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EFFECTS OF SELENIUM SUPPLEMENTATION AND CHRONIC INFLAMMATION ON BONE MICROARCHITECTURE AND STRENGTH IN MICE

By

ABIY GIRMA MELAKU

Bachelor of Arts in Biochemistry and Molecular Biology

Cornell College

Mt. Vernon, Iowa

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Thesis Approved:

Dr. Barbara J. Stoecker

Thesis Adviser

Dr. Brenda J. Smith

Dr. Stephen Clarke

Dr. Sheryl A. Tucker

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Selenium as an Essential Trace Element

Selenium (Se) is an essential trace element for humans, and its appearance in the food supply is closely related to soil selenium, which is highly variable [1]. For example, selenium was first recognized as an essential trace element in 1957 when it was discovered to be crucial as a component of Factor 3, which prevented liver necrosis in rats [2]. In 1973, the University of Wisconsin reported Se as a part of glutathione peroxidase (Gpx) [3], and in 1985, phospholipid hydro-peroxide glutathione peroxidase (p-Gpx) was identified as a second Se-containing enzyme [4]. These two enzymes generally characterize the biochemical functions of Se such that the presence or absence of Se affects the underlying mechanism by which Se contributes to redox balance [4]. In animals, Se deficiency results in pathological conditions manifested as defective growth, hepatic necrosis, myocardial degeneration and muscular dystrophy [5]. In humans, Se deficiency takes on symptoms related to the function of Se in the body. The symptoms range from bone and muscle pain to dry, flaky skin [6].

High levels of free radicals induced through deficiency of Se leads to reactions contributing to pathologies of diabetes, cardiovascular disease, hypertension and related complications [6]. Se is crucial for the antioxidant activity of Gpx, which catalyzes the scavenging of reactive hydrogen peroxide and lipid hydroperoxide [7]. It is also important in the

conversion of the thyroid hormone thyroxine (T_4) to triiodothyronine (T_3), as a component of the selenoprotein 5' iodothyronine deiodinase, the enzyme responsible for the conversion [8]. Low Se intake has also been associated with Kashin-Beck disease, a disease characterized by endemic osteoarthropathy affecting both bones and joints with a typical onset in the first or second decade of life [9, 10].

Despite its requirements for enzymatic function, excess Se may lead to a toxicity associated with negative effects. Short term exposure to excess Se consumed through diet in animals results in abnormal posture, unsteady gait and eventual death. Livestock exhibit blindness, weak legs, paralysis, dullness, anorexia, weight loss, ataxia, and dystrophic hooves from long-term consumption of highly seleniferous grasses and crops [11]. Humans living in areas with excess Se in the soil developed changes in their integumentary system manifested as dermatitis, hair loss and nail changes [12]. These changes were observed at an intake greater than 16 times the optimal level for the recommended dietary allowance (RDA) [12]. As a result, early research on Se focused on the consequences of excessive Se intake. Current research, however, also includes focus on Se deficiency and the resulting pathophysiological conditions after Se was identified as an essential nutrient for balancing the redox system [13]. The emerging evidence is promising in support of the importance of Se in the prevention of chronic diseases, including its impact on bone health [13].

Selenium and Bone

Overview of Bone

Bones constitute a large part of the endoskeleton of vertebrates and support and protect various organs of the body. Bone consists of osseous tissue that provides rigidity and a coral-like three-dimensional internal structure. This tissue is relatively hard and light weight, formed mostly of calcium phosphate in the form of calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [14]. Osseous tissue houses marrow, endosteum, periosteum, nerves, blood vessels and cartilage [14]. Bone is not uniformly solid but consists of two main compartments: cortical bone and trabecular

bone. Cortical bone is compact (5-30% porosity) and makes up the outer compartment of bone, while trabecular bone makes up the interior and is composed of a network of rod and plate-like elements that make the overall tissue lighter and allow room for blood vessels and marrow. Trabecular bone accounts for 20% of total bone mass, and cortical bone accounts for 80% [15]. Aside from a structural and protective role, bones are important in movement, blood production, mineral storage, growth factor production, and fat storage [16].

Cellular Structure of Bone

Two types of bone cells constitute trabecular and cortical bone. Osteoblasts descend from osteoprogenitor cells and form bone. They are located on the surface of osteoid seams (narrow regions of newly formed organic matrix) and make a protein mixture known as osteoid, which mineralizes to become bone. Osteoid is mainly composed of Type I collagen secreted by osteoblasts. Osteoblasts also produce hormones like prostaglandins to act on the bone itself. They also produce other important molecules involved in the mineralization process including alkaline phosphatase [14]. Osteocytes are formed when osteoblasts mature after migrating into bone matrix and get trapped after mineralization. Not only are osteoblasts important in bone formation and matrix maintenance, but are crucial in calcium homeostasis [14].

