Investigating the effects of feeding soy protein and soy isoflavones on bone metabolism in female rats fed low dietary calcium.

By Sara Farnworth

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ABSTRACT

Investigating the effects of feeding soy protein and soy isoflavones on bone metabolism in female rats fed low dietary calcium.

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The effects of feeding soy protein (SP) or SP plus isoflavones (IF) (150 and 400 mg IF/kg diet) on bone metabolism were assessed in female weanling and retired breeder (RB) rats fed low calcium (Ca) for five weeks. Young rats fed SP-based diets had significantly smaller reductions in bone mineral density (BMD) and bone mineral content (BMC) as a result of the low Ca diet compared to those fed casein-based diets. Added IFs had no further benefits. Soy protein also affected bone metabolism in both the young and RB rats as indicated by markers of bone resorption. Neither the SP nor the added IFs had any effects on BMD or BMC in the RB rats. Feeding SP to young rats resulted in beneficial changes in BMD, BMC, and biochemical markers of bone metabolism. This study indicates that SP positively affects bone metabolism and minimizes the negative effects associated with low Ca intakes in young rats.

RÉSUMÉ

Examiner les effets d'une alimentation contenant des protéines de soya et des isoflavones de soya sur le métabolisme osseux des rates, ayant une diète faible en calcium.

Par Sara Farnworth

Les effets d'une alimentation contenant des protéines de soya (PS) ou des PS additionnées d'isoflavones (150 et 400 mg d'isoflavones/kg de diète) sur le métabolisme osseux ont été évalués chez des rates sevrées et de reproduction retraitées (RR), ayant une diète faible en calcium. Les jeunes rates ayant la diète de PS avaient une réduction significativement plus faible de leur densité minérale osseuse (DMO) et de leur contenu minéral osseux (CMO), en raison de la diète faible en calcium, par rapport à celles nourries de la diète à base de caséine. L'ajout d'isoflavones n'a eu aucun autre bénéfice. De plus, les protéines de soya ont eu un effet sur le métabolisme osseux chez les jeunes rates, mais aussi chez les rates RR tel qu'indiqué par les marqueurs de la résorption osseuse. Ni les PS ni l'ajout d'isoflavones n'a eu d'effet sur la DMO ou le CMO chez les rates RR. Chez les jeunes rates, une alimentation contenant les PS a résulté en des changements bénéfiques au niveau de la DMO, du CMO et des marqueurs biochimiques du métabolisme osseux. Cette étude démontre que les PS ont un effet positif sur le métabolisme osseux et minimise les effets négatifs associés à un faible apport en calcium chez les rates.

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Osteoporosis

Osteoporosis is a disease characterized by low bone mass and micro architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. An estimated 1.4 million Canadians are believed to have osteoporosis, of which one in four women and one in eight men are affected (21). Osteoporosis is a significant burden to the health care system in Canada, with an estimated cost of $1.3 billion per year to treat the disease and related fractures (16; 21). In addition, with a growing elderly population the number of osteoporotic fractures and costs associated with the disease will likely increase dramatically in the coming years.

Bone mineral density

Osteoporotic fractures are considered the endpoint of the disease (21) and almost a quarter of patients over the age of fifty die within one year of a hip fracture (16; 85). Fractures can be predicted by several risk factors including but not exclusive to: low BMD, family history of osteoporotic fractures, low Ca intake, tendency to fall, and increasing age (16; 21; 47). The most common and widely accepted definition of osteoporosis is based on the World Health Organization's derived T-scores of BMD (21; 23; 52). Normal BMD is defined as a T-score between +2.5 and –1.0 (the patient's BMD is between 2.5 standard deviations (SDs)[1] above the young adult mean and one SD below the young adult mean). Osteopenia (low BMD) is associated with a T-score between –1.0 and –2.5, inclusive. Lastly, osteoporosis is defined as a T-score lower than –2.5. Although it is not the only tool for diagnosing osteoporosis and for predicting fractures, this system is frequently used and has been incorporated into the Osteoporosis Society of Canada's guidelines for clinical practice for the diagnosis and management of osteoporosis (21).

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[1] Refer to Abbreviations List on page 70

Bone metabolism: a cycle of formation and resorption

Bone is a highly metabolically active tissue that undergoes a constant cycle of formation and breakdown (known as resorption) often referred to as bone turnover (21; 47) or bone remodeling (26; 82). During growth and development, a period known as modeling, bone formation outweighs resorption since the skeleton is rapidly being developed (28; 48). Once maturation is reached, remodeling continues throughout life and bone health is maintained in a balanced cycle of bone formation and resorption. Remodeling is essential for sustaining skeletal homeostasis, for providing elasticity and mechanical integrity to bone, and for producing a steady source of extracellular Ca and phosphate ions (64; 82). The extracellular fluid (ECF) concentration of Ca is under tight regulation, with the major sites of this regulation occurring in bone, as well as in the kidneys and the intestines (23; 42).

The sites at which bone remodeling occurs are termed basic multicellular units (BMUs) or bone remodeling units (26; 28; 63). The BMU comprises of osteoclast cells, osteoblast cells, a central vascular capillary, a nerve supply, and associated connective tissue (63). Each stage of the bone remodeling cycle is outlined in Figure 1. The first stage in bone remodeling is termed the activation phase and involves digestion and retraction of the membranes and cells that line the surface of the bone (26; 28). Osteoclasts are then attracted to the exposed mineralized bone surface, attach, and begin removing bone (28; 63). Bone resorption is carried out by acidification and proteolytic digestion (63), which erodes the bone matrix and creates an open cavity known as a lacunae (26). As the BMU advances, osteoclasts leave the resorption site and osteoblasts move in to cover the lacunae and begin the process of forming new bone by secreting osteoid, which is eventually mineralized into new bone (26; 28; 63). The osteoid matrix or bone matrix is mostly comprised of type 1 collagen and when the lacunae is completely filled with osteoid, the newly formed matrix is mineralized with hydroxyapatite (26). The remodeled area then passes into an inactive stage known as quiescence which signals the end of the bone turnover cycle (26; 28; 63).

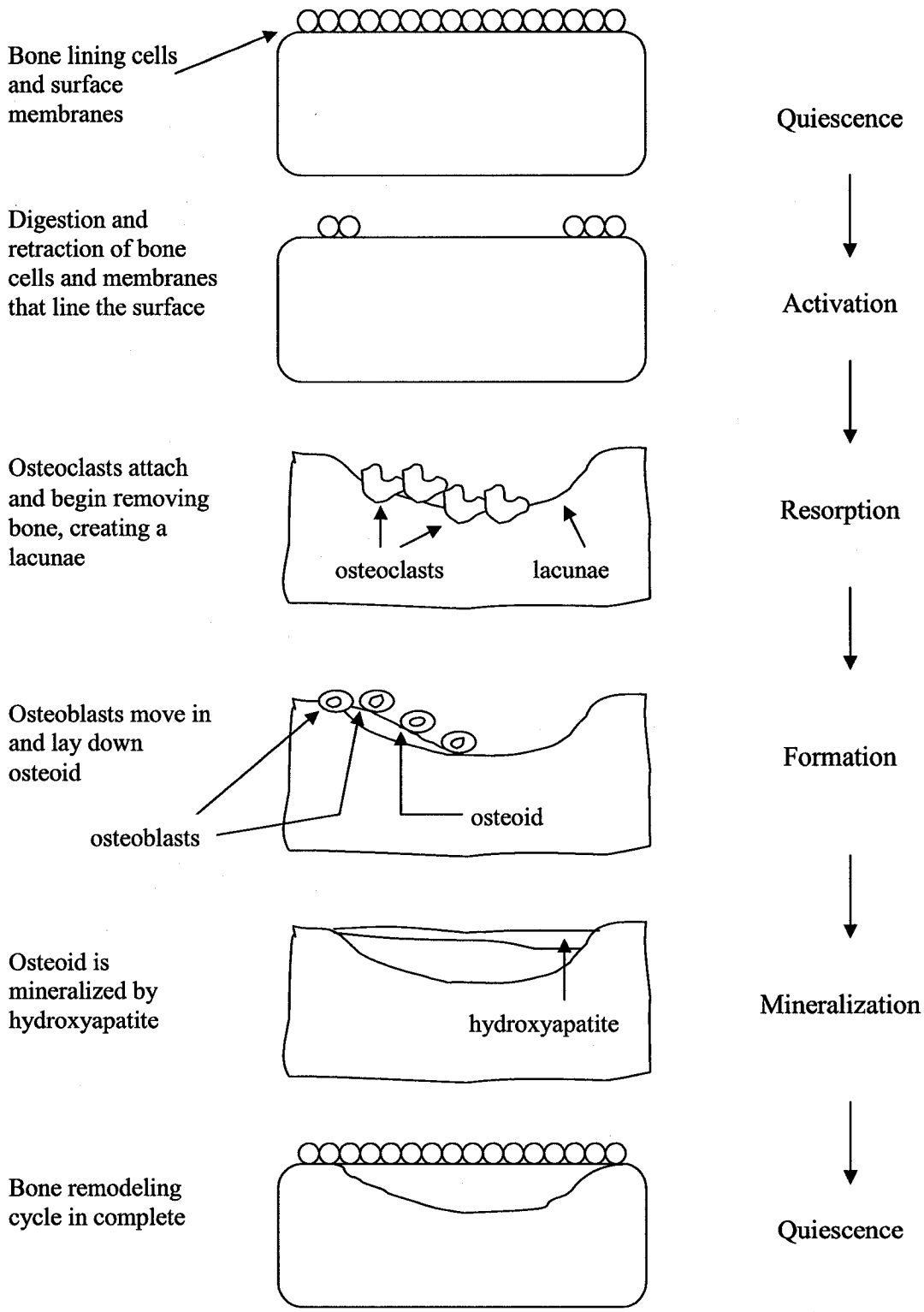


Figure 1. Bone remodeling cycle modified from (26; 28).

Bone loss occurs when there is an imbalance in bone turnover and resorption outweighs formation, when the rate of bone turnover increases significantly, or when bone remodeling occurs at a greater number of sites at any given time (47; 48; 82). In these circumstances Ca and phosphate ions can be released and lost from the bone. Generally, with increasing age, the formation phase fails to keep pace with the resorptive activity of osteoclasts and it is not uncommon for bone loss to occur (47; 48; 82). The prevalence of this type of bone loss is universal, occurs both in men and women, and is referred to as age-related bone loss (47; 48; 82). Women also experience postmenopausal bone loss, which results from the decline in estrogen associated with menopause and as a result osteoporosis is more common in women.

Biochemical markers of bone formation and resorption

Bone metabolism can be measured by assessing biochemical markers of bone formation and resorption that are found in either the blood or urine. Biochemical indices of formation and resorption are reflective of osteoblast and osteoclast activity, respectively. Biochemical markers of bone formation include serum osteocalcin and biochemical markers of bone resorption include urinary levels of deoxypyridinoline (DPD) and pyridinoline (PYD) (26).

Osteocalcin is a small protein that is produced by osteoblasts during the bone matrix mineralization phase (26; 98). While most of this protein is primarily deposited into the bone matrix, where it is the most abundant non-collagenous protein, a small amount can be detected in the blood (26; 98). The amount of osteocalcin in the blood reflects the portion of newly synthesized protein that is not bound to the bone matrix but is released directly into the circulation (26). It has been suggested that because osteocalcin is a good indicator of osteoblast activity it also reflects bone formation (26; 98).

Ninety percent of the bone matrix is type 1 collagen that is cross-linked by molecules such as DPD and PYD that provide strength and rigidity (26; 98). When type 1 collagen is degraded by osteoclasts during the first stages of remodeling, DPD and PYD are released (26). Generally, DPD and PYD cross-links are absent from most

tissues except the bone (26) and as such, are considered to be sensitive measurements of bone resorption (98).

Measuring biochemical markers is important for detecting early changes in bone metabolism, since biochemical events occurring in bone can be detected long before other events occur such as significant changes in BMD or BMC (85).

Calcium and bone health

Calcium is among the most important constituents of bone and is essential for bone growth and formation (23; 42). In humans during the modeling period, from birth until the approximate age of 25, the amount of Ca in bone rapidly increases from 25 g to 1000-1500 g (42; 48). The accumulation of Ca during the first few decades of life comes from the diet (42). Thus, adequate Ca intakes during this period are critical for proper development and mineralization in order for optimal peak bone mass to be achieved. Those with higher peak bone mass may be at lower risk of developing osteoporosis (23; 30; 48). Once maturation is reached, approximately 99% of the body's Ca is found in the bones in the form of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (23; 49). Calcium is also required by cells throughout the body and its homeostasis is tightly regulated by the kidneys, the intestinal cells, and by the bone to meet these demands (23; 42).

Calcium deficiency decreases bone growth and causes bone loss in experimental animals and is often used as a model to study osteoporosis (23; 70; 80). Animal studies have observed significantly lower BMD (87), lower bone Ca content (30; 55; 90) higher bone resorption (30; 55; 87; 90), and lower bone formation (90) in rats fed a Ca restricted diet.

When Ca intakes are low, the ECF concentration of Ca decreases. This results in hypocalcemia and an increase in parathyroid hormone (PTH) secretion (23). An increase in PTH causes Ca resorption to increase in the bone (23). Mechanistically the rise in PTH that results from a low Ca intake causes the number and size of osteoclasts to increase (61). The amount of bone broken down by osteoclasts increases, which results in a deeper lacunae that is insufficiently filled by osteoblasts (Figure 2B). Thus a low Ca diet causes an imbalance in bone turnover as bone resorption outweighs bone formation.

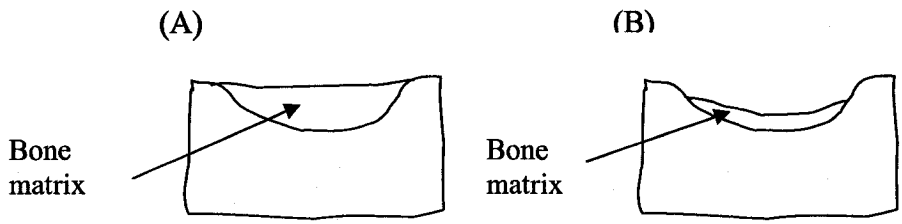


Figure 2. Bone remodeling under normal conditions (A) when bone resorption and formation processes are balanced, and (B) when a low Ca diet causes an increase in bone resorption and a decrease in bone formation, thus, resulting in an unbalanced bone turnover cycle. Modified from (28).

Calcium intakes in Canadian women

North American women, especially middle-aged and elderly, consume diets that are low in Ca (32; 38). Data collected from the Food Habits of Canadians Survey conducted in 1997–1998, which is the most recent national nutrition survey in Canada, reported that a significant number of women consume inadequate intakes of Ca (38). Calcium levels among women at the 25th percentile of intake were under 500 mg for all age groups indicating very low intakes in many women (38). Provincial surveys that have been conducted also indicate that mean Ca intakes of adults 18 to 74 years of age are generally low, with intakes declining with increasing age (57). The most recent provincial food survey, conducted in Ontario, reported that Ca intakes for women were below the Dietary Reference Intake recommendations. The recommended daily Adequate Intake for Ca for women between the ages 19-50 years is 1000 mg/day and over 50 years is 1200 mg/day (49). The mean intakes reported were 795-759 mg/day (ages 19-49 years) and 714-645 mg/day (ages 50-74 years) (66). The health implications of these reported low intakes are important because of the increasing proportion of elderly people in the Canadian population (21; 32).

Bone mineral content: the importance of phosphorus and magnesium

Bone health is dependent, not only on Ca status, but on overall nutrition. Other minerals, such as phosphorus (P) (32; 48; 75) and magnesium (Mg) (48; 75) are important constituents of bone and are required for bone growth and maintenance. Approximately 80–90% of BMC is comprised of Ca and P since they are an integral part

of the hydroxyapatite crystal $Ca_{10}(PO_4)_6(OH)_2$ (48). Hydroxyapatite is an essential compound needed during the final stage of the bone remodeling cycle (26). Phosphorus is second to Ca in abundance in the human body with 85% of the body's P found in the skeleton (48; 49). The primary role of P in bone is to support growth and mineralization of the skeleton (48; 75) and an adequate supply of P is needed throughout life to replace losses that are associated with bone turnover. It is also indirectly involved in regulating PTH secretion (48). Excessive intakes of P, which are commonly associated with Western-style diets (75), lead to increased PTH secretion and increased bone resorption (48). The consumption of P has risen in recent years (48) and may be detrimental to bone. Low intakes of P can also lead to negative effects on bone health since a depletion of P leads to impaired bone mineralization and compromised osteoblast function (75).

Two-thirds of Mg that is found in the body is in the bone, where it accumulates on the surface of the hydroxyapatite crystal (48). Magnesium is involved in bone and mineral homeostasis, bone crystal growth and stabilization, and it plays a role in the vitamin D-PTH feedback system (75). Poor bone development, compromised bone strength, and hypocalcemia often result from Mg deficiency (48).

Soy isoflavones and their relationship to bone health

Description, biochemistry, and biological implications of soy isoflavones

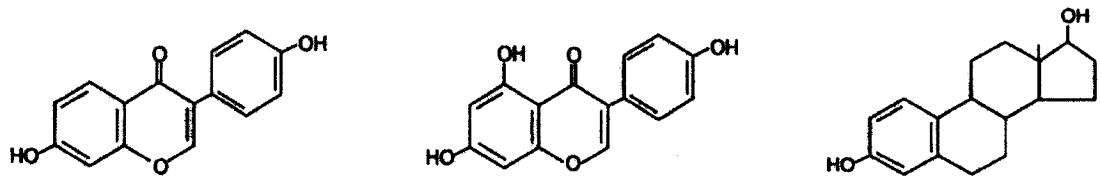
Isoflavones are molecules that belong to the class of compounds known as flavonoids (37; 103) and are often referred to as phytoestrogens. Phytoestrogens are naturally occurring plant–derived compounds which include: IFs, coumestans, and lignans (37; 84; 85). The biological roles of phytoestrogens are to protect plants from stress and to act as part of a plant's defence mechanisms (31; 37).

Isoflavones are commonly found in legumes and are highly concentrated in soybeans and soy-based products. Commercial IF extracts and supplements are also widely available in North America. The primary IFs that are found in soybeans are genistin and daidzin where they are present as beta-glycosides (37; 83). Once ingested, IFs are converted to their aglycone forms, genistein and daidzein, by intestinal bacterial glucosidases (40; 106). Soybeans contain a number of biologically active constituents

(37), however, IFs are the compounds receiving the most attention because of their possible protective role in hormone-dependent conditions, including cancer, menopausal symptoms, cardiovascular disease, and osteoporosis (84).

Isoflavones are structurally similar to mammalian estrogens, such as 17-beta estradiol (E2), and can bind to estrogen receptors (ERs) throughout the body (15; 37; 83; 84). As a result, they mimic estrogens and have estrogenic effects in different tissues (15; 37; 83; 84). Isoflavones are considered to be weak estrogens given that their binding affinities to ERs are low in comparison to E2 (Figure 3) (56; 84).

There are two forms of ERs that exist in the human body, ER alpha and ER beta (37). Both ER subtypes have been identified in bone (56; 74) and it has been demonstrated that genistein acts as an estrogen agonist on human osteoblastic cells through both ERs alpha and beta (78). Others have suggested that it is the beta form that is mostly found in bone (109) and since genistein has a very high binding affinity for ER beta, it is likely having different effects on various E2-related diseases.



Daidzein
ER alpha: 0.1 %
ER beta: 0.5 %

Genistein
ER alpha: 4 %
ER beta: 87 %

17-beta estradiol
ER alpha: 100 %
ER beta: 100 %

Figure 3. Chemical structures of daidzein, genistein, and 17-beta estradiol and their binding affinities to human ER alpha and beta. Modified from (56; 83; 84).

Estrogenic effects of soy on bone: role in the ovariectomized animal model

Estrogens are key regulators of bone growth, maturation and metabolism in males and females (28; 64; 79) and it has been recognized for years that estrogen deficiency plays a central role in the development of postmenopausal osteoporosis (5).

There has been a growing amount of focus on the estrogenic role of soy in osteoporosis since estrogen deficiency is directly associated with postmenopausal osteoporosis (5; 15; 47; 84) and many women are looking for alternatives to hormone replacement therapy (8; 84). This is especially true since the release of the findings of the Women's Health Initiative in 2002 (80).

