monitored PT sessions (typically 2-3 times per week) and were encouraged to perform extracurricular sports activities. Yet, cadets in the present study reported averages of only ~ 3.9 hours of weekly activity. Thus, average exercise level among study participants did not increase during the 13-week study period, and in fact, likely decreased compared to pre-study exercise level.

The level of calcium supplementation used in this study (500 mg/day) was lower than that required to show benefit in several other studies. Research in post-adolescents who used low dose calcium without exercise intervention showed little gain in bone density. A study by Peterson and colleagues (2000) involving women aged 18 to 30 consuming supplements of ~ 500 mg of calcium per day showed only an attenuation in BMC losses, without gains in BMD. Specker (1996) found in a meta-analysis that calcium supplementation levels of 1000 mg/day were required to show BMD improvements when combined with exercise. The 500 mg supplement used in this study may have been too low to either alter dietary calcium intakes between groups or to impact BMD. The 500 mg calcium supplement level was chosen for this study because baseline dietary intakes of calcium were high (mean ± SD = 1198.3 ±

624.0 mg/day).   Additional supplementation would have approached or exceeded the tolerable upper limit of 2500 mg/day for calcium intake (National Research Council 1993).

Vitamin D and vitamin K intake levels did not significantly change during the study. This finding was not surprising, considering the fairly low levels of supplementation. True vitamin D levels are difficult to assess from dietary information alone as the human body synthesizes vitamin D from cholesterol utilizing sunlight. Similarly, vitamin K can be synthesized in the gut by normal flora, contributing to vitamin K stores in the body (National Research Council 1990). Thus, dietary intake accounts for only a part of the body's vitamin D or vitamin K levels.

Without significant increases in exercise or dietary calcium intake, bone density would be expected to change little during a 13-week period in this age group. Rico (1993) found that between the ages of 19 and 24, males typically attain a total gain in BMD of 0.1gm/cm^2. If this gain were spread evenly across time, the 13-week period would be expected to bring changes of 0.004 g/cm^2, without interventions.

It is possible that the time period covered by the study was too short to bring about a significant change in bone density. A complete bone remodeling transit has been measured as ranging anywhere from 3 to 6 months (Favus et al. 1999) or even as long as 16 months (Heaney 1994). The current study covered the minimum time for a bone remodeling transit. Etherington and colleagues (1999) also found that a 10-week exercise regimen was likely too short to show the effects of bone remodeling in military recruits.

The relatively low alteration in exercise levels would also account for the lack of stress fractures reported by study participants. Subjects did not report any loss of exercise or any medical attention required due to stress fractures. Stress fractures have largely been found to be related to sudden increases in exercise intensity, as shown in multiple studies. While both intensity and duration of physical activity likely affect stress fracture rates (Kelly et al. 2000), intensity of activity appears to have more impact on stress fracture occurrence compared to duration of exercise (Branca 1999). High intensity exercise was associated with high stress fracture rates in a study by Lauder and associates (2000). The high levels of pre-study fitness combined with moderate intensity of PT could easily account for the lack of stress fractures occurring during this study.

Significant changes in GH or total testosterone levels during the course of the study were not found.   These findings were not surprising due to the small sample size and wide variability of hormone levels between subjects. The responsiveness of testosterone to exercise has been debated, as a recent study demonstrated a lack of changes in serum testosterone in response to strength or endurance training (Bell et al. 2000). Additionally, the relatively high pre-study levels of exercise would decrease the chance of obtaining any exercise-induced alterations in these hormones. While long-term effects were not seen, there may have been acute elevations in testosterone as supported by Hakkinen and colleagues (2000) and Kraemer and associates (1998). Other factors may also be affected. Alen and associates (1988) have suggested that while total serum testosterone may show little long-term change with exercise, testosterone turnover rates may be affected. While Hakkinen and colleagues (2000) report exercise-induced testosterone increases, they also postulate exercise may cause alterations in the testosterone receptor itself.   Both GH and testosterone are secreted in a pulsatile manner. Although researchers were careful to ensure blood samples were collected at the same time of day for all subjects at all data points, it may not have been at a peak secretion time for GH or testosterone, decreasing the likelihood of detecting significant results.