Osteoclasts are the multinucleated cells responsible for breaking down bone, a process known as bone resorption. These cells are located in resorption pits, also called Howship's lacunae, and need to migrate to the site of resorption. They act by releasing active enzymes such as tartrate resistant acid phosphatase to break down the crystal of bone. They release acids to solublize mineral as well [14].

Molecular Structure of Bone

Bone matrix makes up the majority of bone and consists of both inorganic and organic components [17]. As mentioned above, hydroxyapatite is an important component of bone, and is the inorganic portion of bone matrix [18]. The organic part of matrix is mainly composed of Type I collagen synthesized intracellularly as tropocollagen and then exported to form fibrils

[18]. Various matrix proteins also make up the organic portion of bone matrix including glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin and cell attachment factor. These growth factors are thought to function as growth factors to promote bone formation/mineralization but their full function is not fully known [14].

Bone Remodeling

The purpose of bone remodeling or bone turnover is to maintain plasma calcium (Ca) homeostasis, to repair micro-damages to bone from stress, and to shape and sculpt the bone during growth. Bone turnover occurs continuously throughout life and requires osteoblast and osteoclast activity in tandem. Blood calcium is regulated by parathyroid hormone activity, which stimulates osteoclasts that breakdown bone and release calcium into blood, and osteoblasts that reconstitute the Ca from the blood into bone. Bone volume is determined by the rates of bone formation and bone resorption. Mohan and Baylink report that certain growth factors may work to locally alter bone formation by increasing osteoblast activity [19]. These factors include insulin-like growth factors I and II, transforming growth factor-β, fibroblast growth factor, platelet-derived growth factor, and bone morphogenetic proteins [19]. Research has suggested that trabecular bone volume in postmenopausal osteoporosis may be determined by the relationship between the total bone forming surface and the percent of surface resorption [20]. Bone remodeling is also important in facture and microfracture repair of the skeleton. Repeated stress, such as weight-bearing exercise, or bone healing, results in the bone thickening at the points of maximum stress. It has been hypothesized that this is a result of bone's piezoelectric properties, which cause bone to generate small electrical potentials under stress [21]. Chronic inflammation has been shown to disrupt bone remodeling leading to bone loss [22].

Bone disorders

According to the Institute of Musculoskeletal Health and Arthritis at the National Institutes of Health, more than 400 million people around the world suffer from crippling, chronic pain of joint disease, osteoporosis, spine diseases and musculoskeletal trauma, and this number is

predicted to increase to 570 million people by the year 2020 [23]. Associated risk factors include aging, low estrogen/testosterone levels, low dietary Ca intake, and family history. Epidemiological studies and studies with animal models have suggested that a deficiency of selenium (Se) is associated with bone loss from osteoporosis [23].

Osteoporosis is as metabolic bone disease characterized by low bone mineral density and microarchitectural deterioration of bone leading to its fragility [24]. About 1.5 million hip, spine, and wrist fractures are reported in the United States every year [25]. About 24% of patients with hip fractures die within a year after the incident, either from direct complications or due to the surgical treatment [26]. These complications include pneumonia and blood clots in the lung [25]. The estimated national direct expenditures (hospitals and nursing homes) for treatment of osteoporosis and associated fractures were $19 billion in 2005 ($52 million each day) and the cost is expected to reach $25.3 billion by 2050 [25].

Several factors have been implicated in the etiology of bone diseases including selenium deficiency. Still a major public health concern in many parts of the third world, selenium deficiency has been associated with bone loss [27-29]. However, the mechanism in which Se plays that role is not completely elucidated.

Overall Objective

The long-term research goal is to examine the potential role for selenium supplementation as a prevention strategy for chronic diseases associated with inflammation.

Statement of Problem

The rationale for these studies is that there is not yet (to our knowledge) a systematic investigation of the role of increased dietary intake of selenium as a possible prevention strategy for diseases of chronic inflammation. This research will evaluate potential synergistic effects of selenium deficiency and inflammation on bone loss as well as potential beneficial effects of selenium supplementation. In addition, the research model also provides a basis for future investigation of nutrient, phytochemical, and drug effects on bone loss due to chronic

inflammation. By understanding the role of Se under normal and inflammatory states, this may have a significant impact on the dental, medical, and nutritional fields.