There is evidence suggesting that soy and/or IFs may slow postmenopausal bone loss (8; 15; 83). This has been widely investigated using an ovariectomized (ovx) animal model (15), (83) which is one of the preferred models outlined by the Food and Drug Administration for evaluating therapies used in the treatment and prevention of postmenopausal osteoporosis (95).

Among the ovx animal studies, SP, isolated IFs, and IFs in the context of SP have all shown beneficial bone sparing effects (15; 83; 85). It has also been reported that the bone protective effect of soy may be from its amino acid composition (73). Results from feeding soy and/or IFs to ovx animals have included increases in BMD (10; 20; 50; 73; 81), increases in bone Ca and P content (50; 81), increases in mechanical strength (20; 50; 73), increases in bone formation markers such as osteocalcin (34), alkaline phosphatase (ALP) (10; 80) and insulin growth factors (IGF) (10), and decreases in urinary DPD (50), PYD (50) and Ca (22). The synthetic IF, Ipriflavone has demonstrated similar bone sparing properties (11) and most of the beneficial effects from soy, IFs, and Ipriflavone are comparable to E2 administration in the ovx rat model (11; 22; 50; 81). In these studies, few researchers have investigated the long-term ingestion of soy or IFs on bone health and the primary focus has been to investigate soy's role in bone loss due to estrogen deficiency. Little research has been conducted in a non-ovx animal model or in other models of bone loss that are not related to estrogen deficiency. It was demonstrated that in intact female rats urinary DPD and lumbar BMD were positively affected by soy diets with high and low amounts of IFs (69). Also Ipriflavone has been shown to inhibit bone resorption both in intact and ovx rats (24). These data taken together suggest that bone turnover is positively altered in non-ovx animal models.

Mechanisms: direct and indirect actions on bone cells

Actions at the molecular level indicate that IFs have direct and indirect effects on bone cells (37; 85) (Figure 4). Isoflavones have been consistently shown to have anabolic effects on osteoblast-like cells and that these actions are occurring via ER-dependent mechanisms (78). Culturing osteoblast cells (osteoblast-like cells derived from newborn mouse calvariae) with IFs has been shown to produce increases in: protein content (91; 92), ALP activity (53; 91; 92), and cellular Ca and P content (53). These results are likely associated with proliferation and activity of osteoblasts (53). Similar anabolic effects have been demonstrated with environmental estrogens (53) and E2 (91; 92) and are inhibited when an anti-estrogen or blocking agent was added (91; 92)

Recently Chen *et al* (25) demonstrated that genistein decreased interleukin-6 (IL-6), a cytokine that is produced by osteoblasts (28; 64). Two other products of osteoblasts, osteoprotegerin and the receptor activator of nuclear factor (NF)-κB ligand (RANKL) were also affected by genistein (25). All three cytokines are known to affect osteoclastic differentiation and activity (28; 64). Thus *in vitro* results have demonstrated that osteoblast-like cells respond directly to IFs and that their protein products have the ability to regulate osteoclasts and bone resorption.

The actions of IFs on osteoclast-like cells do not appear to be dependent on ERs (37). Gao and colleagues (35; 36) have reported that genistein may decrease bone resorption through inhibition of tyrosine kinase (4) and topoisomerase II (107). Their role in suppressing osteoclastogenesis may be through their influence on specific enzyme systems and cell signaling pathways that regulate osteoclasts (37; 84).

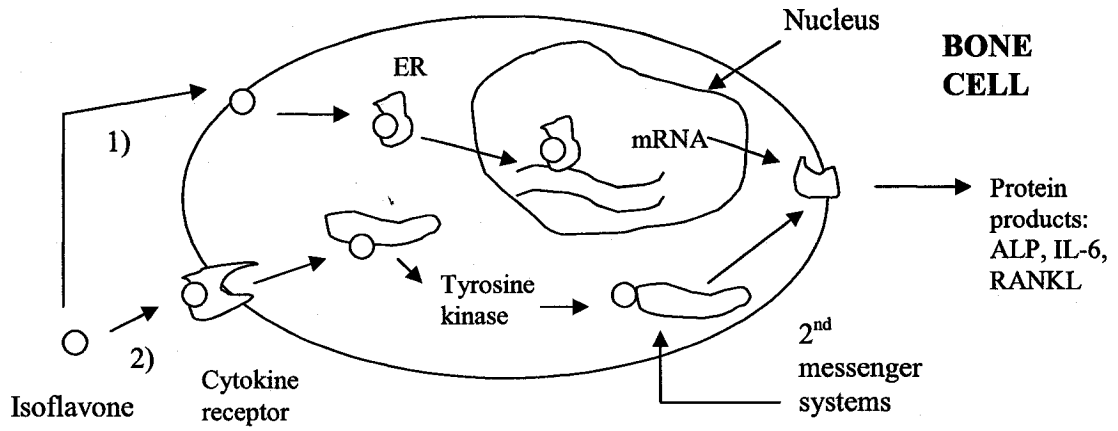


Figure 4. Mechanisms of IFs at the molecular level. Both 1) an ER-dependent pathway and 2) a non-ER dependent pathway are depicted. The ER-dependent pathway involves IFs taken up by passive diffusion across the cell membrane, binding to ERs that are found in bone cells, and up/down regulating messenger ribonucleic acid (mRNA) of certain genes such as alkaline phosphatase (ALP), interleukin-6 (IL-6), and the receptor activator of nuclear factor (NF)-κB ligand (RANKL). The non-ER dependent pathway involves IFs interacting with membrane receptors, inhibiting tyrosine kinase or topoisomerase II, and influencing 2nd messenger systems in the cytoplasm which can affect proteins that are produced by the bone cell. Modified from (37).

Effects of soy and isoflavones in human populations: epidemiological data

Results from the ovx model assessing the protective role of soy and/or IFs on bone loss are fairly consistent although work needs to continue. However, evidence of the effect of soy and soy IFs on bone health in humans is inconsistent. It is possible that soy may have positive effects in other animal models and populations that may or may not have low endogenous estrogen levels.

Epidemiological data indicate that Asian populations have lower rates of fractures and osteoporosis (2; 59; 105) in comparison to Caucasians and these lower rates occur despite low Ca intakes (58). Lower rates of fractures and osteoporosis are considerably reduced when Western eating habits are adopted (2). Traditionally, soy has been a central part of the Asian diet and consumption of soy and soy-based foods is much higher than in North America (2; 37). It has been postulated that soy and/or IFs may be protective by providing beneficial effects on bone health.

The efficacy of soy and/or IFs in reducing bone loss and altering bone turnover has been investigated in young (109), peri-(6) and premenopausal women (101), as well as intact female rats (24; 69). Slightly positive effects of IF-rich SP have been reported

on bone loss in the lumbar spine of perimenopausal women (6). In premenopausal women, bone turnover was significantly affected by a SP plus low IF diet. Markers of bone formation were increased and a corresponding increase in DPD levels was seen in 14 premenopausal women (101). Most recently, investigators showed that feeding soy with added IFs had significant effects on bone markers in young women and that the effects were most pronounced during a stage of the menstrual cycle when estrogens were high (109).

Soy and its role in calcium homeostasis

It has been widely suggested that IFs act directly on bone cells and this mechanism is the basis for soy's protective role against bone loss (15; 37; 85). It has also been hypothesized that soy and/or IFs may be having beneficial effects on bone health partly due to their ability to increase Ca absorption (20; 89). It has been reported that feeding a soymilk-based diet to 6 week-old female rats increased intestinal Ca absorption compared to a casein-based diet (73). Similarly, ovx rats fed a soy diet for 35 days had higher intestinal Ca transport, measured in isolated duodenal and colonic cells, than rats receiving a casein-based diet (14). It was the IF-rich but not the IF-depleted SP that completely prevented the ovx-induced reduction of Ca transport in the duodenal and colonic cells (14).

This hypothesis is further supported by the evidence that estrogen promotes intestinal absorption of Ca *in vivo* (47) through ERs that are present in the intestinal cells (93; 94). Estrogen administration can increase and restore Ca absorption levels in postmenopausal women to levels measured in premenopausal women (89).

Intestinal Ca absorption occurs by two processes, one of which is known as active Ca absorption and is regulated by 1,25 $(OH)_2D_3$ (60; 99). The actions of 1,25 $(OH)_2D_3$ in Ca absorption are dependent on interactions with the nuclear vitamin D receptor (VDR) (99) and the number of VDRs is a primary determinant of 1,25 $(OH)_2D_3$'s biological response (60). Active Ca absorption requires several transport proteins including the Ca transport protein 1 and calbindin-D_{9k} and occurs predominantly in the duodenum (99). Estrogen can have many effects in the body including the ability to upregulate VDR expression and calbindin-D_{9k} content in

duodenal cells of female rats exposed to estrogen (60). Additionally ER knockout mice have lower expression of Ca transport protein 1 mRNA and VDR knockout mice have higher Ca transport protein 1 mRNA when treated with estrogen (99). Thus, it seems that estrogens and vitamin D are important independent regulators of Ca absorption (99). Estrogen's role in intestinal Ca absorption most likely occurs through ERs since estrogen-induced effects, such as an increase in Ca absorption, are blocked by ER antagonists (93). It is not surprising then that estrogen deficiency reduces Ca absorption and causes bone loss in rats and that this effect is prevented by E2 administration (72). Furthermore, the synthetic IF Ipriflavone, significantly enhances Ca absorption in ovx rats and increases are comparable to E2 (13). Since estrogen is involved in Ca absorption and because IFs are known to have estrogenic effects in the body, the hypothesis that soy and/or IFs may enhance Ca absorption is consistent with the experimental data.

Rationale

Several animal and short-term human studies have indicated that SP alone or SP enriched with IFs may be used as an alternative therapy to estrogen replacement therapy. Much of the attention of soy and bone metabolism has been investigated in the ovx rat model which represents a severe form of postmenopausal bone loss. However, very few of the previous studies have investigated soy's estrogenic effect on both Ca and bone metabolism in animals or humans, which is essential in ascertaining the mode of action of IFs. This is important since bone turnover in other populations, perimenopausal women (6), young women (109), and intact rats (24; 69) has also been positively affected by soy and/or IFs.

Preliminary work (Farnworth and L'Abbé, unpublished) showed that when male and female rats of two different ages were fed diets with SP or SP with increasing amounts (5, 50, 250 or 1250 mg/kg diet) of IFs, no clear and consistent SP or IF benefit on BMD, physical parameters, and bone mineral measurements were seen. However, in the above study no manipulations were done to stress the skeleton and cause loss of bone mass and bone minerals. Dietary levels of Ca and other minerals were more than adequate to meet the needs for rat growth and to maintain bone health. Situations where

there is a clear stress on bone such as a low Ca diet and the ovx rat model are the primary areas in which soy and/or IFs are thought to be beneficial. It was not surprising then that neither the SP nor the IFs had any effects in an animal model of adequate Ca. Bone loss induced by a Ca deficient diet has been successfully prevented with estrogen in rats (87) and since IFs are known to have estrogenic effects in the body (15; 37; 83; 84), it is hypothesized that feeding soy and/or IFs can prevent or slow bone loss induced by a low Ca diet.

Objective

The objective of this study was to assess the effects of feeding SP versus casein with or without added IFs on bone loss in 2 animal models with low dietary Ca. Female weanling and RB rats were fed a low Ca diet to decrease bone growth and induce bone loss, respectively. The effects of feeding SP and IFs on bone metabolism were determined under these two experimental conditions by measuring biochemical markers of bone formation and resorption, BMD and BMC, and bone growth parameters.

Hypothesis

It expected that the group of rats being fed the low Ca diets will have altered bone metabolism. Also it is hypothesized that the soy IFs will reduce bone loss and normalize bone growth by altering bone metabolism as measured by BMD, BMC, and biochemical indices of bone formation and resorption. Lastly, it is expected that the group of RB rats will benefit more from the ingestion of soy IFs because their Ca homeostasis and bone mass have been affected by multiple reproductive cycles.

Materials and methods

Animals and diets

Weanling (21 days-old) and RB (approximately 9 months-old) female Sprague-Dawley rats were purchased from Charles River (St. Constant, Qc). Upon arrival, a group of 96 rats, 48 weanling and 48 RBs, were randomly assigned to 1 of 8 diets, described in Table 1 and Table 2: diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet. Animals were individually housed in wire-bottom stainless steel cages and kept in an environmentally controlled room with a 12-hour light: dark cycle.

All 8 diets were formulated according to the AIN-93G diet requirements with the exception that casein was replaced by alcohol washed soy protein isolate (76; 77). A specialized formulation of the AIN-93G mineral mix with Ca omitted was purchased from Harlan Teklad (Madison, WI). Calcium was added back to all diets as Ca_2CO_3. Diets 1 and 2 contained the recommended Ca levels (5 g Ca/kg diet) and diets 3-8 had low Ca levels (1 g Ca/kg diet). Isoflavones were added to the diets as Novasoy (Archer Daniels Midland Company, Decatur, IL). The total aglycone content of Novasoy determined by HPLC (100) was 25%. The Ca levels of the diets were verified by flame atomic absorption spectrophotometry (Perkin-Elmer 5100PC, Perkin-Elmer, Norwalk, CT) and the P levels of the diets were verified by a colourimetric method (68). Diets were mixed thoroughly and pelleted for more accurate measurement of food intake. Weekly food consumption and body weights were recorded.

Table 1. Composition of experimental casein-based diets[1].

DIET	1 (0 mg IF/kg diet)	3 (0 mg IF/kg diet)	4 (150 mg IF/kg diet)	5 (400 mg IF/kg diet)
Casein[2]	222.2	222.2	222.2	222.2
Sucrose	100.0	100.0	100.0	100.0
Cornstarch	362.8	372.8	372.3	371.4
Dextrinized cornstarch	132.0	132.0	132.0	132.0
Soybean oil	70.0	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0	50.0
Mineral mix[3]	35.0	35.0	35.0	35.0
Vitamin mix[4]	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5
L-Cystine	3.0	3.0	3.0	3.0
tert-Butylhydroquinone	0.014	0.014	0.014	0.014
Novasoy[5]	-	-	0.50	1.33
Calcium[6]	12.50	2.50	2.50	2.50

[1] All ingredients in g/kg diet

[2] Casein contained 90% crude protein (ICN Biomedicals, Irvine, CA)

[3] AIN 93-G mineral mix without Ca, a specialized formulation from Harlan Teklad

[4] AIN 93-V vitamin mix

[5] Novasoy isoflavone concentrate contained 25% total isoflavone as aglycones, purchased from Archer Daniels Midland Company (Decatur, IL)

[6] Calcium added back to the diets as Ca_2CO_3, diets 1 and 2 contained normal Ca levels (5 g Ca/kg diet) and diets 3-8 contained 1 g Ca/kg diet

Table 2. Composition of experimental SP-based diets[1].

DIET	2 (0 mg IF/kg diet)	6 (0 mg IF/kg diet)	7 (150 mg IF/kg diet)	8 (400 mg IF/kg diet)
SP[2]	222.2	222.2	222.2	222.2
Sucrose	100.0	100.0	100.0	100.0
Cornstarch	362.8	372.8	372.3	371.4
Dextrinized cornstarch	132.0	132.0	132.0	132.0
Soybean oil	70.0	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0	50.0
Mineral mix[3]	35.0	35.0	35.0	35.0
Vitamin mix[4]	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5
L-Methionine	3.0	3.0	3.0	3.0
tert-Butylhydroquinone	0.014	0.014	0.014	0.014
Novasoy[5]	-	-	0.50	1.33
Calcium[6]	12.50	2.50	2.50	2.50

[1] All ingredients in g/kg diet

[2] alcohol washed SP that contained 90% crude protein

[3] AIN 93-G mineral mix without Ca, a specialized formulation from Harlan Teklad

[4] AIN 93-V vitamin mix

[5] Novasoy isoflavone concentrate contained 25% total isoflavones as aglycones, purchased from Archer Daniels Midland Company (Decatur, IL)

[6] Calcium added back to the diets as Ca_2CO_3, diets 1 and 2 contained normal Ca levels (5 g Ca/kg diet) and diets 3-8 contained 1 g Ca/kg diet

An additional group of 12 rats (6 weanling and 6 RBs) were randomly selected and killed at baseline as controls. Rats had free access to food and water for the duration of the study. At necropsy blood, femur, tissues, and urine were collected. Approval for animal experimental protocols was obtained from the Animal Care Committee of the Health Products and Food Branch of Health Canada and all animal handling and care followed the guidelines of the Canadian Council for Animal Care. Animals were fed for 5 weeks and killed by exsanguination through cardiac puncture under general anesthesia with isoflurane. Samples were immediately frozen on dry ice, and stored at -80°C until analysis.

Chemicals, reagents, and ELISA kits

Alcohol washed soy protein isolate (Pro Fam 930) and Novasoy IF concentrate were purchased from Archer Daniels Midland Company (Decatur, IL). Casein protein was from ICN Biomedicals (Irvine, CA). The modified AIN-93G mineral mix with Ca omitted was purchased from Harlan Teklad (formulation number TD 04374, Madison, WI). All other chemicals and reagents used were from Sigma (St. Louis, MI).

Bone mineral density, bone mineral content, and bone growth measurements

The right femurs were cleaned with a scalpel and gauze and weighed (wet weight was recorded). Femoral crude density was measured by water displacement, based on Archimedes' Principle, and recorded on a volumetric basis modified from the method previously described (51; 54). A specific gravity bottle (KIMAX, Kimble Glass Inc.) was used and whole femurs were placed in the bottle filled with water. Whole bones were used since they are more biologically relevant than hollowed out bones (54).

Femoral BMD was measured by 2 methods: water displacement (referred to as crude density above) and dual energy x-ray absorptiometry (DEXA) using facilities at the University of Manitoba (Hologic QDR 4500A Elite with software version 11.2). The DEXA machine was calibrated daily before femurs were scanned. Each femur was centered in a water bath and all femurs were scanned in the same manner. Each day triplicate scans were conducted to calculate SDs and coefficient of variation (CV) percentages of femoral BMD and BMC. The CVs for the analyses conducted the first

day were 6% for BMD (n = 15) and 3% for BMC (n = 15). The CVs for the analyses conducted the second day were 3% for BMD (n = 7) and 4% for BMC (n = 7).

Femoral length and diameter of the distal epiphysis and mid-diaphysis were measured with a Vernier calliper. Femurs were then dried overnight at 100°C and dry weights were recorded the following day. Femurs were dry ashed at 450°C using concentrated nitric acid for analysis of minerals by flame atomic absorption spectrophotometry and P by a colourimetric method as described below and once a white residue was obtained, ash weights were recorded. Samples were then diluted with 0.3N nitric acid and water.

Bone mineral levels

Femoral minerals (Ca, Mg, Zn, Na, and K) were measured by flame atomic absorption spectrophotometry (Perkin-Elmer 5100PC, Perkin-Elmer, Norwalk, CT) and P was assayed colourimetrically (68). Analytical standards were prepared from certified single-element stock solutions (High Purity Standards, Charleston, SC) which were used to generate calibration curves. Samples were diluted with a La_2O_3 solution for Ca and Mg determinations, with a CsCl solution for Na and K determinations, with 0.3N nitric acid solution for Zn determinations, and with water for P determinations. Final concentrations were corrected for dilutions. All analytical procedures followed have been checked in multi-laboratory quality control studies (41) and have been verified for mineral analyses (27).

A small number of samples were run in triplicate to calculate SDs and CV percentages for within (inter) and between (intra) runs. The inter- and intra-run CVs for atomic absorption spectrophotometry were 12% (n = 5) and 4% (n = 2), respectively and for P determination were 7% (n = 10) and 4% (n = 4), respectively.

Bone resorption measurements: urinary deoxypyridinoline and pyridinoline

Urine levels of DPD and PYD were measured using the Metra PYD and DPD ELISA kits. The DPD and PYD ELISA kits were purchased from Quidel Inc. (San Diego, CA). Urine samples were diluted with an assay buffer (if required) to bring concentrations within the range of the standard curve. Both DPD and PYD

concentrations were corrected for dilutions and creatinine levels. All other aspects of the protocols were followed according to manufacturer's instructions. A small number of samples were run in triplicate to calculate SDs and CV percentages. The inter- and intra-assay CVs for DPD were 6% (n = 10) and 6% (n = 2), respectively, and for PYD were 7% (n = 10) and 7% (n = 2), respectively. Urine creatinine was measured by a colourimetric kit purchased from Oxford Biomedicals (Oxford, MI).