Serum levels of IGF-1 significantly increased. This finding was somewhat unexpected given the low sample number for the study, and especially in light of the lack of changes in GH. Circulating IGF-1 is primarily produced by the liver, under the control of GH (Canalis 1996, Greenspan and Gardner 2001, Rosen et al. 1994). However, skeletal IGF-1 may also play a factor in serum IGF levels (Rosen et al. 1994), and could be affected by bone turnover. Tissue-specific production of IGF-1 has been documented (Weber et al. 1998). Skeletal secretion of IGF-1 is increased by PTH and second-messenger systems and inhibited by glucocorticoids (Canalis 1996), which were not measured in the current study. Thus, IGF-1 level may have direct effects on bone, rather than a modulated effect through GH. It is also possible that there were increases in GH which were undetected due to the low sample size, which affected IGF-1 levels over time.

Serum osteocalcin showed a significant increase. This finding corroborates the postulate that BMD changes might possibly have been detected during a longer study. On the

other hand, as osteocalcin is released during bone resorption as well as during bone formation, (Favus et al. 1999) the finding may be a sign of increased bone turnover rates, without any accompanying significant increase in bone formation.

There were several significant dietary changes. Subjects showed significant decreases in caloric, fat, carbohydrate, and protein intakes. Intake levels may have decreased due to a lack of variety in meals. Most of the student meals were consumed in a military dining hall, and by the end of the study, many subjects had developed very standardized food selections. Although overall intake decreased, the macronutrient breakdown of the diet remained constant—with 31 to 33% of total calories from fat, 53 to 56% from carbohydrate, and 14 to 15% consumed as protein sources.

Another likely factor in the lower reported nutrient intakes may have been poor compliance with food records. While 100% of the initial food data collected was in the form of paper and pencil 3-day diet records, compliance with record-keeping declined sharply. Approximately 50% of the mid-study and final food record information was obtained as 3-day dietary recalls via telephone. Subjects did not respond to repeated e-mails or telephone reminders to provide their food diary data. Despite efforts to ensure accuracy and complete reporting during the recalls, it is possible that participants neglected to report their full intakes—resulting in the decreases displayed in the study.

Future research

There are many questions unanswered by the current study. Opportunities for future research lay in several directions. A study of longer duration would allow additional time for BMD changes to develop and could cover greater than one bone remodeling period.

A research protocol with more controlled exercise regimens would be valuable. A study could focus on trainees with lower pre-study fitness levels, ensuring larger exercise affects. The typical ROTC PT regimen does not offer the extreme physical stresses seen during military basic training. One option would be to duplicate the study in a group of basic trainees. The exercise regimen used in initial military basic training has been documented to alter BMD and to cause stress fractures (Kelly et al. 2000, Cline et al. 1998).

Higher supplement levels have the possibility of providing more divergent results. Calcium supplementation of 500 mg/day was chosen for this study due to fairly high initial

calcium intakes.   A supplement of 1000 mg/day has been required in many studies to provide alterations in BMD (Welten et al. 1995). It would be valuable to study the effectiveness of the combined supplement in a group of subjects with lower baseline vitamin and mineral intakes. Use of a separate vitamin D and vitamin K supplement would allow closer control over dosages of each nutrient.

Summary

The effects of calcium, vitamin D, and vitamin K supplementation during initial military cadet training were assessed during this study. Thirty incoming freshmen cadets received DXA scans and were randomly stratified into control and supplement groups. Subjects in the supplement group received one supplement per day, containing 500 mg calcium, 100 IU vitamin D, and 40 µg vitamin K. Effects on BMD, osteocalcin, and specific hormones related to bone density were monitored.

Significant changes in BMD were not found. Serum osteocalcin and IGF-1 levels increased significantly across time during the study, but without significant differences between groups. Significant differences in GH and testosterone were not found, either between groups or across time. Caloric intake, dietary carbohydrate, fat, and protein levels all decreased significantly during the study period, at similar rates across both groups, while n-telopeptide levels (n = 10), calcium, vitamin D, and vitamin K intakes all remained relatively constant throughout the study.

The expected outcome of an increase in BMD across time, with significantly higher increases amongst the supplemented group, was not supported. High levels of pre-study activity, combined with moderate ROTC PT programs likely account for the lack of changes seen in bone density. The moderate dose of dietary calcium contained in the supplement may not have been high enough to induce changes in these groups of males and females with relatively adequate baseline dietary calcium intake.

Combining nutrients vital to bone growth in one supplement is a logical step towards improving bone health. While the current study did not find significant improvements with supplementation, there may still be benefits to this strategy. Future research should focus on higher levels of supplementation, effects on groups with lower baseline calcium intakes, and combining the supplementation with a more controlled exercise regimen.

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