Objective

The objective in this research project was to evaluate the effects of selenium depletion and supplementation on the skeletal response using an *in vivo* model of chronic inflammation.

Hypothesis

The central hypothesis was that selenium adequate and selenium supplemented diets will have better bone quality than selenium deficient diets. The inflammatory treatment is expected to reduce bone quality.

Specific Aims

The specific aims were:

Specific Aim 1: to assess bone microarchitecture of animals consuming different levels of dietary selenium. The hypothesis is that higher dietary selenium will result in better bone microarchitecture parameters.

Specific Aim 2: to assess bone strength of animals consuming different levels of dietary selenium. The hypothesis is that higher dietary selenium will result in bone that is stronger.

Significance of the Study

Selenium deficiency has been associated with osteoporosis in growing individuals [30]. This experiment is innovative as the animal model provides a framework for research to apply to human models. The effects of Se supplementation on chronic inflammation have implications on human conditions associated with inflammation such as osteoarthritis and periodontitis. The results of the study apply to Se and its effect on bone quality. If this study is confirmed by more animal and controlled clinical human studies, it may serve as a basis for dietary recommendation for preventing or decreasing the incidence of a selenium deficiency-related bone disorder. Previous medicinal and dental conditions of inflammation that have required continual

hospitalization and extended drug therapy will have a potential treatment. This treatment can be adapted to current dietary habits, reducing the cost of medicinal care in the healthcare industry.

CHAPTER II

LITERATURE REVIEW

Metabolism of Selenium

Selenium is classified as a metalloid and has metallic forms such as selenate, inorganic forms such as selenite, and organic forms such as the amino acids selenomethionine (SeMet) and selenocysteine (SeCys) [31]. SeCys and SeMet are most widely found in plant and animal sources in protein form [31]. SeCys is a modified amino acid found in selenoproteins in both flora and fauna food sources, playing a role in redox reactions as active centers of selenoenzymes [32]. SeCys does not have a free form due to a selenol (-SHe) group that is highly reactive. Se in SeMet residues is largely found indiscriminately within the protein, i.e. a specific codon for methionyl is not found in proteins containing Se in the form of SeMet residues [32]. The accumulation of Se in plants often takes inactive forms such as SeMet, methyl selenocysteine (MeSeCys) and γ-glutamyl-Se-methylselenocysteine [32].

Selenium as a Component of Protein

The amino acid forms that Se takes are SeCys or SeMet [33]. The term selenoprotein refers to proteins containing SeCys, while proteins with Se as SeMet are called Se-containing proteins [6, 31]. Se is incorporated within the protein early during the translation of the primary structure through SeCys. This incorporation of Se at this stage

protein translation makes Se slightly different among other trace elements [34]. For example, both Cu and Zn and other metals are integrated into their respective proteins after the primary structure has been formed [34]. SeCys is coded for with a UGA codon in the selenoprotein mRNA [35]. SeCys is located in the active site of the majority of selenoproteins that scavenge reactive oxygen species (ROS) [36]. Cysteine is structurally similar to SeCys except the S atom in cysteine is replaced by Se in SeCys [37].