Bone formation measurements: serum osteocalcin

Serum osteocalcin was assessed using the Biotechnologies Inc. Rat osteocalcin EIA kit BT-490 purchased from Biomedical Technologies Inc. (Stoughton, MA). Serum samples were diluted with sample buffer (if required) to bring concentrations within the range of the standard curve and final concentrations were corrected for dilutions. All other aspects of the protocol were followed according to manufacturer's instructions. A small number of samples were run in triplicate to calculate SDs and CV percentages. The sera samples for the young and RB rats were analyzed in separate assays and the inter-assay CVs were 9% (n = 8) for the young rat samples and 10% (n = 8) for the RB rat samples.

Hematology

Immediately following necropsy, whole blood samples were centrifuged at 4°C for ten minutes to separate plasma and serum. Hematology was conducted on plasma samples using a Beckman Coulter Analyzer (Fullerton, CA).

Statistical analyses

Data were analyzed using Statistica Version 6.1 (StatSoft, Tulsa, OK). All data were expressed as the mean ± standard error of the mean (SEM). A p-value of < 0.05 was considered statistically significant. Descriptive statistics, including means and standard errors were calculated. Normal distribution was tested by the Shapiro-Wilk W test and homogeneity of variance was tested by Levene's test.

The effect of Ca, protein source, and added IFs on physical parameters (length, diameter, wet, dry, ash, and body weights), BMD, BMC, femoral minerals (Ca, Mg, P,

Zn, Na, and K), DPD, PYD, osteocalcin, food consumption, and hematology parameters were tested by a 3-way ANOVA, where interaction terms were included. Differences between means were determined by Tukey's *poc hoc* test. Where no statistical significant differences or interactions were found, 2-way and 1-way ANOVA using the same main effects were used to assess differences in pooled data for dependent variables listed above.

Results

The study was 5 weeks in duration and on day 4 of the study one weanling rat died and was replaced. The total number of young rats remained at 48. On day 12 one RB was euthanized because of poor health. The total number of RB rats for the duration of the study was 47.

Pooled data for each of the main effects (Ca levels, protein source, and added IFs) are also presented where significant differences are seen. These pooled data for the main effects are described in Table 3.

Table 3. Pooled diet groupings used for the main effects tested on all measurements in young and retired breeder rats.

Main effect	Levels	Diets[1]	n
Young rats (n = 48 in total)			
Ca	High Ca	1 and 2	12
	Low Ca	3-8	36
Protein	Casein	3, 4, and 5	18
	SP	6, 7, and 8	18
IF	0	3 and 6	12
	150	4 and 7	12
	400	5 and 8	12
RB rats (n = 47 in total)			
Ca	High Ca	1 and 2	12
	Low Ca	3-8	35
Protein	Casein	3, 4, and 5	17
	SP	6, 7, and 8	18
IF	0 IF	3 and 6	12
	150 IF	4 and 7	12
	400 IF	5 and 8	11

[1] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

There were no differences between diet groups in food consumption in the young rats throughout the 5 weeks of the study and in total (Table 4). Young rats fed the low Ca diets generally consumed more food (consumed more food in total), however there were no differences in food consumption over the 5 weeks when comparing Ca levels, protein source, or added IFs (Table 5). Total food consumption of diets 2 and 5 in the RB rats were statistically different (Table 4). Retired breeder rats on the low Ca diets ate much less in total ($p < 0.05$) and at week 4 ate less ($p < 0.01$) than rats fed control diets. Food consumption was statistically higher with SP at week 2 and there was a statistically significant difference between 150 and 400 mg/kg diet of IFs at week 4 in the RB rats.

Table 4. Weekly food consumption of each diet group for young and retired breeder rats[1, 2]

Diet[3]	n	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Week 5 (g)	Total (g)
Young rats							
1	6	50.6 ± 7.3[a]	92.2 ± 8.8[a]	110.9 ± 15.5[a]	138.5 ± 10.6[a]	147.0 ± 20.8[a]	539.2 ± 42.6[a]
2	6	61.8 ± 10.9[a]	100.3 ± 8.7[a]	143.6 ± 14.5[a]	137.7 ± 9.0[a]	136.0 ± 13.1[a]	579.5 ± 41.5[a]
3	6	58.4 ± 10.5[a]	95.4 ± 8.7[a]	132.1 ± 12.8[a]	137.9 ± 10.4[a]	146.3 ± 17.6[a]	570.0 ± 48.9[a]
4	6	52.6 ± 6.3[a]	87.9 ± 8.7[a]	133.0 ± 17.3[a]	173.1± 17.0[a]	185.5 ± 19.8[a]	632.1 ± 38.8[a]
5	6	53.7 ± 9.3[a]	91.2 ± 5.5[a]	127.9 ± 9.9[a]	163.8 ± 15.0[a]	168.7 ± 11.9[a]	605.3 ± 45.5[a]
6	6	68.6 ± 13.3[a]	87.7 ± 6.1[a]	126.4 ± 5.6[a]	137.4 ± 13.7[a]	140.3 ± 12.9[a]	560.5 ± 34.8[a]
7	6	59.7 ± 9.5[a]	91.2 ± 7.7[a]	132.2 ± 7.7[a]	158.2 ± 15.5[a]	176.8 ± 21.6[a]	618.0 ± 47.4[a]
8	6	38.5 ± 12.0[a]	73.6 ± 9.6[a]	135.6 ± 15.5[a]	160.6 ± 10.4[a]	151.8 ± 16.6[a]	560.1 ± 45.3[a]
RB rats							
1	6	160.3 ± 31.4[a]	171.2 ± 9.2[a]	168.7 ± 12.0[a]	209.1 ± 29.9[a]	139.0 ± 19.4[a]	848.3 ± 51.5[a,b]
2	6	206.1 ± 24.3[a]	199.1 ± 6.7[a]	198.1 ± 9.4[a]	202.4 ± 10.1[a]	178.5 ± 21.4[a]	984.1 ± 53.6[a]
3	6	168.2 ± 3.8[a]	162.7 ± 8.2[a]	175.6 ± 6.9[a]	180.8 ± 5.6[a]	152.9 ± 21.7[a]	840.2 ± 33.2[a,b]
4	6	157.6 ± 11.2[a]	174.3 ± 7.2[a]	177.5 ± 7.4[a]	180.4 ± 6.3[a]	158.1 ± 20.0[a]	847.8 ± 25.1[a,b]
5	5	151.9 ± 10.6[a]	137.8 ± 7.1[a]	158.4 ± 4.5[a]	157.6 ± 10.5[a]	135.5 ± 18.7[a]	745.4 ± 37.7[b]
6	6	178.6 ± 10.8[a]	176.3 ± 6.9[a]	168.5 ± 5.6[a]	166.3 ± 5.0[a]	161.7 ± 22.2[a]	851.5 ± 33.7[a,b]
7	6	163.2 ± 13.7[a]	190.5 ± 28.8[a]	175.2 ± 11.8[a]	182.2 ± 5.2[a]	157.8 ± 13.4[a]	868.8 ± 37.7[a,b]
8	6	180.3 ± 31.1[a]	198.1 ± 13.7[a]	180.7 ± 16.8[a]	164.6 ± 12.1[a]	145.9 ± 9.8[a]	869.5 ± 66.4[a,b]

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 5. Summary of data for main effects on weekly food consumption for young and retired breeder rats[1,2]

Main effect*	n	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Week 5 (g)	Total (g)
Young rats							
High Ca	12	56.3 ± 6.5[a]	96.3 ± 6.0[a]	127.2 ± 11.2[a]	138.1 ± 6.6[a]	141.5 ± 11.9[a]	559.3 ± 29.0[a]
Low Ca	36	55.2 ± 4.2[a]	87.8 ± 3.2[a]	131.2 ± 4.6[a]	155.2 ± 5.7[a]	161.6 ± 7.0[a]	591.0 ± 17.2[a]
p-value		ns	ns	ns	ns	ns	ns
Casein	18	54.9 ± 4.8[a]	91.5 ± 4.2[a]	131.0 ± 7.4[a]	158.3 ± 8.6[a]	166.8 ± 9.9[a]	602.5 ± 25.0[a]
SP	18	55.6 ± 7.0[a]	84.2 ± 4.7[a]	131.4 ± 5.8[a]	152.1 ± 7.7[a]	156.3 ± 10.1[a]	579.5 ± 24.2[a]
p-value		ns	ns	ns	ns	ns	ns
0 IF	12	63.5 ± 8.2[a]	91.6 ± 5.2[a]	129.2 ± 6.7[a]	137.6 ± 8.2[a]	143.3 ± 10.5[a]	565.2 ± 28.6[a]
150 IF	12	56.1 ± 5.5[a]	89.6 ± 5.5[a]	132.6 ± 9.0[a]	165.7 ± 11.2[a]	181.2 ± 14.0[a]	625.0 ± 29.3[a]
400 IF	12	46.1 ± 7.6[a]	82.4 ± 5.9[a]	131.7 ± 8.8[a]	162.2 ± 8.7[a]	160.3 ± 10.1[a]	582.7 ± 31.4[a]
p-value		ns	ns	ns	ns	ns	ns
RB rats							
High Ca	12	183.2 ± 20.1[a]	185.1 ± 6.9[a]	183.4 ± 8.5[a]	205.8 ± 15.1[a]	158.7 ± 15.0[a]	916.2 ± 40.9[a]
Low Ca	35	167.0 ± 6.4[a]	174.3 ± 6.4[a]	173.0 ± 4.0[a]	172.4 ± 3.3[b]	152.4 ± 7.0[a]	839.8 ± 17.0[b]
p-value		ns	ns	ns	0.0028	ns	0.0411
Casein	17	159.7 ± 5.2[a]	159.5 ± 5.5[a]	171.2 ± 4.1[a]	173.8 ± 4.8[a]	149.6 ± 11.3[a]	815.0 ± 20.5[a]
SP	18	174.0 ± 11.3[a]	188.3 ± 10.4[b]	174.8 ± 6.8[a]	171.0 ± 4.8[a]	155.1 ± 8.8[a]	863.2 ± 26.2[a]
p-value		ns	0.023	ns	ns	ns	ns
0 IF	12	173.4 ± 5.7[a]	169.5 ± 5.5[a]	172.0 ± 4.4[a]	173.6 ± 4.2[a,b]	157.3 ± 14.8[a]	845.8 ± 22.6[a]
150 IF	12	160.4 ± 8.6[a]	182.4 ± 14.3[a]	176.3 ± 6.7[a]	181.3 ± 4.0[a]	157.9 ± 11.4[a]	858.3 ± 21.8[a]
400 IF	11	167.3 ± 17.4[a]	170.7 ± 12.3[a]	170.6 ± 9.7[a]	161.4 ± 7.8[b]	141.2 ± 9.6[a]	813.1 ± 42.9[a]
p-value		ns	ns	ns	0.044	ns	ns

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

* As described in table 3

As designed, there was a significant difference in Ca levels in the two control diets and in the low Ca diets (Table 6). The Ca levels in the SP-based diets were higher than the Ca levels in the casein-based diets. There were no differences in the P levels.

Table 6. Measured calcium and phosphorus levels in each diet. [1,2]

Diet	Ca (g/kg diet)	P (g/kg diet)
Control diets*		
Casein	4346.23[a]	3494.46[a]
SP	4537.14[b]	3582.14[a]
Low Ca diets*		
Casein	925.09[a]	3523.58[a]
SP	1026.08[b]	3435.59[a]

[1] Mean values are reported
[2] Groupings with different letters are significant, $p < 0.05$
* Control diets (diet 1 and diet 2) and Low Ca diets (diets 3-8)

Body weights

There were no differences among diet groups for the young and the RB rat body weights at baseline, evidence that randomization was successful (Table 7). Body weights were lower over the 5 weeks in both young and RB rats fed the low Ca diets compared to controls (Table 8). Body weights of the young rats were significantly lower by week 4 ($p < 0.05$) compared to normal Ca controls. Body weights in both the young and RB rats each week were higher in the rats fed SP, however the differences were non significant. Within the controls (diet 1 versus diet 2) rats fed SP had higher, but not significantly higher, body weight compared to casein after week 0 and this observation is seen in both young and RB rats (Table 7). No other differences were detected in either the young or RB rats.

Table 7. Weekly body weights of each diet group for young and retired breeder rats[1,2]

Diet[3]	n	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5
Young rats							
1	6	43 ± 0.6[a]	83 ± 2.0[a]	115 ± 2.8[a]	152 ± 4.9[a]	183 ± 6.3[a]	210 ± 5.7[a]
2	6	43 ± 1.1[a]	86 ± 2.8[a]	119 ± 5.1[a]	157 ± 9.7[a]	187 ± 2.0[a]	215 ± 16.5[a]
3	6	43 ± 1.1[a]	85 ± 2.4[a]	112 ± 2.7[a]	145 ± 3.4[a]	170 ± 5.3[a]	197 ± 8.4[a]
4	6	43 ± 0.9[a]	88 ± 2.2[a]	116 ± 3.0[a]	146 ± 6.0[a]	169 ± 7.9[a]	188 ± 10.0[a]
5	6	44 ± 1.0[a]	85 ± 4.3[a]	115 ± 4.0[a]	145 ± 3.3[a]	169 ± 6.2[a]	188 ± 7.1[a]
6	6	44 ± 1.0[a]	85 ± 4.5[a]	117 ± 4.8[a]	155 ± 5.9[a]	183 ± 6.1[a]	208± 8.2[a]
7	6	43 ± 0.9[a]	85 ± 2.9[a]	112 ± 3.8[a]	143 ± 4.8[a]	167 ± 6.0[a]	192 ± 8.7[a]
8	6	44 ± 1.0[a]	89 ± 2.3[a]	118 ± 4.3[a]	154 ± 6.8[a]	177 ± 9.5[a]	202 ± 11.1[a]
RB rats							
1	6	341 ± 12.9[a]	365 ± 23.0[a]	383 ± 17.7[a]	394 ± 13.1[a]	403 ± 12.7[a]	412 ± 12.9[a]
2	6	362 ± 13.0[a]	401 ± 17.2[a]	419 ± 15.4[a]	436 ± 16.1[a]	458 ± 21.8[a]	477 ± 22.8[a]
3	6	337 ± 10.4[a]	367 ± 8.0[a]	385 ± 9.9[a]	398 ± 11.6[a]	395 ± 19.8[a]	425 ± 13.0[a]
4	6	328 ± 9.4[a]	359 ± 9.5[a]	384 ± 5.9[a]	402 ± 7.3[a]	414 ± 8.6[a]	429 ± 9.7[a]
5	5	331 ± 9.4[a]	365 ± 7.5[a]	373 ± 7.7[a]	385 ± 6.7[a]	391 ± 7.4[a]	394 ± 12.8[a]
6	6	337 ± 8.0[a]	387 ± 9.0[a]	400 ± 8.9[a]	414 ± 11.5[a]	423 ± 13.2[a]	439 ± 17.1[a]
7	6	326 ± 9.7[a]	362 ± 11.6[a]	376 ± 11.4[a]	395 ± 12.1[a]	410 ± 13.0[a]	422 ± 13.8[a]
8	6	351 ± 14.6[a]	391 ± 26.4[a]	411 ± 26.2[a]	427 ± 28.7[a]	410 ± 21.8[a]	440 ± 30.0[a]

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 8. Summary of data for main effects on weekly body weights of tested effects for young and retired breeder rats[1,2]

Main effect*	n	Baseline[3] (g)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Week 5 (g)
Young rats							
High Ca	12	43.0 ± .06[a]	84.1 ± 1.7[a]	116.6 ± 2.8[a]	154.3 ± 5.2[a]	185.0 ± 6.5[a]	212.5 ± 8.4[a]
Low Ca	36	43.9 ± 0.4[a]	86.5 ± 1.3[a]	115.1 ± 1.5[a]	148.0 ± 2.1[a]	172.4 ± 2.8[b]	195.8 ± 3.6[b]
p-value		ns	ns	ns	ns	0.0472	0.0389
Casein	18	44.1 ± 0.5[a]	86.2 ± 1.7[a]	114.5 ± 1.8[a]	145.4 ± 2.4[a]	169.2 ± 3.6[a]	191.0 ± 4.8[a]
SP	18	43.6 ± 0.6[a]	86.8 ± 1.9[a]	115.8 ± 2.4[a]	150.5 ± 3.5[a]	175.5 ± 4.3[a]	200.6 ± 5.4[a]
p-value		ns	ns	ns	ns	ns	ns
0 IF	12	42.9 ± 0.7[a]	85.3 ± 2.4[a]	114.9 ± 2.7[a]	149.9 ± 3.6[a]	176.1 ± 4.3[a]	202.3 ± 5.8[a]
150 IF	12	44.1 ± 0.7[a]	86.9 ± 1.8[a]	113.8 ± 2.4[a]	144.5 ± 3.7[a]	167.8 ± 4.8[a]	189.9 ± 6.3[a]
400 IF	12	44.5 ± 0.6[a]	87.4 ± 2.4[a]	116.7 ± 2.8[a]	149.5 ± 3.9[a]	173.2 ± 5.5[a]	195.2 ± 6.6[a]
p-value		ns	ns	ns	ns	ns	ns
RB rats							
High Ca	12	351.8 ± 9.3[a]	382.8 ± 14.7[a]	401.2 ± 12.4[a]	415.0 ± 11.8[a]	430.6 ± 14.6[a]	444.3 ± 15.9[a]
Low Ca	35	335.1 ± 4.2[a]	371.8 ± 5.7[a]	388.6 ± 5.7[a]	404.1 ± 6.2[a]	407.5 ± 6.1[a]	425.8 ± 7.1[a]
p-value		ns	ns	ns	ns	ns	ns
Casein	17	332.0 ± 5.4[a]	363.4 ± 4.7[a]	380.9 ± 4.6[a]	395.7 ± 5.2[a]	400.4 ± 7.9[a]	417.4 ± 7.4[a]
SP	18	337.9 ± 6.5[a]	379.8 ± 9.9[a]	395.8 ± 10.0[a]	412.1 ± 10.9[a]	414.2 ± 9.1[a]	433.7 ± 11.8[a]
p-value		ns	ns	ns	ns	ns	ns
0 IF	12	336.7 ± 6.3[a]	376.7 ± 6.5[a]	392.5 ± 6.8[a]	406.2 ± 8.1[a]	408.6 ± 12.1[a]	431.8 ± 10.4[a]
150 IF	12	326.9 ± 6.5[a]	360.6 ± 7.2[a]	380.2 ± 6.2[a]	398.4 ± 6.8[a]	411.7 ± 7.5[a]	425.9 ± 8.1[a]
400 IF	11	342.2 ± 9.2[a]	378.8 ± 14.7[a]	393.3 ± 15.3[a]	408.2 ± 16.6[a]	401.6 ± 12.2[a]	419.1 ± 18.1[a]
p-value		ns	ns	ns	ns	ns	ns

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

[3] Indicates baseline data (week = 0)

* As described in table 3

Bone mineral density and bone mineral content

Young rats fed the low Ca diets had lower BMD and BMC than the rats fed normal Ca (Table 9 and Figure 5). Bone mineral density and BMC of young rats fed SP-based diets were significantly higher than for young rats fed the casein-based diets, when Ca intakes were low (Table 10 and Figure 6). Soy protein also produced higher BMD and BMC in the young rats fed normal Ca (diet 1 versus diet 2), however differences were not statistically significant (Table 9). There were no differences due to Ca levels, protein source, or added IFs on BMD measured in the RB rats (Table 10). The RB rats fed SP had higher BMD and BMC compared to casein, although the differences were not significant. There were no differences detected in BMC of the RB rats with the exception of a significant difference found between diet 8 and diet 4 (Table 9).

Table 9. Bone mineral density and bone mineral content of each diet group for young and retired breeder rats[1,2].