Absorption and Transport of Selenium

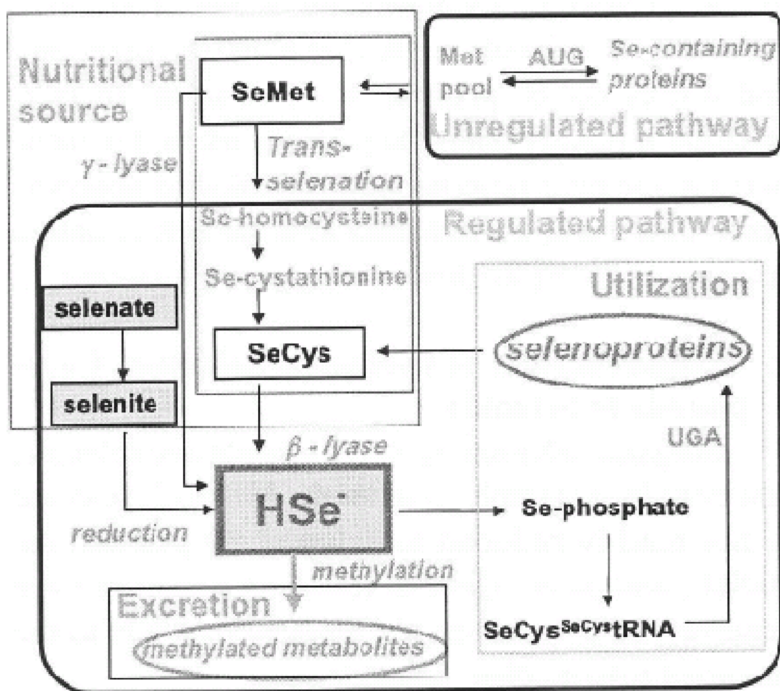
Se consumed through food is readily absorbed at a rate that ranges from 50 to 100% [38]. The major dietary form is SeMet, and it is readily absorbed at about 90% through a similar mechanism to that which absorbs methionine [38]. Inorganic Se is relatively poorly absorbed, and the mechanism of selenocysteine absorption is not well known [39]. Selenate absorption uses a mechanism common to the one used by sulfate. It depends on the Na^+ gradient, and absorption is maintained by the Na^+/K^+ ATPase [40]. Selenate is absorbed almost completely with some loss occurring through urine. Selenite absorption, however, is less consistent, due to interactions in the gut. Once absorbed, selenite is relatively well retained without the partial loss through urine seen with selenate [39]. Se membrane transporters have yet to be reported, but SeMet uses the same mechanism as methionine [6] (Fig. 2.1).

Metabolic Pathways for Conversion of Se to a Common Intermediate: Selenide

Varying forms of Se are consumed through diet to be eventually transformed into a common active intermediate for the synthesis of SeCys [41] (Fig. 2.1). Inorganic forms of Se, selenite and selenate, are reduced by glutathione (GSH) and thioredoxin reductase (TrxRs) to selenide [42, 43]. Transportation of Se uses bicarbonate and phosphate buffer systems. Selenite is directly taken up by red blood cells (RBCs), while selenate ions are taken up by hepatocytes [44]. Selenite is readily reduced to selenide, in RBCs and intestinal cells [45]. It is released into the blood stream and is bound to albumin and transported to the liver [45]. The reduced forms of inorganic Se are used to synthesize selenoprotein P and glutathione peroxidase (Gpx) in the liver

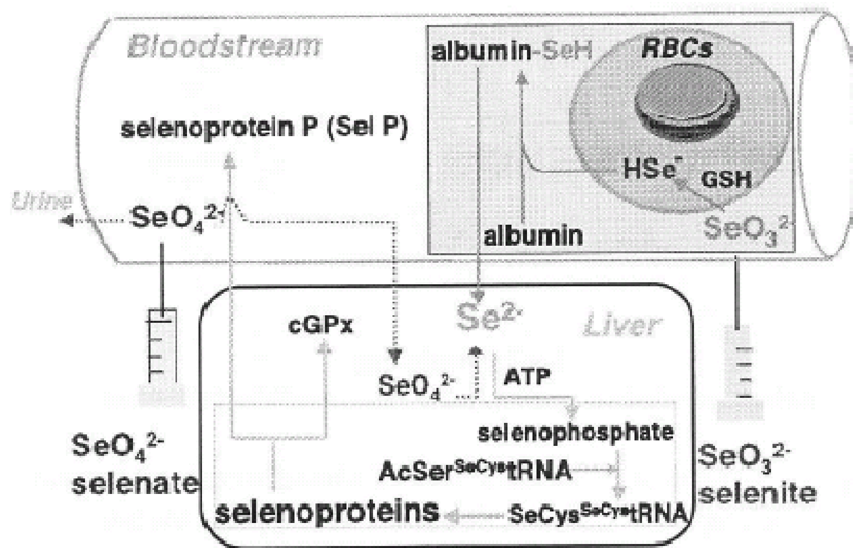
for release into the bloodstream [46]. The organic forms of Se, SeCys and SeMet, (as selenoamino acids) are transformed to selenide by a lyase reaction [47]. β-lyase transforms SeCys directly to selenide while SeMet transforms to selenide by a trans-selenation pathway in the cells **(Fig 2.1)**. Excessive Se intake causes the C-Se bond to be cleaved at the γ position by γ-lyase of Se Met. This results in the formation of selenide for synthesis of selenoproteins [48].