DIET[3]	n	BMD (Crude) (g/cm^3)	BMD (DEXA) (g/cm^2)	BMC (g)
Young rats				
1	6	1.352 ± 0.012[a]	0.160 ± 0.002[a]	0.2142 ± 0.006[a]
2	6	1.385 ± 0.027[a]	0.168 ± 0.007[a]	0.2287 ± 0.022[a]
3	6	1.167 ± 0.021[b]	0.067 ± 0.003[b]	0.1053 ± 0.007[b]
4	6	1.154 ± 0.014[b]	0.063 ± 0.001[b]	0.0919 ± 0.005[b]
5	6	1.154 ± 0.013[b]	0.061 ± 0.002[b]	0.0961 ± 0.003[b]
6	6	1.152 ± 0.025[b]	0.071 ± 0.001[b]	0.1157 ± 0.003[b]
7	6	1.164 ± 0.026[b]	0.068 ± 0.003[b]	0.1016 ± 0.003[b]
8	6	1.163 ± 0.005[b]	0.066 ± 0.001[b]	0.1066 ± 0.005[b]
RB rats				
1	6	1.597 ± 0.019[a]	0.241 ± 0.007[a]	0.5006 ± 0.025[a,b]
2	6	1.632 ± 0.027[a]	0.253 ± 0.009[a]	0.5214 ± 0.022[a,b]
3	6	1.601 ± 0.050[a]	0.236 ± 0.006[a]	0.4685 ± 0.011[a,b]
4	6	1.563 ± 0.032[a]	0.230 ± 0.008[a]	0.4326 ± 0.011[a]
5	5	1.520 ± 0.044[a]	0.230 ± 0.010[a]	0.4684 ± 0.030[a,b]
6	6	1.571 ± 0.007[a]	0.238 ± 0.005[a]	0.4926 ± 0.013[a,b]
7	6	1.535 ± 0.029[a]	0.229 ± 0.005[a]	0.4486 ± 0.016[a,b]
8	6	1.598 ± 0.020[a]	0.250 ± 0.003[a]	0.5105 ± 0.021[b]

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

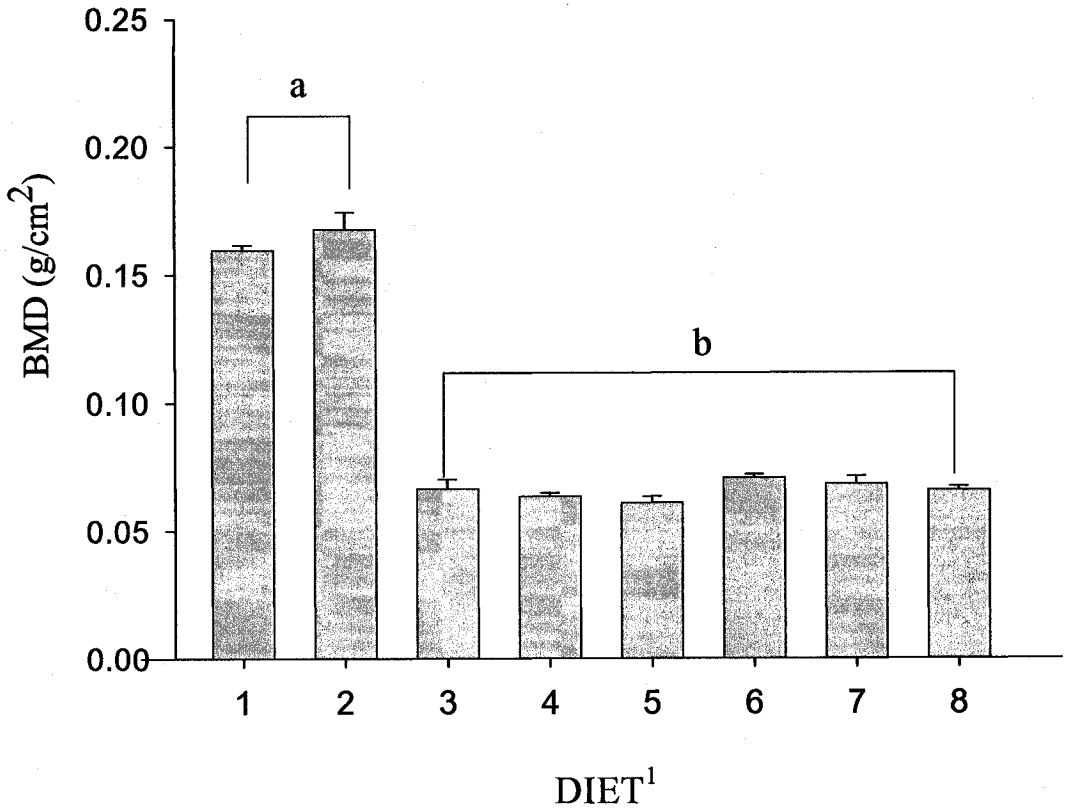


Figure 5. Effects of dietary calcium on bone mineral density in young rats. Diet groupings with different letters are significant, $p < 0.05$

[1] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 10. Summary of data for main effects on bone mineral density and bone mineral content for young and retired breeder rats[1,2].

Main effect*	n	BMD (Crude) (g/cm^3)	BMD (DEXA) (g/cm^2)	BMC (g)
Young rats				
High Ca	12	1.369 ± 0.015^a	0.164 ± 0.003^a	0.221 ± 0.011^a
Low Ca	36	1.159 ± 0.007^b	0.066 ± 0.001^b	0.103 ± 0.002^b
p-value		0.0001	0.0001	0.0001
Casein	18	1.159 ± 0.009^a	0.064 ± 0.002^a	0.098 ± 0.004^a
SP	18	1.160 ± 0.010^a	0.068 ± 0.001^b	0.108 ± 0.002^b
p-value		ns	0.0434	0.0327
0 IF	12	1.160 ± 0.015^a	0.068 ± 0.002^a	0.110 ± 0.004^a
150 IF	12	1.159 ± 0.014^a	0.066 ± 0.002^a	0.097 ± 0.003^a
400 IF	12	1.159 ± 0.006^a	0.064 ± 0.001^a	0.102 ± 0.004^a
p-value		ns	ns	ns
RB rats				
High Ca	12	1.615 ± 0.017^a	0.247 ± 0.006^a	0.511 ± 0.016^a
Low Ca	35	1.567 ± 0.012^a	0.236 ± 0.003^a	0.472 ± 0.008^a
p-value		ns	ns	0.0141
Casein	17	1.565 ± 0.024^a	0.232 ± 0.004^a	0.455 ± 0.010^a
SP	18	1.568 ± 0.013^a	0.239 ± 0.003^a	0.484 ± 0.011^a
p-value		ns	ns	ns
0 IF	12	1.584 ± 0.022^a	0.237 ± 0.004^a	$0.482 \pm 0.009^{a,b}$
150 IF	12	1.547 ± 0.021^a	0.230 ± 0.004^a	0.441 ± 0.010^a
400 IF	11	1.569 ± 0.024^a	0.243 ± 0.005^a	0.495 ± 0.018^b
p-value		ns	ns	0.0157

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

* As described in table 3

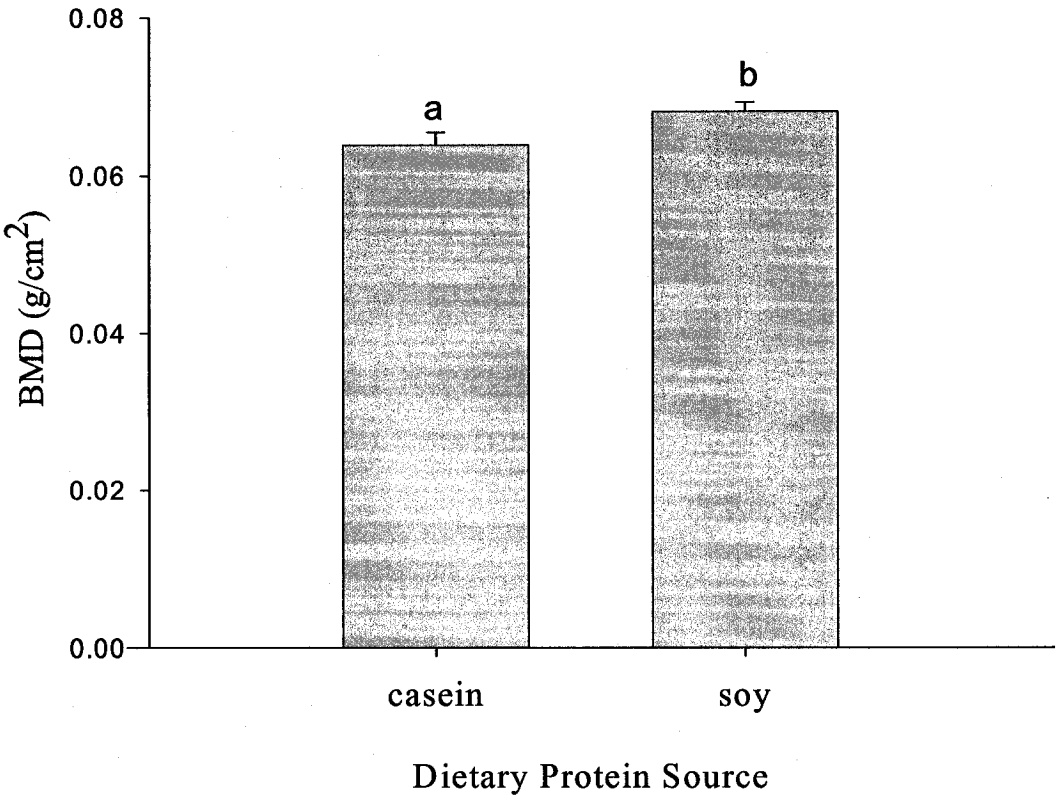


Figure 6. Effects of dietary protein (casein versus soy) on bone mineral density in young rats fed low calcium. Bars with different letters are significant, $p < 0.05$

Bone minerals

The young rats fed the low Ca diets had lower femoral Ca, Mg, and P levels (mg per gram dry wt) and higher Na levels than the normal Ca diets ($p < 0.001$) (Table 11 and 12). Within diets 3-8, SP significantly ($p < 0.05$) decreased Zn levels, and non-significantly increased Ca, Mg, K, and P levels compared to casein in the young rats (Table 12). Also within the controls (diet 1 versus diet 2) rats fed the SP-based diets had higher Ca, Mg, and P levels in comparison to the casein fed animals, but values were not statistically higher (Table 11). Calcium levels decreased with IFs ($p < 0.01$ and $p < 0.001$) but no other effects due to added IFs were seen. There were no differences due to dietary Ca level, protein source, or added IFs on femoral mineral (Ca, Mg, P, Zn, Na, and K) levels measured in the RB rats.

Table 11. Bone minerals of each diet group for young and retired breeder rats[1,2].

Diet[3]	n	Ca (mg/g dry wt)	Mg (mg/g dry wt)	P (mg/g dry wt)	Zn (mg/g dry wt)	Na (mg/g dry wt)	K (mg/g dry wt)
Young rats							
1	6	159.45 ± 2.27^{a}	2.94 ± 0.043^{a}	79.26 ± 1.72^{a}	0.177 ± 0.003^{a}	6.69 ± 0.279^{a,b}	0.332 ± 0.017^{a}
2	6	160.24 ± 1.33^{a}	2.96 ± 0.065^{a}	79.29 ± 1.45^{a}	0.150 ± 0.003^{b,c}	6.42 ± 0.335^{a}	0.322 ± 0.015^{a}
3	6	127.82 ± 1.32^{b}	2.37 ± 0.106^{b}	64.75 ± 1.83^{b}	0.188 ± 0.004^{a}	7.72 ± 0.772^{a,b}	0.291 ± 0.034^{a}
4	6	117.03 ± 2.24^{b,c}	2.26 ± 0.088^{b}	63.89 ± 1.68^{b}	0.198 ± 0.008^{a}	8.83 ± 0.678^{a,b}	0.302 ± 0.042^{a}
5	6	115.98 ± 3.83^{c}	2.23 ± 0.085^{b}	61.48 ± 1.76^{b}	0.187 ± 0.009^{a}	8.97 ± 0.599^{b}	0.300 ± 0.041^{a}
6	6	130.27 ± 3.45^{b}	2.56 ± 0.043^{b}	69.29 ± 1.30^{b}	0.158 ± 0.008^{a,b,c}	8.36 ± 0.396^{a,b}	0.323 ± 0.044^{a}
7	6	122.66 ± 2.52^{b}	2.35 ± 0.046^{b}	62.78 ± 0.57^{b}	0.157 ± 0.004^{a,b,c}	8.66 ± 0.654^{a,b}	0.271 ± 0.033^{a}
8	6	120.41 ± 2.40^{b}	2.35 ± 0.025^{b}	64.49 ± 2.68^{b}	0.166 ± 0.005^{a,b,c}	8.12 ± 0.385^{a,b}	0.351 ± 0.042^{a}
RB rats							
1	6	204.22 ± 17.8^{a}	2.86 ± 0.043^{a}	79.75 ± 1.35^{a}	0.154 ± 0.004^{a}	5.38 ± 0.244^{a}	0.296 ± 0.031^{a}
2	6	174.92 ± 4.68^{a,b}	2.93 ± 0.051^{a}	80.78 ± 1.56^{a}	0.155 ± 0.004^{a}	5.61 ± 0.314^{a}	0.303 ± 0.027^{a}
3	6	155.97 ± 3.06^{b}	2.87 ± 0.092^{a}	81.02 ± 1.91^{a}	0.161 ± 0.006^{a}	4.80 ± 0.668^{a}	0.278 ± 0.021^{a}
4	6	173.97 ± 4.62^{a,b}	2.80 ± 0.065^{a}	81.26 ± 1.47^{a}	0.156 ± 0.003^{a}	5.70 ± 0.175^{a}	0.273 ± 0.017^{a}
5	5	173.36 ± 5.41^{a,b}	2.70 ± 0.085^{a}	78.32 ± 1.76^{a}	0.154 ± 0.006^{a}	5.51 ± 0.173^{a}	0.268 ± 0.021^{a}
6	6	173.85 ± 4.03^{a,b}	2.80 ± 0.052^{a}	79.72 ± 1.67^{a}	0.154 ± 0.003^{a}	5.53 ± 0.139^{a}	0.355 ± 0.013^{a}
7	6	173.96 ± 4.82^{a,b}	2.74 ± 0.032^{a}	78.77 ± 0.95^{a}	0.152 ± 0.002^{a}	5.39 ± 0.186^{a}	0.281 ± 0.014^{a}
8	6	175.12 ± 2.44^{a,b}	2.90 ± 0.109^{a}	78.51 ± 0.95^{a}	0.159 ± 0.003^{a}	5.33 ± 0.161^{a}	0.264 ± 0.019^{a}

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 12. Summary of data for main effects on bone minerals of tested effects for young and retired breeder rats[1,2].

Main effect*	n	Ca (mg/g dry wt)	Mg (mg/g dry wt)	P (mg/g dry wt)	Zn (mg/g dry wt)	Na (mg/g dry wt)	K (mg/g dry wt)
Young rats							
High Ca	12	159.8 ± 1.3^a	2.95 ± 0.04^a	79.3 ± 1.1^a	0.164 ± 0.004^a	6.55 ± 0.20^a	0.327 ± 0.011^a
Low Ca	36	121.9 ± 1.4^b	2.34 ± 0.03^b	64.2 ± 0.8^b	0.177 ± 0.004^b	8.46 ± 0.25^b	0.304 ± 0.015^a
p-value		0.0001	0.0001	0.0001	0.0166	0.0002	ns
Casein	18	120.3 ± 2.0^a	2.29 ± 0.05^a	63.4 ± 1.0^a	0.191 ± 0.004^a	8.51 ± 0.40^a	0.298 ± 0.02^a
SP	18	123.9 ± 1.8^a	2.41 ± 0.03^a	65.1 ± 1.1^a	0.160 ± 0.003^b	8.40 ± 0.30^a	0.312 ± 0.02^a
p-value		ns	ns	ns	0.0001	ns	ns
0 IF	12	128.8 ± 1.5^a	2.44 ± 0.07^a	66.6 ± 1.4^a	0.176 ± 0.006^a	7.98 ± 0.50^a	0.304 ± 0.03^a
150 IF	12	119.8 ± 1.8^b	2.31 ± 0.05^a	63.3 ± 1.0^a	0.177 ± 0.007^a	8.74 ± 0.45^a	0.287 ± 0.03^a
400 IF	12	118.0 ± 2.4^b	2.29 ± 0.05^a	62.9 ± 1.5^a	0.118 ± 0.006^a	8.59 ± 0.38^a	0.324 ± 0.03^a
p-value		0.007 and 0.002	ns	ns	ns	ns	ns
RB rats							
High Ca	12	189.6 ± 9.9^a	2.90 ± 0.03^a	80.3 ± 1.0^a	0.155 ± 0.002^a	5.50 ± 0.19^a	0.300 ± 0.02^a
Low Ca	35	170.8 ± 2.9^a	2.81 ± 0.03^a	79.7 ± 0.6^a	0.156 ± 0.002^a	5.37 ± 0.13^a	0.289 ± 0.01^a
p-value		ns	ns	ns	ns	ns	ns
Casein	17	167.4 ± 5.3^a	2.80 ± 0.05^a	80.3 ± 1.0^a	0.157 ± 0.003^a	5.32 ± 0.25^a	0.273 ± 0.01^a
SP	18	174.3 ± 2.2^a	2.82 ± 0.04^a	79.0 ± 0.7^a	0.155 ± 0.002^a	5.42 ± 0.09^a	0.305 ± 0.01^a
p-value		ns	ns	ns	ns	ns	ns
0 IF	12	164.9 ± 7.1^a	2.83 ± 0.05^a	80.4 ± 1.2^a	0.158 ± 0.003^a	5.17 ± 0.34^a	0.317 ± 0.02^a
150 IF	12	174.0 ± 3.2^a	2.77 ± 0.04^a	80.0 ± 0.9^a	0.154 ± 0.002^a	5.54 ± 0.13^a	0.277 ± 0.01^a
400 IF	11	176.3 ± 3.3^a	2.81 ± 0.07^a	91.2 ± 12.8^a	0.162 ± 0.006^a	5.59 ± 0.20^a	0.266 ± 0.01^a
p-value		ns	ns	ns	ns	ns	ns

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

* As described in table 3

Biochemical markers of bone formation and resorption

The young rats fed the low Ca diets had higher serum osteocalcin ($p < 0.001$), and slightly, but not significantly, higher urinary DPD and PYD levels compared to controls (Table 13 and Table 14). There was a significant ($p < 0.05$) effect of protein source on DPD and PYD in the rats fed low Ca diets (Figure 7). Values from rats fed the SP-based diets were higher than those from the rats fed the casein-based diets for both DPD and PYD. Generally, the SP diet tended to increase osteocalcin levels but they were not significantly different from casein fed rats. No differences were detected due to added IFs on DPD, PYD or osteocalcin levels in the young rats. In the RB rats, the low Ca diets also tended to increase serum osteocalcin, urinary DPD and PYD compared to the controls, although the differences were not significant. There was a significant protein effect on DPD levels, but no other differences in DPD, PYD or osteocalcin values were detected in the RB rats.

Table 13. Biochemical indicators of bone formation and resorption of each diet group in young and retired breeder rats[1,2].

Diet[3]	n	Osteocalcin (ng/mL)	DPD (nmol/mmol creatinine)	PYD (nmol/mmol creatinine)
Young rats				
Baseline	6	8.81 ± 0.873^{a,b}	576.80 ± 62.31^{a}	247.56 ± 25.96^{a}
1	6	6.28 ± 0.975^{a}	114.92 ± 27.00^{b}	36.41 ± 7.37^{b}
2	6	6.12 ± 0.856^{a}	90.69 ± 14.87^{b}	36.13 ± 5.81^{b}
3	6	10.43 ± 1.007^{a,b}	88.47 ± 15.48^{b}	34.39 ± 6.52^{b}
4	6	10.94 ± 1.733^{a,b}	85.53 ± 10.41^{b}	37.61 ± 4.33^{b}
5	6	12.03 ± 0.979^{b}	88.90 ± 15.96^{b}	35.46 ± 5.55^{b}
6	6	12.50 ± 1.342^{b}	131.12 ± 25.37^{b}	47.57 ± 6.00^{b}
7	6	11.72 ± 0.879^{b}	138.61 ± 17.98^{b}	62.07 ± 7.19^{b}
8	6	11.53 ± 1.081^{b}	88.35 ± 22.63^{b}	45.40 ± 13.95^{b}
RB rats				
Baseline	6	2.85 ± 0.434^{a}	52.46 ± 9.11^{a}	21.33 ± 3.94^{a}
1	6	2.77 ± 0.333^{a}	105.03 ± 10.54^{b}	27.47 ± 2.44^{a}
2	6	2.37 ± 0.281^{a}	81.66 ± 5.89^{a}	22.20 ± 1.40^{a}
3	6	2.62 ± 0.373^{a}	122.35 ± 10.32^{a}	30.87 ± 2.53^{a}
4	6	2.05 ± 0.090^{a}	115.48 ± 11.36^{a}	30.69 ± 3.52^{a}
5	5	2.01 ± 0.168^{a}	116.51 ± 13.15^{a}	31.59 ± 3.86^{a}
6	6	2.88 ± 0.556^{a}	88.90 ± 16.85^{a}	25.75 ± 4.77^{a}
7	6	2.96 ± 0.379^{a}	98.10 ± 7.50^{a}	27.73 ± 2.00^{a}
8	6	1.92 ± 0.161^{a}	102.12 ± 13.51^{a}	27.88 ± 4.24^{a}

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 14. Summary of data for main effects on biochemical indicators of bone formation and resorption of tested effects in young and retired breeder rats[1,2].

Main effect*	n	Osteocalcin (ng/mL)	DPD (nmol/mmol creatinine)	PYD (nmol/mmol creatinine)
Young rats				
High Ca	12	6.18 ± 0.62[a]	4.53 ± 0.14[a]	3.52 ± 0.11[a]
Low Ca	36	11.52 ± 0.47[b]	4.54 ± 0.07[a]	3.69 ± 0.07[a]
p-value		0.0001	ns	ns
Casein	18	11.13 ± 0.72[a]	4.41 ± 0.08[a]	3.52 ± 0.08[a]
SP	18	11.92 ± 0.61[a]	4.67 ± 0.12[a]	3.86 ± 0.10[b]
p-value		ns	ns	0.0153
0 IF	12	11.47 ± 0.86[a]	4.60 ± 0.13[a]	3.64 ± 0.11[a]
150 IF	12	11.33 ± 0.93[a]	4.65 ± 0.11[a]	3.84 ± 0.11[a]
400 IF	12	11.78 ± 0.70[a]	4.38 ± 0.03[a]	3.58 ± 0.13[a]
p-value		ns	ns	ns
RB rats				
High Ca	12	2.57 ± 0.22[a]	93.3 ± 6.7[a]	3.19 ± 0.06[a]
Low Ca	35	2.60 ± 0.18[a]	107.0 ± 5.1[a]	3.33 ± 0.05[a]
p-value		ns	ns	ns
Casein	17	2.41 ± 0.22[a]	118.2 ± 6.3[a]	3.41 ± 0.06[a]
SP	18	2.79 ± 0.29[a]	96.4 ± 7.3[b]	3.26 ± 0.07[a]
p-value		ns	0.0403	ns
0 IF	12	2.75 ± 0.32[a]	105.6 ± 10.7[a]	3.30 ± 0.09[a]
150 IF	12	2.75 ± 0.30[a]	106.8 ± 7.0[a]	3.35 ± 0.07[a]
400 IF	11	2.29 ± 0.33[a]	107.2 ± 10.1[a]	3.35 ± 0.09[a]
p-value		ns	ns	ns

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

* As described in table 3

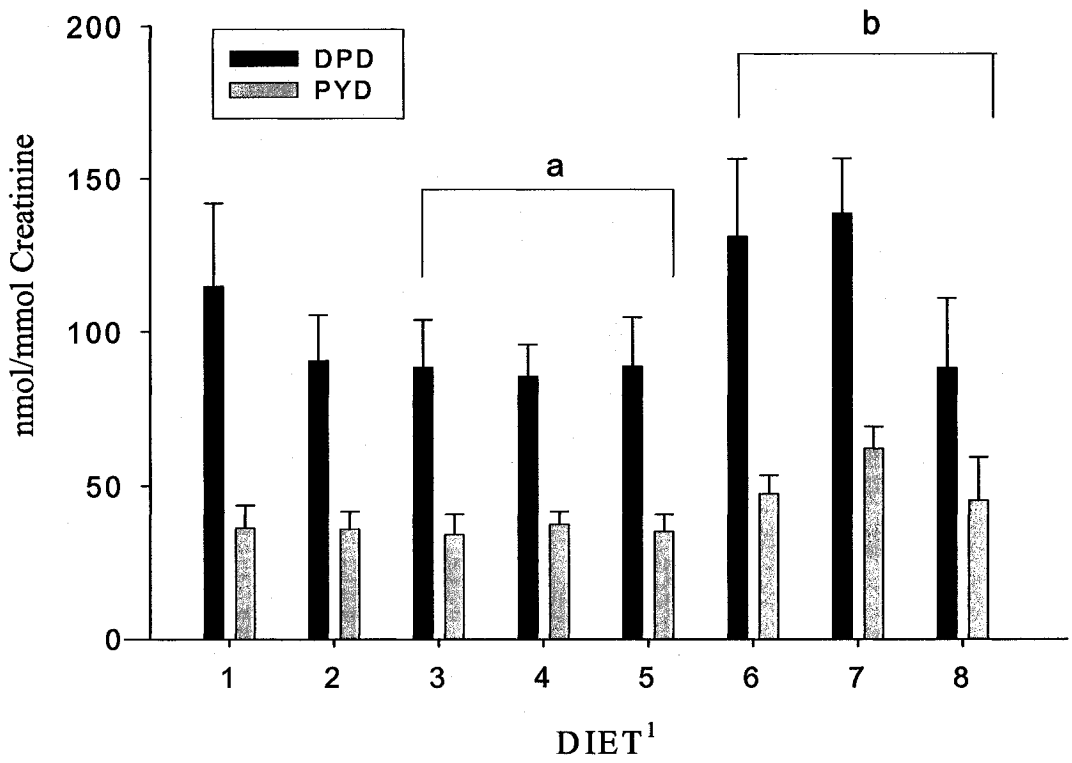


Figure 7. Effects of dietary protein (casein versus soy) on urinary DPD and PYD (nmol/mmol creatinine) in young rats fed low calcium. Diet groupings with different letters are significant for DPD and PYD, $p < 0.05$

[1] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Bone growth parameters

The young rats fed the low Ca diets had lower wet, dry, and ash weights than controls ($p < 0.001$), smaller values for lengths and diameters (distal and mid diaphysis) than controls ($p < 0.05$), and higher moisture percent than controls ($p < 0.001$) (Table 15 and Figure 8). There were no further significant differences due to dietary protein (soy versus casein) or added IFs on any of the physical parameters, although some of the physical parameters (wet, dry, and ash weights, length and diameter) tended to be higher in the SP fed animals compared to casein fed animals (Table 16). The RB rats tended to have lower wet, dry, and ash weights, smaller lengths and diameters (distal and mid diaphysis), and higher moisture percent in the low Ca diet groups but none of these values were statistically different from controls (Table 15). There were no other differences in bone growth parameters detected in the RB rats.

Table 15. Bone growth parameters of each diet group for young and retired breeder rats[1,2].

Diet[3]	n	Wet wt (g)	Dry wt (g)	Ash wt (g)	Length (cm)	Distal diameter (cm)	Mid diaphysis diameter (cm)	Moisture (%)
Young rats								
1	6	0.615 ± 0.028^{a}	0.334 ± 0.010^{a}	238.17 ± 8.57^{a}	3.14 ± 0.031^{a}	0.739 ± 0.059^{a}	0.375 ± 0.006^{a}	45.61 ± 1.13^{a}
2	6	0.621 ± 0.038^{a}	0.347 ± 0.025^{a}	234.64 ± 11.69^{a}	3.15 ± 0.056^{a}	0.678 ± 0.006^{a}	0.383 ± 0.005^{a}	44.29 ± 0.74^{a}
3	6	0.470 ± 0.019^{b}	0.191 ± 0.008^{b}	110.87 ± 8.23^{b}	3.03 ± 0.044^{a}	0.645 ± 0.011^{a}	0.354 ± 0.01^{a}	59.42 ± 0.69^{b}
4	6	0.478 ± 0.026^{b}	0.188 ± 0.008^{b}	107.38 ± 9.06^{b}	3.01 ± 0.039^{a}	0.656 ± 0.016^{a}	0.353 ± 0.008^{a}	60.38 ± 1.06^{b}
5	6	0.483 ± 0.006^{a,b}	0.194 ± 0.006^{b}	98.21 ± 3.78^{b}	3.04 ± 0.060^{a}	0.659 ± 0.008^{a}	0.331 ± 0.008^{a}	60.00 ± 0.77^{b}
6	6	0.515 ± 0.019^{a,b}	0.205 ± 0.004^{b}	117.38 ± 2.08^{b}	3.10 ± 0.045^{a}	0.676 ± 0.007^{a}	0.371± 0.004^{a}	60.14 ± 0.86^{b}
7	6	0.468 ± 0.020^{b}	0.191 ± 0.006^{b}	105.65 ± 4.08^{b}	3.05 ± 0.037^{a}	0.654 ± 0.007^{a}	0.305 ± 0.011^{a}	59.07 ± 0.60^{b}
8	6	0.507 ± 0.027^{a,b}	0.202 ± 0.007^{b}	113.89 ± 5.13^{b}	3.08 ± 0.039^{a}	0.680 ± 0.009^{a}	0.354 ± 0.008^{a}	59.97 ± 1.10^{b}
RB rats								
1	6	0.965 ± 0.035^{a}	0.698 ± 0.027^{a}	566.00 ± 31.92^{a}	3.88 ± 0.042^{a}	0.720 ± 0.005^{a}	0.400 ± 0.005^{a}	27.72 ± 0.86^{a}
2	6	0.964 ± 0.029^{a}	0.705 ± 0.021^{a}	574.32 ± 43.73^{a}	3.90 ± 0.039^{a}	0.715 ± 0.007^{a}	0.404 ± 0.011^{a}	26.81 ± 0.61^{a}
3	6	0.921 ± 0.014^{a}	0.660 ± 0.017^{a}	550.92 ± 25.73^{a}	3.87 ± 0.052^{a}	0.713 ± 0.007^{a}	0.413 ± 0.005^{a}	28.35 ± 1.23^{a}
4	6	0.850 ± 0.012^{a}	0.603± 0.013^{a}	478.87 ± 26.10^{a}	3.88 ± 0.020^{a}	0.703 ± 0.013^{a}	0.386 ± 0.004^{a}	29.08± 0.68^{a}
5	5	0.941 ± 0.040^{a}	0.672 ± 0.026^{a}	509.38 ± 26.10^{a}	3.88 ± 0.019^{a}	0.715 ± 0.006^{a}	0.413 ± 0.004^{a}	28.63 ± 0.73^{a}
6	6	0.967 ± 0.023^{a}	0.691 ± 0.022^{a}	551.61 ± 18.56^{a}	3.89 ± 0.037^{a}	0.722 ± 0.011^{a}	0.416 ± 0.011^{a}	28.65 ± 0.61^{a}
7	6	0.896 ± 0.023^{a}	0.642 ± 0.020^{a}	519.79 ± 24.11^{a}	3.84 ± 0.044^{a}	0.700 ± 0.009^{a}	0.400± 0.005^{a}	28.46 ± 0.61^{a}
8	6	0.975 ± 0.050^{a}	0.562 ± 0.111^{a}	592.89 ± 19.33^{a}	3.85 ± 0.053^{a}	0.712 ± 0.011^{a}	0.416 ± 0.015^{a}	28.16 ± 0.43^{a}

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 16. Summary of data for main effects on bone growth parameters for young and retired breeder rats[1,2].

Main effect*	n	Wet wt (g)	Dry wt (g)	Ash wt (g)	Length (cm)	Distal diameter (cm)	Mid diaphysis diameter (cm)	Moisture (%)
Young rats								
High Ca	12	0.618 ± 0.022^a	0.340 ± 0.013^a	245 ± 10.6^a	3.14 ± 0.03^a	0.709 ± 0.030^a	0.379 ± 0.004^a	45.0 ± 0.7^a
Low Ca	36	0.487 ± 0.009^b	0.195 ± 0.00^b	109 ± 2.6^b	3.05 ± 0.02^b	0.662 ± 0.005^b	0.352 ± 0.004^b	59.9 ± 0.3^b
p-value		0.0001	0.0001	0.0001	0.0105	0.0349	0.0014	0.0001
Casein	18	0.477 ± 0.010^a	0.191 ± 0.004^a	106 ± 4.3^a	3.02 ± 0.03^a	0.653 ± 0.007^a	0.347 ± 0.006^a	60.0 ± 0.5^a
SP	18	0.497 ± 0.014^a	0.200 ± 0.004^a	112 ± 2.6^a	3.08 ± 0.02^a	0.671 ± 0.005^a	0.358 ± 0.005^a	59.7 ± 0.5^a
p-value		ns	ns	ns	ns	ns	ns	ns
0 IF	12	0.490 ± 0.015^a	0.197 ± 0.005^a	114 ± 4.6^a	3.06 ± 0.03^a	0.659 ± 0.009^a	0.362 ± 0.007^a	59.7 ± 0.5^a
150 IF	12	0.473 ± 0.015^a	0.190 ± 0.005^a	107 ± 4.6^a	3.02 ± 0.03^a	0.656 ± 0.008^a	0.351 ± 0.007^a	59.9 ± 0.6^a
400 IF	12	0.497 ± 0.015^a	0.198 ± 0.005^a	107 ± 4.2^a	3.07 ± 0.03^a	0.671 ± 0.007^a	0.344 ± 0.007^a	60.0 ± 0.7^a
p-value		ns	ns	ns	ns	ns	ns	ns
RB rats								
High Ca	12	0.965 ± 0.021^a	0.702 ± 0.016^a	570 ± 25.1^a	3.89 ± 0.03^a	0.718 ± 0.004^a	0.402 ± 0.006^a	27.3 ± 0.5^a
Low Ca	35	0.919 ± 0.012^a	0.656 ± 0.010^b	535 ± 10.8^a	3.86 ± 0.02^a	0.709 ± 0.004^a	0.405 ± 0.003^a	28.6 ± 0.3^a
p-value		ns	0.0367	ns	ns	ns	ns	ns
Casein	17	0.900 ± 0.017^a	0.642 ± 0.013^a	513 ± 14.9^a	3.87 ± 0.02^a	0.710 ± 0.006^a	0.403 ± 0.005^a	28.7 ± 0.5^a
SP	18	0.933 ± 0.017^a	0.668 ± 0.013^a	552 ± 14.1^a	3.84 ± 0.02^a	0.709 ± 0.006^a	0.406 ± 0.005^a	28.4 ± 0.3^a
p-value		ns	ns	ns	ns	ns	ns	ns
0 IF	12	0.947 ± 0.016^a	0.677 ± 0.015^a	551 ± 14.5^a	3.88 ± 0.03^a	0.718 ± 0.007^a	0.414 ± 0.006^a	28.5 ± 0.6^a
150 IF	12	0.876 ± 0.016^a	0.624 ± 0.014^a	502 ± 15.5^a	3.85 ± 0.03^a	0.701 ± 0.007^a	0.394 ± 0.004^a	28.7 ± 0.4^a
400 IF	11	0.938 ± 0.026^a	0.671 ± 0.016^a	557 ± 22.7^a	3.84 ± 0.02^a	0.708 ± 0.005^a	0.407 ± 0.005^a	28.4 ± 0.4^a
p-value		ns	ns	ns	ns	ns	ns	ns

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

* As described in table 3

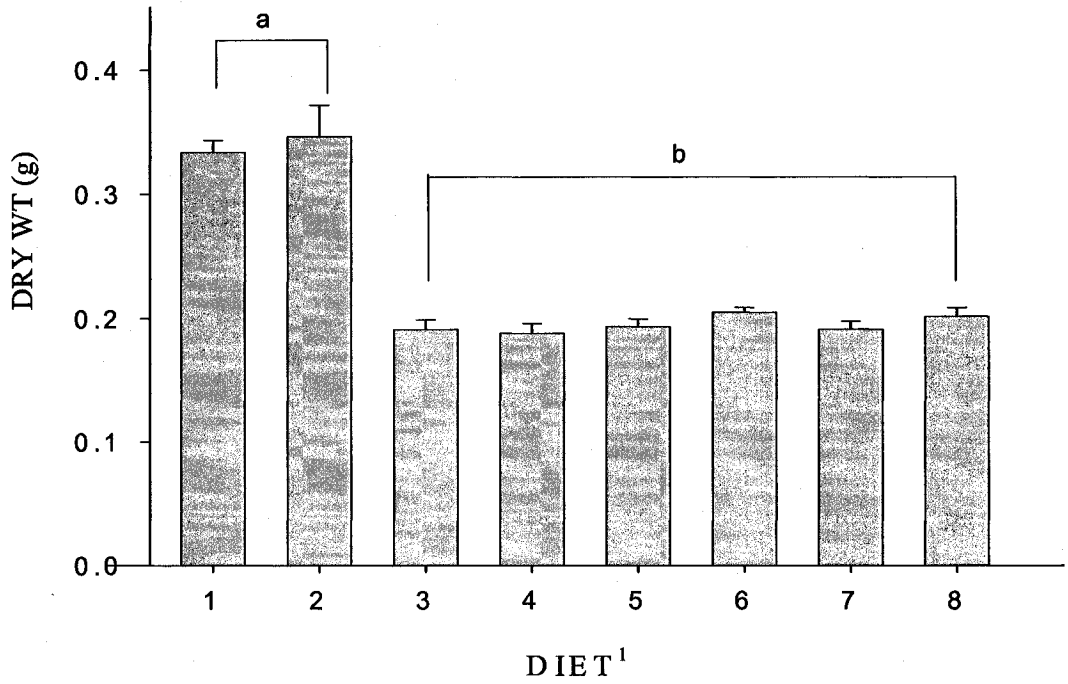


Figure 8. Effect of dietary calcium on dry weights (g) in young rats. Diet groupings with different letters are significant, $p < 0.05$

[1] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Hematology

The young rats fed the low Ca diets had statistically higher hemoglobin (HGB), hematocrit (HCT) and statistically lower mean platelet volume (MPV) values compared to controls (Table 18). There was a significant protein effect on platelets (PLT levels) ($p < 0.01$) and MPV levels ($p < 0.01$). Values for PLT and MPV were higher for the SP fed rats compared to casein fed rats. There were no differences due to Ca levels or added IFs on hematology parameters in the RB rats (Table 18). There was a significant protein effect on red blood cell (RBC), PLT, and MPV levels. The SP fed rats has higher values for PLT and lower values for RBC and MPV compared to the casein fed rats.

Table 17. Hematology of each diet group for young and retired breeder rats[1,2].

DIET[3]	n	WBC (10^3/L)	RBC (10^{12}/L)	HGB (g/L)	HCT (%)	MCV (fl)	MCH (pg)	RDW (%)	PLT (10^3/L)	MPV (fl)
Young rats										
1	6	5.92 ± 0.67[a]	7.17 ± 0.11[a]	146 ± 2.0[a]	0.419 ± 0.006[a]	58.33 ± 0.56[a]	20.32 ± 0.26[a]	11.33 ± 0.20[a]	870 ± 63[a]	7.83 ± 0.19[a]
2	6	6.17 ± 0.62[a]	7.26 ± 0.16[a]	147 ± 1.4[a]	0.421 ± 0.006[a]	58.17 ± 0.70[a]	20.28 ± 0.32[a]	11.68 ± 0.13[a]	931 ± 28[a]	7.27 ± 0.11[a,b]
3	6	6.33 ± 0.86[a]	7.45 ± 0.16[a]	151 ± 2.3[a]	0.436 ± 0.006[a]	58.67 ± 1.02[a]	20.27 ± 0.44[a]	11.13 ± 0.07[a]	869 ± 31[a]	7.47 ± 0.16[a,b]
4	6	6.33 ± 1.29[a]	7.70 ± 0.20[a]	157 ± 3.2[a]	0.453 ± 0.009[a]	59.00 ± 0.52[a]	20.38 ± 0.10[a]	11.28 ± 0.33[a]	822 ± 23[a]	7.45 ± 0.21[a,b]
5	6	5.20 ± 0.75[a]	7.50 ± 0.14[a]	152 ± 1.3[a]	0.435 ± 0.005[a]	58.17 ± 0.40[a]	20.18 ± 0.23[a]	11.17 ± 0.16[a]	863 ± 32[a]	7.25 ± 0.08[a,b]
6	6	5.60 ± 0.77[a]	7.21 ± 0.10[a]	147 ± 2.0[a]	0.423 ± 0.007[a]	58.67 ± 0.33[a]	20.33 ± 0.06[a]	11.23 ± 0.13[a]	972 ± 48[a]	7.20 ± 0.07[b]
7	6	6.98 ± 0.23[a]	7.41 ± 0.20[a]	151 ± 3.1[a]	0.436 ± 0.010[a]	58.67 ± 0.56[a]	20.42 ± 0.23[a]	11.27 ± 0.20[a]	906 ± 25[a]	7.17 ± 0.11[b]
8	6	6.72 ± 1.28[a]	7.33 ± 0.15[a]	153 ± 3.6[a]	0.433 ± 0.012[a]	59.17 ± 0.83[a]	20.78 ± 0.18[a]	11.28 ± 0.19[a]	943 ± 49[a]	6.92 ± 0.09[b]
RB rats										
1	6	3.37 ± 0.30[a]	7.81 ± 0.09[a]	145 ± 2.4[a]	0.420 ± 0.005[a]	53.83 ± 0.83[a]	18.52 ± 0.24[a]	11.65 ± 0.50[a]	742 ± 50[a]	8.10 ± 0.15[a,b,c,d]
2	6	3.50 ± 0.57[a]	7.40 ± 0.15[a]	142 ± 2.6[a]	0.408 ± 0.001[a]	55.33 ± 1.15[a]	19.15 ± 0.30[a]	11.47 ± 0.34[a]	738 ± 63[a]	7.72 ± 0.17[a,b,c,d]
3	6	3.20 ± 0.28[a]	7.62 ± 0.08[a]	142 ± 2.3[a]	0.407 ± 0.014[a]	54.83 ± 0.48[a]	18.67 ± 0.19[a]	12.15 ± 0.28[a]	650 ± 39[a]	8.32 ± 0.14[a,b,c,d]
4	6	3.48 ± 0.55[a]	7.78 ± 0.06[a]	144 ± 1.7[a]	0.425 ± 0.004[a]	54.83 ± 0.40[a]	18.47 ± 0.11[a]	11.98 ± 0.22[a]	670 ± 20[a]	8.10 ± 0.17[a,b,c,d]
5	5	2.50 ± 0.32[a]	7.61 ± 0.12[a]	142 ± 4.2[a]	0.418 ± 0.012[a]	55.00 ± 0.71[a]	18.60 ± 0.28[a]	11.68 ± 0.32[a]	673 ± 35[a]	8.28 ± 0.26[b,c]
6	6	2.93 ± 0.50[a]	7.44 ± 0.13[a]	140 ± 1.7[a]	0.407 ± 0.004[a]	54.83 ± 0.65[a]	18.82 ± 0.25[a]	11.65 ± 0.32[a]	836 ± 23[a]	7.33 ± 0.14[a,b,d]
7	6	2.55 ± 0.29[a]	7.60 ± 0.14[a]	143 ± 3.8[a]	0.418 ± 0.012[a]	55.00 ± 1.03[a]	18.87 ± 0.38[a]	11.57 ± 0.38[a]	823 ± 43[a]	7.48 ± 0.10[a,b,d]
8	6	2.15 ± 0.38[a]	7.34 ± 0.10[a]	138 ± 2.1[a]	0.400 ± 0.005[a]	54.50 ± 0.43[a]	18.72 ± 0.15[a]	11.78 ± 0.22[a]	765 ± 43[a]	7.48 ± 0.10[a,b,d]

[1] Values are mean ± SEM

[2] Diets with different letters are significant, $p < 0.05$

[3] Diet 1 a casein-based diet, diet 2 a SP-based diet, diet 3 a casein-based diet with low Ca, diet 4 a casein-based diet with low Ca and 150 mg IFs/kg diet, diet 5 a casein-based diet with low Ca and 400 mg IFs/kg diet, diet 6 a SP-based diet with low Ca, diet 7 a SP-based diet with low Ca and 150 mg IFs/kg diet, and diet 8 a SP-based diet with low Ca and 400 mg IFs/kg diet

Table 18. Summary of data for main effects on hematology parameters for young and retired breeder rats[1,2].

Main effect*	n	WBC (10^9/L)	RBC (10^{12}/L)	HGB (g/L)	HCT (%)	MCV (fl)	MCH (pg)	RDW (%)	PLT (10^9/L)	MPV (fl)
Young rats										
High Ca	12	6.04 ± 0.44[a]	7.22 ± 0.09[a]	146 ± 1.2[a]	0.420 ± 0.004[a]	58.3 ± 0.43[a]	20.3 ± 0.19[a]	11.5 ± 0.12[a]	900 ± 34[a]	7.55 ± 0.13[a]
Low Ca	36	6.19± 0.41[a]	7.43 ± 0.06[a]	152 ± 1.1[b]	0.436 ± 0.004[b]	58.7 ± 0.25[a]	20.4 ± 0.10[a]	11.2 ± 0.08[a]	896 ± 16[a]	7.24 ± 0.06[b]
p-value		ns	ns	0.0145	0.0187	ns	ns	ns	ns	0.0083
Casein	18	5.96 ± 0.55[a]	7.55 ± 0.55[a]	153 ± 1.5[a]	0.441 ± 0.004[a]	58.6 ± 0.39[a]	20.3 ± 0.16[a]	11.2 ± 0.12[a]	851 ± 17[a]	7.39 ± 0.09[a]
SP	18	6.43 ± 0.61[a]	7.31 ± 0.09[a]	150 ± 1.7[a]	0.430 ± 0.006[a]	58.8 ± 0.34[a]	20.5 ± 0.11[a]	11.3 ± 0.10[a]	940 ± 24[b]	7.09 ± 0.06[b]
p-value		ns	ns	ns	ns	ns	ns	ns	0.0053	0.0094
0 IF	12	5.97 ± 0.56[a]	7.33 ± 0.10[a]	149 ± 1.6[a]	0.429 ± 0.005[a]	58.7 ± 0.51[a]	20.3 ± 0.21[a]	11.2 ± 0.07[a]	920 ± 31[a]	7.33 ± 0.09[a]
150 IF	12	6.66 ± 0.83[a]	7.55 ± 0.14[a]	154 ± 2.3[a]	0.444 ± 0.007[a]	58.8 ± 0.37[a]	20.4 ± 0.12[a]	11.3 ± 0.19[a]	846 ± 21[a]	7.31 ± 0.12[a]
400 IF	12	5.96 ± 0.74[a]	7.41 ± 0.10[a]	152 ± 1.9[a]	0.434 ± 0.006[a]	58.7 ± 0.47[a]	20.5 ± 0.17[a]	11.2 ± 0.12[a]	903 ± 30[a]	7.08 ± 0.08[a]
p-value		ns	ns	ns	ns	ns	ns	ns	ns	ns
RB rats										
High Ca	12	3.43 ± 0.31[a]	7.60 ± 0.10[a]	143 ± 1.8[a]	0.414 ± 0.006[a]	54.6 ± 0.71[a]	18.8 ± 0.21[a]	11.6 ± 0.29[a]	740 ± 39[a]	7.91 ± 0.12[a]
Low Ca	35	2.81 ± 0.17[a]	7.57 ± 0.05[a]	141 ± 1.1[a]	0.412 ± 0.004[a]	54.8 ± 0.25[a]	18.7 ± 0.09[a]	11.8 ± 0.12[a]	738 ± 19[a]	7.82 ± 0.09[a]
p-value		ns	ns	ns	ns	ns	ns	ns	ns	ns
Casein	17	3.09 ± 0.24[a]	7.67 ± 0.05[a]	143 ± 1.5[a]	0.417 ± 0.006[a]	54.6 ± 0.70[a]	18.8 ± 0.21[a]	11.6 ± 0.29[a]	740 ± 39[a]	7.91 ± 0.12[a]
SP	18	2.54 ± 0.23[a]	7.46 ± 0.07[b]	140 ± 1.6[a]	0.408 ± 0.005[a]	54.8 ± 0.41[a]	18.8 ± 0.15[a]	11.7 ± 0.17[a]	808 ± 22[b]	7.43 ± 0.06[b]
p-value		ns	0.0260	ns	ns	ns	ns	ns	0.0001	0.0001
0 IF	12	3.07 ± 0.28[a]	7.53 ± 0.08[a]	141 ± 1.4[a]	0.407 ± 0.007[a]	54.8 ± 0.39[a]	18.7 ± 0.15[a]	11.9 ± 0.21[a]	743 ± 36[a]	7.83 ± 0.17[a]
150 IF	12	3.02 ± 0.32[a]	7.69 ± 0.08[a]	144 ± 2.0[a]	0.422 ± 0.006[a]	54.92 ± 0.53[a]	18.7 ± 0.20[a]	11.8 ± 0.22[a]	747 ± 32[a]	7.79 ± 0.13[a]
400 IF	11	2.31 ± 0.25[a]	7.46 ± 0.09[a]	140 ± 2.2[a]	0.408 ± 0.006[a]	54.7 ± 0.38[a]	18.7 ± 0.14[a]	11.7 ± 0.18[a]	723 ± 31[a]	7.85 ± 0.18[a]
p-value		ns	ns	ns	ns	ns	ns	ns	ns	ns

[1] Values are mean ± SEM

[2] Groupings of main effects with different letters are significant, $p < 0.05$

* As described in table 3

Discussion

Consistent with data from previous studies, the low Ca diet used in this study resulted in increases in urinary DPD and PYD, and serum osteocalcin, and decreases in BMD and BMC in the young rats. However, unlike Creedon and Cashman (30) whose experimental design was slightly different, there were large decreases in femoral Ca, Mg and P levels in the young rats fed the low Ca diet in the present study. The rat data is also consistent with human studies which have shown that healthy young women fed a low Ca diet (< 300 mg/day), have increased serum osteocalcin and urinary DPD levels (3).

In addition to significantly affecting BMD, BMC, and femoral Ca, Mg and P levels, the low Ca rat model produced adverse effects on physical parameters such as bone mass (wet, dry, and ash weights), bone length, and body weights. It can be concluded that the low Ca diet resulted in reduced bone growth since it predominantly affected the young growing rats as compared to the RB rats in this study.

It has been well documented that a low Ca diet can decrease bone growth, cause bone loss, and may lead to the development of osteoporosis (21; 46; 70; 88) and animal models of low dietary Ca are often used as models to study osteoporosis (45; 86). Osteoporosis is a significant health problem in Canada (21) and will continue to burden the health care system as the number of elderly grows and as Ca intakes remain low (32; 38) in the coming years.

Animal studies investigating the negative effects of a low Ca diet on bone health have been conducted for years (30; 55; 87; 90) and have been supported by human studies (3) that also show the deleterious effects of low Ca intakes on bone metabolism. Since a low Ca diet contributes to an increased risk of fracture, low Ca intakes have been identified as a minor risk factor for developing osteoporosis (21). Risk of fractures can also be determined by low BMD and thus it is the primary predictor for the diagnosis and management of osteoporosis (21).

Development of new software permits BMD of small animals to be measured by DEXA (54). Until recently, the standard method for measuring BMD of bones from

small animals had been the application of Archimedes' Principle (54). This method is based on water displacement and BMD is recorded on a volumetric basis. There are several studies assessing bone metabolism that have reported BMD by this technique (10; 11; 12; 51) and their results are consistent with those reported here. Dual energy x-ray absorptiometry BMD is generally recorded in g/cm^2 which is based on two dimensions rather than a true volumetric measurement, g/cm^3, as in the case with Archimedes' Principle (33). Although it is more expensive than Archimedes' Principle, DEXA is established as the standard for measuring BMD and can be applicable to *in vivo* analysis (33; 54). Both methods of measuring BMD are equally effective and are highly correlated to each other (54); other variables such as femur ash weight and Ca content are also highly correlated to BMD with both techniques (54). Therefore, both techniques were used in this study to measure BMD.

Virtually all cells throughout the body require Ca. Calcium homeostasis is tightly regulated by the kidneys, the intestines, and the skeleton (23). A low Ca diet causes the ECF concentration of Ca to decrease which results in hypocalcemia and an increase in PTH secretion (23). An increase in PTH causes Ca resorption to increase in the bone (23). Mechanistically the rise in PTH causes the number and size of osteoclasts to increase (61).

There is strong and consistent evidence that the low Ca induced rise in the number and size of osteoclasts increases bone resorption. Bone resorption can increase by as much as 400% (90) and it has been shown to increase both by histomorphometric methods (55; 90) and by biochemical indices (3; 30; 87). Repletion with adequate Ca has been shown to completely reverse these effects, sometimes within one day of repletion (61).

Unlike bone resorption, a low Ca diet causes bone formation to decrease. Bone formation has been shown to decrease as measured by histomorphometric methods in rats (90) and in mice (55) and by biochemical markers measured in humans (3). Also IGFs are often used as indices of bone formation (28; 79) because they are abundant growth factors produced by bone cells (108) that can stimulate bone formation (28). Insulin growth factors can bind to a group of specialized IGF binding proteins (IGFBPs)

that in turn negatively regulate IGFs. At high concentrations, IGFBPs are known to inhibit IGFs and as a result the stimulation of bone formation by IGFs is decreased. It was reported that a low Ca diet fed to weanling rats increased levels of IGFBPs by as much as 2- and 4-fold (108), indicating decreased bone formation.

The results obtained here indicate that bone growth was extensively lowered despite increases in osteocalcin, a well-known bone formation marker (26; 98). However because osteocalcin is an indicator of osteoblast activity, it may not be representative of the entire formation process (26). This would explain why some investigators detect increases in the number of osteoblasts (96), and associated increases in osteocalcin (3; 87) while observing decreases in bone formation. Recently, women fed a low Ca diet showed increases in serum osteocalcin but decreases in another bone formation marker, procollagen I carboxyterminal propeptide (PICP) levels (3).

The discrepancy in the circulating levels of different bone formation markers could suggest that there are several aspects of bone formation (3) and each biomarker would reflect one aspect and not necessarily the entire process. In this regard, because PICP is a product of bone collagen synthesis (98), it is presumed that the PICP concentration reflects the total amount of bone matrix synthesized (3). On the other hand, it is possible that osteocalcin may be reflective of osteoblastic activity and bone matrix turnover because it is degraded and released into the circulation during bone resorption (3). This supports evidence from animal studies that indicate bone formation is decreasing even though osteoblasts and subsequent osteocalcin levels are increasing.

Akesson *et al* (3) suggested that there are different aspects of bone formation. The results obtained from this study support this hypothesis. The first aspect of bone formation is formation of the bone matrix, which occurs when osteoblasts fill the lacunae and begin the process of forming new bone by secreting osteoid (26; 28). The second aspect is the mineralization of the osteoid, which is the final step of the cycle and involves the hydroxyapatite compound (26; 28). Thus the bone matrix and the mineral fraction of bone are formed separately or in two distinct stages.

Approximately 80–90% of BMC is comprised of Ca and P since they are an integral part of the hydroxyapatite compound $Ca_{10}(PO_4)_6(OH)_2$ (48). These two minerals, along with Mg represent the majority of the mineral fraction. It can be

concluded that the low Ca model used here was affecting the mineral fraction of the bone since marked decreases in BMD, BMC, Ca, P and Mg levels were observed. The reduced bone growth occurred because there was a decrease in the mineralized fraction of the bone.

Identification of the different aspects of bone formation also explains why the levels of osteocalcin remained high despite the low Ca diet. During growth and development, a period known as bone modeling, formation is much greater than resorption. The young rats used in this study were still undergoing bone modeling since they were under the age of 3 months (22; 102). As stated, the mineralized portion of the bone was greatly affected by the low Ca diet, but the formation of the bone matrix was likely not as affected and it is possible that it remains intact. Thus there are a large number of osteoblasts forming the bone matrix during this period of growth and the measured levels of osteocalcin, a marker of osteoblastic activity, are subsequently high.

The results also indicate that some of the young rats were able to adapt to or compensate for low Ca diets, as in general, the young rats fed the low Ca diets had higher food intakes, higher femoral moisture percent, and higher femoral Na levels.

Interestingly, femoral Na levels increased in the low Ca diet groups. It has been reported that the composition of bone in older women appears to change in that there is a higher Na content relative to Ca because more Ca is lost (7). Rats fed the low Ca diet had greater Ca losses since most of the BMC is comprised of Ca. Sodium remains high because, perhaps, it is found in other compartments such as the bone matrix. Human bone is approximately 65% mineralized (82) and the remaining 35% is comprised of tissues and cells that form the matrix. Unlike the mineral faction, the bone matrix was presumably not as affected by the low Ca diet and could still have high levels of Na present. Additionally, it appears that the amount of Na in bone is influenced by other factors such as vitamin D nutrition and PTH status (7), which in the case of the low Ca diet is increased.

Also it is well documented that Na, mostly in the form of salt, increases urinary Ca in experimental animals and humans (48; 65; 71; 75). The movements of Ca and Na through the kidneys are linked and a direct relationship exists between Na and Ca

excretion (75). This correlation is generally Na driven since it is the Na that influences urinary Ca excretion (71); however the opposite may occur, as in the case with a large Ca load or a low Ca diet. Renal tubular reabsorption of Ca increases when PTH secretion rises from a low Ca diet (23; 42). Thus the role of Ca in regulating Na excretion could indicate that the low Ca diet is causing less Na to be lost and, hence more Na is available and present in the bone.

The RB rat can be used for bone research because it has been through multiple reproductive cycles, which can have a profound effect on bone structure and Ca homeostasis (18; 19; 48; 97). For example lactation often depletes Ca stores and decreases female skeletal mass in both humans and rats (18). Female rats, in particular, have very pronounced bone loss during the first lactation and this phenomenon has been well reported (19). Also it has been demonstrated that RB rats had significantly lower BMD compared to age-matched virgin rats and this occurred at all sites of the femur that were measured (39). Cancellous bone volume decreases by as much as 79% in the tibia (97), which can be greater than bone loss observed following estrogen deficiency (67). Thus the RB was a useful animal model for bone studies because of the impact of reproductive cycles on the skeleton and Ca homeostasis.

The low Ca model in this study had little to no effect in the RB rats since there were only small changes detected. This lack of effect would indicate that the RB had skeletal Ca stores that were able to compensate for the lower intakes without any major changes occurring in the bone. Decreases that often appear during pregnancy and lactation are transient and bone mass is mostly restored post-lactation (48). Skeletal changes during and after reproductive cycles (19; 97) in female rats occur to protect the maternal skeleton for subsequent reproductive cycles. Thus it seems that the RB rats had sufficient Ca stores and substantial bone loss did not occur in the RB rats fed the low Ca diets used in this present study.

Overall, the rats were healthy as indicated by the hematological measurements. There were small differences in some of the parameters in the young rats which are most likely indicative of the negative effects of the low Ca diet. Requirements for Ca during a

stage of rapid growth and development are additionally significant and the low Ca diet used in this study was, as stated earlier, very effective in reducing bone growth. Low Ca intakes cause serum Ca to decrease and PTH levels to increase (23) and the changes in hematological parameters are likely compensating for the hypocalcemia and/or the increases in PTH.

Soy protein exerts effects on platelet activation and aggregation which may improve cardiovascular health (37). Differences of platelet parameters between the SP-based diet and the casein-based diets were detected in both the young and RB rats and are consistent with previous reports (81).

The effect of feeding SP versus casein was found to have some beneficial effects since it was able to minimize, but not fully reverse, some of the adverse effects caused by the low Ca diets in the young rats.

It has been hypothesized that soy exhibits bone-sparing effects because of its ability to decrease bone resorption (12; 50) and/or increase bone formation (10; 34; 50; 81) in the ovx rat model. *In vitro* data has also demonstrated that soy decreased bone resorption (25; 35; 36) and increased bone formation (53; 91; 92).

The protective role of soy and/or IFs has been extensively studied in the ovx animal model (15) and there is a fairly good agreement that when endogenous estrogens are low, soy and/or IFs has a positive effect on bone health. This has been found to be especially true in young growing animal models (22; 102). The role of soy in Ca and bone metabolism has been less well studied but it is an important relationship that should be examined to establish a potential mode of action of soy. Shen *et al* (87) found that estrogen replacement was effective in preventing bone loss in both a low Ca rat model and an ovx rat model simultaneously. Thus, soy, a well known estrogenic compound (8; 15; 37; 83; 84; 85), may possibly prevent bone loss in a Ca deficient animal model.

The young rats in the low Ca groups fed SP had significantly higher BMD and BMC values than the young rats fed casein. The levels of femoral Ca, P, and Mg, as well as other physical parameters (wet, dry, and ash weights, length and diameter) also

tended to be higher in comparison to casein fed rats, although differences were not statistically significant. These data provide evidence that a low dietary Ca induced reduction in the mineral fraction of bone is minimized by dietary SP. Also the Ca control rats fed SP had higher values for BMD, BMC, and femoral Ca, Mg and P levels, although the differences compared to casein fed rats were not statistically significant. Thus feeding SP to the young rats positively affects the formation of the mineral fraction of bone. These results, are consistent with those reported by Blum *et al* (17), where they showed SP, devoid of IFs, to be protective against bone loss because of stimulation and/or maintenance of bone formation (9; 12). Other investigators have detected estrogenic actions of SP isolate in other target tissues as well (104). In conclusion SP, regardless of its IFs content was found to be protective in young rats fed low dietary Ca.

Normally, an increase in bone formation is followed by a compensatory increase in bone resorption since the two processes are balanced during the bone remodeling cycle. However, during growth and development, a period known as bone modeling, formation outweighs resorption since the skeleton is being rapidly developed (28; 48). Once maturation is reached, remodeling continues throughout life and is a balanced cycle to ensure that no net bone mass is lost (28; 47; 82). It was concluded earlier that the low Ca diet used in this animal model slowed bone growth since the mineral fraction of the bone was extensively affected.

In addition, the results of the present study indicated that SP increased bone resorption. Biochemical indices of bone resorption were significantly higher in the young rats fed SP as measured by both DPD and PYD compared to casein fed rats. The increase in bone formation that was accompanied by an increase in bone resorption confirms that bone turnover was likely much more balanced with SP. Therefore, the beneficial effects of feeding SP are in part possibly due to restoring balance to the bone remodeling cycle.

The notably reduced bone growth that occurred in the young rats is additionally significant because they are undergoing bone modeling, a period of rapid bone growth and development. Unlike bone remodeling, the emphasis during bone modeling is on bone formation and growth. It has been reported that rats under the age of 3 months are undergoing bone modeling (22; 102) and bone formation outweighs bone resorption.

The need for adequate Ca, as well as other bone building minerals is essential during this part of life. Minerals such as Ca, Mg, and P are all important constituents of bone and are required for bone growth. Therefore the protective role of SP in restoring balance between formation and resorption during this stage of growth and development is additionally significant and demonstrated by some of the effects on the levels of these other minerals.

Mechanistically, soy may be protective against bone loss because it may increase Ca absorption (29; 89). It has been reported that feeding soymilk and soymilk with small and large peptides to 6 week-old female rats increased intestinal Ca absorption compared to a casein-based diet (73). Along with an increased absorption, the accumulation of Ca was enhanced since femoral Ca levels were much higher than controls (73). This study was an extension of a previous study that had been conducted by the same lab where they report increased BMD, mechanical strength, and intestinal Ca absorption with soybean milk (73). Similarly, ovx rats fed a soy diet for 35 days had higher intestinal Ca transport, measured in isolated duodenal and colonic cells, than rats receiving a casein-based diet (14). It was the IF-rich but not the IF-deplete SP that completely prevented the ovx-induced reduction of Ca transport in the duodenal and colonic cells (14). However, the authors observed a small increase in Ca transport with the IF-deplete soy (14) suggesting that perhaps the SP itself can increase Ca absorption.

Estrogen promotes active intestinal absorption of Ca *in vivo* (47) since it can act as a regulator of Ca transport proteins and receptors in the duodenum (60). The role of estrogen in active Ca absorption is directly through ERs that are present in the intestine (93; 94). In intact and ovx female rats, pharmacological doses of E2 administered by subcutaneous injections increased intestinal Ca absorption, whereas endogenous estrogen had no effect on Ca absorption (93). Similarly, in humans, estrogen administration can increase and restore Ca absorption levels in postmenopausal women to levels measured in premenopausal women (89). Furthermore, the synthetic IF, Ipriflavone, significantly enhances Ca absorption in ovx rats and increases are comparable to E2 (13). The most likely mechanism by which soy is enhancing Ca levels

in the mineral fraction of bone and subsequently increasing BMD and BMC in this study is consistent with its role in increasing Ca absorption in the intestine via ERs.

Soy might also be providing protection against bone loss because of its ability to increase the amount of Ca that is reabsorbed in the kidneys. The kidneys actively increase Ca reabsorption when Ca intakes or absorption are low and this helps regulate Ca homeostasis (23; 42). Adami *et al* (1) suggested that estrogen promotes tubular reabsorption of Ca through ERs that are present in the kidney. It has been previously reported that women who were fed soy diets, with and without added IFs for 28 days, had lower Ca excretion compared to controls (89), which would indicate an improvement in Ca retention. Most recently SP reduced urinary Ca excretion in ovx rats fed diets with and without added IFs (22). The investigators noted that SP itself was able to significantly affect urinary Ca excretion regardless of its IF content and that this ability to improve Ca retention was more pronounced than in the estrogen treated rats (22). Therefore, soy's estrogenic properties are likely responsible for increasing intestinal Ca absorption and renal Ca reabsorption resulting in benefits on bone health.

The positive effects of estrogen and soy on Ca homeostasis and bone metabolism have been well established to be more pronounced in young growing rats (22; 102). Generally, studies conducted in a younger growing rat model support the bone sparing ability of soy, whereas studies conducted in older and skeletally mature rats report inconsistent results (22; 102). The results obtained here are clearly supportive that young growing animals are benefiting more from soy administration.

Despite reports of fewer effects in older skeletally mature rats (22; 102) and the observation that the RB rats were not as affected by the low Ca diet from this study, SP was mildly beneficial on bone metabolism in this group of rats. A small decrease in bone resorption with SP was detected since DPD decreased significantly and PYD decreased non-significantly in comparison to casein. Decreases in bone resorption could be considered a positive outcome since age-related bone loss occurs in rats when bone resorption becomes larger than bone formation. Generally, with increasing age, the formation phase fails to keep pace with the resorptive activity of osteoclasts and it is not

uncommon for bone loss to occur in humans (47; 48; 82). The prevalence of this type of bone loss is universal and is referred to as age-related bone loss (47; 48; 82). Similarly rats can experience a progressive age-related decline in bone mass (29) and soy's ability to decrease bone resorption can be beneficial in reducing age-related bone loss.

A second positive effect from the SP detected in the RB rats was that BMD and BMC values were higher in all of the diets in comparison to rats fed casein. Although none of the differences were statistically significant, there was a general trend towards numerically higher BMD and BMC values among the RB rats fed SP. Thus, soy's effect on bone metabolism in the RB rats was minor but may still be biologically important.

Femoral Zn levels were significantly lower in the groups fed the SP diets, predominantly in the young rats. The reduced femoral Zn levels are because of soy's phytate content which can affect Zn bioavailability (62). At the same time, the phytate content in soy could also affect the bioavailability of Ca. Mineral analyses indicated that there were significant higher levels of Ca in the soy-based diets compared to casein-based diets for both the control and low Ca groups. However, the bioavailability of Ca in soy, like Zn, is lowered because of its phytate content (43; 44; 89). Thus the differences that were detected may not actually be that significant and unlikely to account for the significant differences seen in the bone BMD and metabolism data with the soy diets.

Conclusions

The low Ca diet effectively reduced bone growth in the young growing rats, whereas, perhaps, because of adequate Ca stores, was not as effective in causing bone loss in the older skeletally mature RB rats. The low Ca diet significantly decreased formation of the mineral fraction of bone and at the same time increased bone resorption as measured through biochemical indicators. Poor bone growth which results from low Ca intakes has been well reported in the literature and is consistent with the effects observed in this study. Inadequate Ca intakes have negative consequences on bone health and can increase the risk of fractures and osteoporosis in humans.

Although negative effects of the low Ca diet were not fully reversed, feeding SP to both the young and RB rats resulted in positive changes in BMD, BMC, femoral minerals, and bone metabolism markers. The role of soy in minimizing the negative effects of the low Ca diet was especially significant and biologically important in young rats that were undergoing a stage of rapid bone growth and development. The data from the present study suggests that soy's estrogenic properties are likely responsible for increasing intestinal Ca absorption and renal Ca reabsorption, and subsequently increasing BMD and BMC by providing more Ca to bone. Data from this study clearly indicated that SP, rather than IFs, positively affected bone metabolism in both growing rats and RB rats and can reduce the deleterious effects on bone associated with low Ca intakes especially in young growing rats.

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Appendix A: Abbreviations list

ALP: alkaline phosphatase

ANOVA: analysis of variance

BMC: bone mineral content

BMD: bone mineral density

BMU: basic multicellular unit

Ca: calcium

Ca_2CO_3: calcium carbonate

CsCl: cesium chloride

CV: coefficient of variation

DEXA: dual energy x-ray absorptiometry

DPD: deoxypyridinoline

ECF: extracellular fluid

ELISA: enzyme-linked immunoassay

ER: estrogen receptor

E2: 17-beta estradiol

HCT: hematocrit

IF: isoflavone

IGF: insulin growth factor

IGFBP: insulin growth factor binding protein

IL-6: interleukin-6

K: potassium

La_2O_3: lanthanum oxide

MCH: mean corpuscular hemoglobin

MCV: mean corpuscular volume

Mg: magnesium

HPLC: high performance liquid chromatography

MPV: mean platelet volume

mRNA: messenger ribonucleuic acid

Na: sodium

ns: not statistically significant

ovx: ovariectomized

P: phosphorus

PICP: procollagen I carboxyterminal propeptide

PLT: platelet

PTH: parathyroid hormone

PYD: pyridinoline

RANKL: receptor activator of nuclear factor (NF)-κB ligand

RB: retired breeder

RBC: red blood cell

RDW: red cells distribution width

SD: standard deviation

SEM: standard error of the mean

SP: soy protein

VDR: vitamin D receptor

WBC: white blood cell

wt: weight

Zn: zinc

Appendix B: Animal Ethics Approval

Version 3

Health Canada / Santé Canada
Health Products and Food Branch / Direction générale des produits de santé et des aliments
Animal Care committee / Comité de protection des animaux

HCO-ACC. PROTOCOL NO.
N° DE PROTOCOLE DE C.P.A.
2004-033

PROTOCOL FOR PROJECTS INVOLVING THE USE OF LABORATORY ANIMALS
ÉVALUATION DE PROTOCOLES DE RECHERCHES COMPORTANT L'UTILISATION D'ANIMAUX DE LABORATOIRE

Review Due Date
Date limite de la revue
08/25/2004

HC-Protected

Title of project - Titre du projet	Submitted For Review Envoyé pour revue
6-Soy EFFECT OF SOY ON BONE METABOLISM	

INVESTIGATOR DATA - DONNÉES SUR LE CHERCHEUR PRINCIPAL	
Responsible investigator - Chercheur principal Mary L'Abbe	**Telephone number** **Office - Bureau** 948-8476 Email mary_l'abbe@hc-sc.gc.ca
Organisation Bureau of Nutritional Scienices	**Unit** Directors office/Nutrition Research Division
Technician - Technicien(ne) Keith Trick	**Telephone number** **Office - Bureau** 957-0925; 946-9483
Collaborator - Collaborateur(trice) Jesse Bertinato Sara Farnworth	**Telephone number** **Office - Bureau** 946-2424
Statistician - Statisticien(ne)	**Telephone number** **Office - Bureau**
Directorate ACC Member - Nom du membre du C.P.A. Gerard Cooke	**Telephone number - N° de téléphone** **Office - Bureau** 957-0990
Responsible Manager - Gestionnaire Responsable Peter Fischer	**Telephone number - N° de téléphone** **Office - Bureau** 957-0919

IF UNDER CONTRACT, NAME OF CONTRACT ORGANIZATION - DONNER LE NOM DE L'ORGANISATION LIÉE PAR CONTRAT	
N.B. If under contract or if a multicentre collaborative project, please provide the protocol approved by the other institutions.	**N.B. Si lié par contrat ou dans le cas d'une collaboration avec plusieurs centres, fournir s.v.p., le protocole approuvé par les autres.**
ARD Staff? Yes	**S.V.P. mentionner les titres et qualités des personnes appelée à manipuler les animaux.**
Please provide qualifications of those who are involved in animal manipulation.	**Nom, adresse et numéro de téléphone d'affaires de(s) la personne(s) responsable(s) s'il y a lieu**
Name, business address and telephone number of person(s) responsible.	
Dr. Mary L'Abbe, AL 2203C Banting Bldg. 948-8476	

RELATED PROTOCOL - PROTOCOLE APPARENTÉ
a) Has this or similar protocol been approved in the past?

☐ Yes ☒ No	Est-ce que ce projet ou un projet similaire a déja été approuvé dan le passé? ☐ Oui ☒ Non
b) If yes, provide previous protocol number and attach any documentation regarding A.C.C. questions and responses as well as any modification to procedures originally proposed.	**Si oui, fournir le numéro de protocole précédent et attacher toute documentation concernant les questions et réponses du CPA ainsi que toutes modifications aux procédures proposées.**
Previous protocol number:- Numéro de protocole précédent::	
Attachment:	

ALTERNATE METHODOLOGIES - MÉTHODOLOGIES DE REMPLACEMENT

Have alternate methodologies which address the 3-R philosophy for the project been explored? In the case of regulatory protocol, the 3-R philosophy still needs to be addressed.	**A-t-on étudié la possibilité d'autres méthodologies respectant la philosophie des 3-R pour un tel projet? Dans le cas d'un protocole faisant l'objet d'une réglementation, la philosophie des 3-R doit également être respectée.**

a) Replacement - Remplacement:

Intact animals are required for dietary studies.

b) Reduction - Réduction:

Six is the mininum number of animals per treatment group needed to yield significant results. Several diet groups are required to obtain data for graded levels of isoflavones and to determine if the effects on bone are related to soy or the isoflavone component of soy protein.

c) Refinement - Raffinement:

Four dietary treaments are planned for both young and aged rats using two different basal diets - soy and casein (see above).

Is this protocol regulatory? ☐ Yes - Oui ☒ No - Non

Act and Regulation of the most recent revision date

SECTION I			
Duration of the project - Durée du projet	☒ ACUTE - COURTE DURÉE (<= 8 Weeks) ☐ CHRONIC - LONGUE DURÉE (> 8 Weeks)	From -Du 09/15/2004	To - Au 10/20/2004

GENERAL PURPOSE OF ANIMAL USE - NATURE DU PROJET ET RAISON D'UTILISATION DES ANIMAUX

Species of animals Espèces animales	Strain	Quantity Nombre	Research Recherche	Diagnosis	Regulatory Réglementation
Rat	Sprague-Dawley	108	Yes		
		0			
		0			
		0			
		Total: 108			

Animal Resources Division/Division des ressources animales - Rodent Environmental Enrichment Program

Version No. / No. de la version: **4**

Approved By / Approuvé par: **Mr. Normand Turcotte**
Director, Animal Resources Division

Date: **01-02-00**
(Replaces 03-03-98)

○ Acceptable/Acceptable ● Unacceptable/Inacceptable	**Pair or Group Housing:** The most beneficial enrichment "device" is housing with conspecifics. Mice, and guinea pigs of the same sex except for breeding purposes, will be paired or group housed. Male hamsters and mice are normally exempt from group housing since these animals tend to fight. Please indicate if there are any special instructions regarding setting up the group housing program. Exercise runs for guinea pigs. **Hébergement en paire ou en groupe:** La méthode d'enrichissement la plus efficace est l'hébergement avec des individus de la même espèce. Les souris, les rats et les cobayes de même sexe seront hébergés en paire ou en groupe, à l'exception des hamsters et souris mâles. Veuillez préciser s'il y a des instructions spéciales concernant le programme d'hébergement collectif. Cages d'exercice pour les cobayes. **Reason:** Pair or group housing is unacceptable as food consumption data are required from individual rats.
○ Acceptable/Acceptable ● Unacceptable/Inacceptable	**Shelters:** A shelter constructed of material, such as stainless steel, aluminum, ceramic or PVC tubing, will be placed in each cage. **Abris:** Un abri constitué de matériaux inertes, tels que l'acier inoxydable, l'aluminium ou la céramique, sera placé dans chaque cage. Des bouts de tuyaux en P.V.C. seront aussi placés dans la cage. **Reason:** ACCEPTABLE BUT - Shelter must be constructed of stainless steel. Other materials may provide a source of trace element contamination.
○ Acceptable/Acceptable ● Unacceptable/Inacceptable	**Nesting Material:** Commercially purchased nesting material made of sterilized cotton fibres (Nestlets ®, Anacare Corp.) or wood shaving will be placed in the cages along with the normal bedding material. **Matériel pour nidification:** Du matériel disponible commercialement, fait de fibres de coton stérilisées (Nestlets ®, Anacare Corp.) ou de fibres de maïs (Corn Husk Nesting ®, The Andersons), sera placé dans les cages avec la litière normale.

	Reason: Nesting material is unacceptable as it may introduce trace element contamination.
○ Acceptable/Acceptable ● Unacceptable/Inacceptable	**Dietary Variety:** In addition to the basic ration of rodent chow, animals will occasionally be offered selected foodstuffs to add variety to their diet. At this time supplemental feedstuffs include washed apple and/or carrots. **Variété d'aliments:** Outre leur diète habituelle, on donnera occasionnellement aux animaux des aliments choisis afin de varier leur menu. À l'heure actuelle, ces aliments sont des pommes ou des carottes lavées. **Reason:** Dietary variety is unacceptable as this is a nutritional study with controlled and analyzed nutritional composition.
○ Acceptable/Acceptable ● Unacceptable/Inacceptable	**Gnawing Sticks:** A sanitized nylon object will be placed in each animal cage. Guinea pigs will be given the commercially purchased Rodent Nylabone® (BioServ, NJ) while mice will be given a segment of a softer, nylon dowelling. **Bâton à ronger:** Objet de nylon désinfecté qui sera placé dans la cage de chaque animal. On donnera aux rats, aux hamsters et aux cochons d'Inde du Rodent Nylabonez, BioServ, N.J.) acheté commercialement et on donnera aux souris un bout de tige de nylon plus mou. **Reason:** Gnawing sticks are unacceptable as it may introduce trace element contamination.
○ Acceptable/Acceptable ● Unacceptable/Inacceptable	**Cage Toys:** Stainless steel, nuts and bolts and washers and marbles. **Jouets pour les cages:** Écrous, boulons, rondelles en acier inoxydable et billes. **Reason:** ACCEPTABLE BUT NOTE - Marbles which are acid washed and rinsed in demineralised water are permissible. Stainless steel nuts and bolts are acceptable (must be stainless steel, not other metals, as they introduce a source of trace element contamination when chewed on, which would compromise the design and results of the study).
● Acceptable/Acceptable ○ Unacceptable/Inacceptable	**Music:** Music will be provided in each room. **Musique:** La musique sera transmise dans chaque chambre.

Other suggestions/ Autres suggestion

Please list any other suggestions you may have regarding ways in which to enrich the environment of the animals on your study. In addition, please record any enrichment procedure inherent to your particular study. Thank you for your assistance.

Veuillez inscrire toute autre suggestion concernant la façon d'enrichir l'environnement des animaux qui participent à votre étude.

JUSTIFICATION OF THE SPECIES SELECTED AND NUMBER OF ANIMALS TO BE USED - JUSTIFICATION DE L'ESPÈCE ANIMALE SÉLECTIONNÉE ET LE NOMBRE D'ANIMAUX À ÊTRE UTILISÉ

Rats are ideal for this study and we have already established sensitive markers of calcium status using rats in the multi-generational rat study. They are inexpensive and allows for rapid results. 6 animals per group is the mininimum number of animlas to obtain statistically valid results

DISPOSITION OF SURPLUS ANIMALS - DISPOSTION DES ANIMAUX EXCÉDENTAIRES

Tout animal qui peut par inadvertance devenir excédentaire sera éliminé de la façon suivante:

None planned - if so, as appropriate by ARD

SECTION II	
Describe the objective of the proposed study in which laboratory animals are to be used and a brief explanation of benefits expected A complete description of the animal phase of the study must be attached.	Décrire les objectifs des travaux proposés comportant l'utilisation d'animaux de laboratoire et expliquer brièvement les avantages attendus. Une description complète du protocole de recherche doit être incluse.

a) KEY WORDS - MOTS CLÉS

calcium deficiency, soy, isoflavones, bone metabolism, osteoporosis

b) LAY SUMMARY - SOMMAIRE VULGARISÉ

Osteoporosis is a disease characterized by low bone mass and deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk. An estimated 1.4 million Canadians are believed to have osteoporosis which represents a serious public health concern. It has been suggested that soy protein and/or soy isoflavones may slow the loss of bone because of their estrogenic effect in the body (1, 2) and their effect on calcium metabolism (3, 4). To date, most of the animal studies that have been conducted have used ovariectomized (ovaries removed) young female rats, a model of postmenopausal bone loss. Most of the data show that soy may be protective against bone loss when estrogen levels are low, however the specific effect and mechanisms involved are unclear and still under investigation. One hypothesis that has been examined is soy's potential ability to increase calcium absorption (3, 4), thus providing more calcium in bones. Calcium deficiency decreases bone growth and causes bone loss in experimental animals and is often used as a representative model of osteoporosis. North American women, especially middle-aged and elderly, consume diets that are low in calcium (5, 6). According to a recent provincial food survey, calcium intakes for women were below the Dietary Reference Intake (DRI) recommendations. The recommended daily Adequate Intake for calcium for women between the ages 19-50 is 1000 mg/day and over 50 is 1200 mg/day (7). The mean intakes reported were 795-759 mg/day (ages 19-49) and 714-645 mg/day (ages 50-74) (8). Our objective is to feed adult female rats a low calcium diet to decrease bone growth and induce bone loss. Soy, as soy protein isolate (SPI) and SPI with different amounts of isoflavones will be fed to assess whether it reduces bone loss and normalizes bone growth and if it has any effect on calcium accretion. This study will provide valuable information regarding the potential protective role of soy and/or isoflavones against bone loss in female rats.

c) OBJECTIVE - OBJECTIF

A low calcium diet decreases bone growth and causes bone loss in experimental animals and is often used as a representative model of osteoporosis. Since many Canadian women consume diets low in calcium, we will feed female rats a low calcium diet to slow or stop bone accretion and induce bone loss. There will be two control groups, a control casein group and a control soy group, both receiving a diet with normal calcium levels. Both soy, as alcohol extracted SPI, and casein with low calcium and different levels of added isoflavones (low and high), will be fed and compared to the casein- and soy-based controls. Our goals are to determine if feeding dietary soy and/or isoflavones to female rats reduces bone loss and returns bone growth to normal despite the effects of a low calcium diet. Another aim is to assess if the potential benefit on bone is a result of an increase in calcium accretion into bones versus a decrease in calcium loss. Another objective will be to include a group of retired breeders. These rats will have depleted calcium stores because of a decreased ability to absorb calcium and because of calcium losses from pregnancy. They will serve as a representation of adult females that have a decreased ability to absorb calcium and have lower calcium intakes. These results will help us

further understand soy's effect on bone and calcium metabolism.

d) PROPOSED EXPERIMENTAL ENDPOINTS (IF APPLICABLE, PLEASE DESCRIBE) - PARAMÈTRES EXPÉRIMENTAUX CHOISIS (SI APPLICABLE SVP DÉCRIRE)

Adult (5 months) and retired breeder (11 months) Sprague-Dawley rats will be fed a low calcium diet, to represent the typical North American female diet which will slow bone accretion and induce bone loss. A total of 108 rats will be required for the study. Upon arrival, 6 (from each population) will be randomly chosen for necropsy (to be conducted the following morning), 6 will be randomly assigned to receive a control casein diet, another 6 will be randomly assigned to receive a control soy diet, and the remaining 36 will receive a low calcium diet (see the table below). The rats receiving the low calcium diet will also be randomly divided into 6 treatment groups consisting of 6 rats each. Group 1 will be fed a casein-based diet, group 2 a casein-based diet with 150 mg isoflavones/kg diet, group 3 a casein-based diet with 400 mg isoflavones/kg diet, group 4 a SPI-based diet, group 5 a SPI-based diet with 150 mg isoflavones/kg diet, and group 6 a SPI-based diet with 400 mg isoflavones/kg diet. After 5 weeks of feeding the 6 treatment diets and the 2 control diets, all 96 rats will be killed. Other studies that have looked at the effects of feeding a low calcium diet on bone metabolism in rats have lasted for approximately 4-6 weeks (9, 10). In one experiment, the low calcium diet that was fed for 4 weeks to 6 month old female rats resulted in significantly lower bone mineral density and significantly higher bone resorption (9). As part on their conclusions, the authors stated that their low calcium model was sufficient in inducing bone loss in rats (9). Thus based on previous research, the low calcium diets for our study will be fed for approximately 5 weeks. After that time, rats will be necropsied, blood and tissue samples will be collected and stored until analysis. Endpoints that will be measured include: bone mineral density, concentration of bone and tissue minerals (Ca, Mg, P), and biochemical markers of bone formation (ex. osteocalcin) and bone resorption (ex. deoxypyridinoline).

Diet	Number	Calcium level	Casein	SPI	Isoflavones
1 (control)	6 (from each group)	normal	x		
2 (control)	6	normal		x	
3	6	low	x		
4	6	low	x		150
5	6	low	x		400
6	6	low		x	
7	6	low		x	150
8	6	low		x	400

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e) A COMPLETE DESCRIPTION OF THE ANIMAL PHASE OF THE STUDY

see attached animal protocol.doc

SECTION III - RESPOND TO ALL QUESTIONS - RÉPONDRE À TOUTES LES QUESTIONS

1. This project will use concious animals? Ce projet utilise-t-il des animaux conscients?	☒ Yes - Oui	☐ No - Non
2. Will this animal project involve one of the following techniques? If "yes" justify: Ce projet implique-t-il le recours a l'une des techniques suivantes? Si "oui", justifier:		
a) Prolonged physical restraint Contiention physique prolongée	☐ Yes - Oui	☒ No - Non
b) Food and/or water deprivation (excluding overnight fasting). Privation d'eau et/ou nourriture (excepté de la privation au cours d'une nuit).	☐ Yes - Oui	☒ No - Non
c) Variations in the environment (temperature, humidity, air pressure, noise, light). Modifications importantes de l'environnement (température, humidité, pression d'air, bruit, lumière).	☐ Yes - Oui	☒ No - Non
d) The use of immobilizing agents or muscle relaxants without anesthesia. Utilisation d'agents paralysants ou de relaxants musculaires sans anesthésie.	☐ Yes - Oui	☒ No - Non
e) LD50 testing Épreuves de DL50	☐ Yes - Oui	☒ No - Non

SECTION IV

a) Is distress probable or possible for any of the animals during the project? a) Estimez-vous que l'animal souffra, probablement ou possiblement, lors de ce projet?	☐ Yes - Oui	☒ No - Non
If "yes", what procedures will be used to ameliorate or end distress? Si "oui", quelles sont les procédures utilisées pour atténuer ou pour éliminer cette soussrance?		
If "yes", what anasthetic, dose rate and route will be used? Quel sera l'agent anastehétique, le dosage et la route d'administration	☐ Yes - Oui	☒ No - Non
If "yes", how will post operative pain be alleviated? Par quel moyen atténuerez-vouz les douleurs post-opératoires?	☐ Yes - Oui	☒ No - Non

Methods of euthansia (specify)
Méthodes d'euthanasie (préciser)
necropsy while under isofluorane anaesthesia

SECTION V

CATEGORIES OF INVASIVENESS IN ANIMAL EXPERIMENTS	CATÉGORIES D'INTERVENTIONS INVASIVES EN EXPÉRIMENTATION ANIMALE
- Categorize where your project fits as described.	- Veuillez indiquer la catégorie à laquelle appartient votre projet de recherche.

CATEGORY A	CATÉGORIE A

☐ STUDIES OR EXPERIMENTS ON MOST INVERTEBRATES, OR ON INCOMPLETE LIVING MATERIAL.
ÉTUDE OU EXPÉRIENCES AVEC LA PLUPART DES INVERTÉBRÉS OU AVEC DU MATÉRIEL VIVANT INCOMPLET.

These might include: tissue culture, tissues obtained at autopsy, necropsy or from the slaughterhouse; eggs, protozoa and related single celled organisms; studies or experiments involving containment, incision or other invasive action on metazoa. It is acknowledged that cephalopods and some higher invertebrates have nervous systems as well developed as some vertebrates and therefore Categories of invasiveness B, C, D and E may apply.

Se sont; la culture de tissus, les tissus prélevés lors d'autopsies, de nécropsies ou à l'abattoir; les oeufs, les protozoaires et les organismes unicellulaires apparentés; les études ou les expériences impliquant de l'isolement, des incisions ou d'autres interventions invasives sur des métazoaires. Les céphalopodes et d'autres invertébrés plus évolués possèdent un système nerveux aussi bien développé que celui de certains vertébrés et, en conséquence, on peut les classer dans les catégories d'interventions invasives B, C, D et E.

CATEGORY B | CATÉGORIE B

☒ STUDIES OR EXPERIMENTS ON VERTEBRATES CAUSING LITTLE OR NO DISCOMFORT OR STRESS.
ÉTUDES OU EXPÉRIENCES SUR LES VERTÉBRÉS CAUSANT PEU OU PAS D'INCONFORT OU DE STRESS.

These might include: holding animals captive for observation or physical examination; blood sampling; injection of non-toxic material by the following routes: intravenous, subcutaneous, intramuscular, intraperitoneal or oral, excluding intrathoracic or intracardiac; acute non-survival experiments in which the animals are completely anesthetized and do not regain consciousness; standard methods of euthanasia that induce rapid unconsciousness, such as anesthetic overdose or decapitation preceeded by sedation or light anesthesia, short periods (few hours) of food and/or water deprivation.

Ce sont: la garde d'animaux en captivité pour effectuer des observations ou des examens physiques; les prises de sang; les injections de produits non toxiques par les voies suivantes: intraveineuse, sous-cutanée, intramusculaire, intrapéritonéale ou orale, excluant les voies intrathoracique et intracardiaque; les expériences aiguës sans survie au cours desquelles les animaux sont complètement anesthésiés et ne se réveillent pas, les méthodes standard d'euthanasie qui provoquent une inconscience rapide comme des surdoses d'anesthésiques ou la décapitation précédée d'une sédation ou d'une légère anesthésie; les expériences comportant de courtes périodes (quelques heures) de privation de nourriture et/ou d'eau.

CATEGORY C | CATÉGORIE C

☐ STUDIES OR EXPERIMENTS ON VERTEBRATES INVOLVING MINOR STRESS OR PAIN OF SHORT DURATION.
ÉTUDES OU EXPÉRIENCES SUR LES VERTÉBRÉS IMPLIQUANT UN STRESS OU UNE DOULEUR DE COURTE DURÉE.

These might include: cannulation or catheterization of blood vessels or body cavities performed under anesthesia; minor surgical procedures under anesthesia, such as biopsies, laparoscopy; short periods of restraint consistent with minimal distress; overmight food and/or water deprivation; behavioural experiments on awake animals that involve short-term, stressful restraint. These would not cause significant change in coat appearance, ocular or nasal discharges, abnormal respiratory or cardiac rate, reduction of fecal or urinary output, isolation or crowding.

Comment: During or after category C studies animals must not show self mutilation, anorexia, dehydration, hyperactivity, increased recumbency, or dormancy, increased vocalization, aggressive-defensive behaviour or demonstrate social withdrawal and self-isolation.

Ce sont; la canulation ou le cathétérisme de vaisseaux ou de cavités corporelles sous anesthésie; les procédures chirurgicales mineures sous anesthésie comme des biopsies, les laparoscopies, les périodes courtes d'immobilisation causant un stress léger; la privation de nourriture et/ou d'eau pendant une nuit; les expériences de comportement avec des animaux éveillés comportant une immobilisation brève et stressante. Toutes ces interventions ne doivent pas causer de changements importants dans l'apparence du pelage, les sécrétions oculaires ou nasales, ni de rythmes respiratoires ou cardiaques anormaux, ni de dimunitions de matières fécales ou d'urine, ni d'isolement ou d'entassement.

Commentaire: Au cours ou après les études de la catégorie C, les animaux ne doivent pas manifester de signes d'automutilation, d'anorexie, de déshydratation, d'hyperactivité, de prostration ou d'ensommeillement prolongés, d'augmentation de vocalisation, de comportement aggressif-défensif, ou démontrer un état de repli sur soi et d'isolement volontaire.

CATEGORY D	CATÉGORIE D

☐ STUDIES OR EXPERIMENTS ON VERTEBRATES THAT INVOLVE MODERATE TO SEVERE DISTRESS OR DISCOMFORT.
ÉTUDES OU EXPÉRIENCES SUR LES VERTÉBRÉS IMPLIQUANT UN STRESS OU UN INCONFORT DE MODÉRÉ À INTENSE.

These might include: Major surgical procedures conducted under anesthesia permitting recovery, with adherence to acceptable veterinary practices, adequate post- operative analgesia, fluid therapy and required veterinary nursing practices; exposure of animals to noxious stimuli for periods not above the minimal level required to demonstrate the required clinical effect; prolonged (several hours or more) periods of physical restraint applied in compliance with CCAC guidelines; induction of behavioural stresses such as maternal deprivation, aggression, predatory-prey interactions, procedures which alter perceptual or motor functions which consequently affect locomotion and behavioural activity; immunization employing Freund's complete adjuvant adminstered subcutaneously or intramuscularly; induction of an anatomical or physiological deficit that will result in pain or distress; application of noxious stimuli from which escape is impossible; procedures that produce pain in which anesthetics are not used, such as toxicity testing with death as an end point; production of radiation sickness; certain injections, and stress and shock research that would result in pain approaching the pain tolerance threshold..

Comment: Animals used in Category D studies should not have signs of prolonged clinical distress, such as marked abnormalities in behavioural patterns or attitudes; lack of grooming, dehydration, abnormal vocalization, prolonged anorexia, circulatory collapse or decreased cardiac activity, increased signs of infectious processes (peritonitis, pleurisy, pneumonia, diarrhea, etc). If the clinical abnormalities cannot be alleviated, the animals should be destroyed using an acceptable method of euthanasia.

Ce sont: les interventions chirurgicales majeures faites sous anesthésie, avec survie, selon des pratiques vétérinaires reconnues, suivies d'analgésie postopératoire adéquate, de fluidothérapie et d'application de soins vétérinaires requis; l'exposition des animaux à des stimuli nocifs pendant des périodes n'excédant pas le niveau minimum requis pour atteindre les effets cliniques recherchés; les périodes prolongées (plusieurs heures et davantage) d'immobilisation physique exécutée selon les lignes directrices du CCPA; l'induction de stress comportementaux comme la privation maternelle, l'agression, les interactions prédateur-proie, les interventions qui modifient les fonctions de perception ou motrices qui affectent subséquemment la locomotion et les comportements; l'immunisation à l'aide d'injections sous-cutanées ou intramusculaires de l'adjuvant complet de Freund; l'induction d'une déficience anatomique ou physiologique qui engendre de la douleur ou de la détresse; l'application de stimuli nocifs que l'animal ne peut éviter; les interventions douloureuses pour lesquelles on utilise aucun anesthésique comme des tests de toxicité devant entrainer la mort de l'animal; l'induction mal des rayons, certaines injections, des recherches sur le stress et le choc qui causeraient de la douleur pouvant atteindre le seuil de la tolérance.

Commentaires: Les animaux utilisés dans les études de la catégorie D ne doivent pas manifester de signes de détresse clinique prolongée comme des anomalies importantes dans leurs attitudes ou leurs types de comportement; absence d'autotoilettage, déshydratation, vocalisation anormale, anorexie prolongée, collapsus circulatoire ou diminution de l'activité cardiaque, augmentation des signes d'infections (péritonite, pleurésie, pneumonie, diarrhée, etc). Si on ne peut pas éviter les anomalies cliniques, on doit euthanasier les animaux d'une manière humanitaire.

CATEGORY E	CATÉGORIE E

☐ PROCEDURES THAT INVOLVE INFLICTING SEVERE PAIN NEAR, AT, OR ABOVE THE PAIN TOLERANCE THRESHOLD OF UNANESTHETIZED, CONSCIOUS ANIMALS.
LES INTERVENTIONS QUI PROVOQUENT DE LA DOULEUR INTENSE PRÈS, ÉGALE OU AU-DESSUS DU SEUIL DE TOLÉRANCE DE LA DOULEUR CHEZ LES ANIMAUX CONSCIENTS NON ANESTHÉSIÉS.

Such studies may not be confined to surgical pratices, but may include exposure to noxious stimuli or agents whose effect are unknown; intradermal or foot pad injection using Freund's complete adjuvant; completely new biomedical experiments which have a high degree of invasiveness; behavioural studies about which the effects of the degree of distress are not known; use of muscle relaxants or paralytic drugs without the use of anesthetics; burn or trauma infliction on anesthetized animals; a euthanasia method not approved by the CCAC or Canadian Veterinary Medical Association.

Comment: Category E experiments are considered highly questionable or unacceptable, irrespective of the significance of anticipated results. Many of these procedures are specifically prohibited because of conflict with CCAC's "Ethics of Animal Experimentation".

De telles études ne sont pas nécessairement limitées aux pratiques chirurgicales mais elles peuvent comprendre une exposition à des stimulis nocifs ou à des agents dont les effets sont inconnus, les injections intradermiques ou intraplantaires d'adjuvant complet de Freund, les expériences biochimiques tout à fait nouvelles et hautement invasives; les études comportementales pour lesquelles les effets des degrés de détresse ne sont pas connus; l'utilisation de relaxants musculaires ou de drogues paralysantes sans anesthésie. L'infliction de brûlures ou de traumatismes à des animaux non anesthésiés; une méthode d'euthanasie non approuvée par le CCPA ou l'Association canadienne des vétérinaires.

SECTION VI

Does the animal component of the project involve exposure to?: - Le projet implique-t'il?:

a) Biohazards? - Des risques biologiques?

☒ No - Non ☐ Yes - Oui **If "yes", identify - Si "oui", préciser.**

b) Hazardous or potentially hazardous chemical agents? - Utilisation de substances chimiques posant un danger?

☒ No - Non ☐ Yes - Oui **If "yes", identify the nature of the hazard, if known - Si "oui", identifier la nature du danger, si connue.**

c) In VIVO use of radioisotopes? / Utilisation des radio-isotopes in VIVO?

☒ No - Non ☐ Yes - Oui **If "yes", state which isotope and licence no. - Si "oui", préciser la nature de l'isotope et le n°.de licence.**

Isotope Quantity - Quantité: **Licence no. - N° de licence:**

d) Infectious agents? - Agents infectieux?

☒ No - Non ☐ Yes - Oui **If "yes", identify. - Si "oui", identifier.**

SAFETY MEASURES - MESURES DE SÉCURITÉ

a) Containment (Ref, Orrice of Bio-Safety guidelines) - Confinement (directive de CRM)

☒ No - Non ☐ Yes - Oui **If "yes", indicate level. - Si "oui", indiquer le niveau.**

b) Other safety measures - Autres mesures de sécurité

☒ No - Non ☐ Yes - Oui **If "yes", stipulate. - Si "oui", stipuler.**

c) Special procedures for disposal of animal waste and carcasses - Procédures spéciales pour l'élimination des déchets et carcasses

☒ No - Non ☐ Yes - Oui **If "yes", stipulate. - Si "oui", stipuler.**

d) Occupational exposure necessitating - special medical procedures / to be determined by Medical Services Branch.
Exposition à des substances dangereuses nécessitant des mesures médicales particulières / à être déterminées par la Direction générale des services médicaux.

☒ No - Non ☐ Yes - Oui

SECTION VII APROVALS - APPROBATIONS

As the investigator responsible for this project, I declare: "that all animals used in this research project will be cared for in accordance with the Guidelines of the Canadian Council on Animal Care, described in the **Guide to the Care and Use of Experimental Animals**. In addition, I am using the appropriate animal species and will minimize the animal numbers, extent and duration of any discomfort, if applicable, and will observe all current safety and occupational regulations".

En tant que responsable de ce projet, je déclare: "que tous les animaux utilisés dans ce projet de recherche seront traités selon les directives contenues dans le **manuel sur le Soin et l'utilisation des animaux d'expérimentation** publié par le Conseil Canadien de protection des animaux. De plus, j'utilise l'espèce animale adéquate et réduirai au minimum le nombre d'animaux, l'intensité et la durée de tout inconfort, s'il y a lieu, de même j'observerai les politiques courantes de sécurité professionnelle".

Signature of Responsible Investigator Signature du resposable du projet	Date
Mary L'Abbe/HC-SC/GC/CA	07/23/2004

As responsible manager, I certify that scientific review has been performed and I approve this project.

En tant que gestionnaire responsable, je certifie qu'une revue scientifique fut effectuée et j'approuve ce projet.

Signature of Responsible Manager Signature du gestionnaire responsable	Date
Peter Fischer/HC-SC/GC/CA	08/11/2004

As the Directorate representative of the Animal Care Committee, I have conducted a preliminary review of this protocol and recommend its circulation to the Committee for review.

En tant que représentant de la Direction au C.P.A., j'ai effectué une revue préliminaire de ce protocole et recommande sa revue par le comité.

Category of Invasiveness - Catégorie d'intervention invasive
Category B

Name of the A.C.C. Member Nom du membre du C.P.A.	Signature	Date
	Gerard Cooke/HC-SC/GC/CA	08/11/2004

MANAGER & A.C.C. MEMBER COMMENTS