Fig.2.1: Metabolic Pathways for Selenium [42]



SeCys is synthesized during protein formation and converted to selenide. Selenide is used to make selenophospate for the synthesis of selenoproteins [49, 50]. This ATP-requiring reaction is catalyzed by selenophosphate synthetase [51]. The carbon skeleton required for SeCys is derived from serine, and dietary SeCys or SeMet are not used [52]. Serine is esterified to the 3′ end of the terminal adenoside of tRNAsec UCA to produce Ser-TRNAsec UCA by seryl-tRNA synthases [53]. The next step involves the production of selenocysteine-rRNASec UCA by the substitution of serine-OH with SeH from selenophosphate by selenocysteine synthase [54]. Degradation of SeCys is catalyzed by selenocysteine lyase which releases elemental Se that converts to selenide to complete the cycle [55].

Fig. 2.2: Differences in metabolic pathways for selenite and selenate [42]



SeMet does not have a specific codon but uses the same AUG codon as Met to be incorporated into general proteins until degradation and release **(Fig. 2.2)** [56]. Once released, it is converted to selenide by trans-selenation or directly by the γ-lyase pathway. The concentration of SeMet in total body protein is proportional to the concentration of Se in the food.

Mammalian Selenoproteins

The first SeCys containing protein was discovered in mammals in 1973 [3]. Since then, more types of selenoglutathione peroxidases (Gpx) have been identified and characterized [57]. Glutathione peroxidase protects against oxidative damage by reducing hydrogen peroxide and other hydro-peroxides [58]. Phospholipid glutathione peroxidases (P-Gpx) also function in the reduction of phospholipid, cholesterol, and cholesteryl ester to prevent cell membrane lipid peroxidations [58]. P-Gpx plays a role in the structural function of male spermatozoa and offers a plausible explanation for the male infertility seen in Se deficiency [59]. There are also three TrxRs that function by reducing thioredoxin and helping to maintain cellular thiol redox status [60]. All of these TrxRs are pyridine nucleotide-disulphide oxidoreductases that contain selenium [61]. Specifically, these TrxRs catalyze the NADPH-dependent reduction of the redox protein thioredoxin [61]. Hill and co-workers compared thioredoxin reductase activity in liver,

kidney and brain of rats fed selenium-deficient and control diets for 14 weeks after weaning [62]. Liver and kidney of selenium deficient mice showed a 4.5% and 11% reduced activity respectively, but inhibition of thioredoxin activity was not seen in brain [62].

Other selenoproteins include a family of deiodinases (three in total) involved in thyroid hormone metabolism [63]. Types I and II are important in the conversion of T_4 to T_3, while the Type III enzyme inactivates T_3 [63]. Selenophosphate synthetase 2 (SPS2), is also a selenoprotein which synthesizes the Se donor for SeCys biosynthesis [64]. Other selenoproteins important in oxidative defense include selenoprotein-W, selenoprotein-P and methionine sulfoxide reductase. Selenoprotein P also serves to transport Se to peripheral tissues [64].

Selenium Concentration in the Body

The total Se content of the human body is estimated from various cadaver studies to range from 13-23 mg [6]. Total Se in US subjects was estimated at 30 mg using stable isotope methodology [38]. About 60% of body selenium is stored in various tissues such as muscle, liver, blood and kidney, while about 30% is found in the skeletal system alone [6]. Immune cells, erythrocytes and platelets have a relatively higher concentration of Se in the body [6]. Normal levels of Se are reported to be 0.1 – 0.34 mg/L (1.27 – 4.32 µmol/L) for white blood cells; 0.04 – 0.60 mg/L (0.11 – 7.6 µmol/L) in serum; 0.03 mg/L (<0.38 µmol/L) in urine and <0.4 µg/g (0.01 µmol/L) in hair [33]. The National Health and Nutrition Examination Survey (NHANES) III for US young adults 19-30 years of age reported the mean serum Se concentration to be 127 and 124 µg/L for males and females respectively [39]. European adults from different countries have different values [65] which ranged from 86 µg/L in Sweden, France, and Italy to 43 µg/L in Serbia. Values for adults in New Zealand are reported to range from 62 – 69 µg/L [66]. Individuals in low Se areas like in China have plasma Se concentration of 11 – 16 µg/L.

Excretion of Selenium

The primary mode of Se excretion after intestinal absorption is urine [67]. Dietary intake, if within normal physiological doses, influences the amount of Se excreted in the urine.

When intake is excessive, Se tends to be exhaled out into breath in addition to the urinary route [68]. Se is methylated sequentially before excretion to produce monomethylated Se and trimethylselenonium as urinary and dimethylselenide as expiratory metabolites [6, 68]. The concentrations of the two urinary metabolites differ by the Se intake: at lower dietary Se intake, monomethylated Se is mostly excreted while at high level of dietary Se intake, the trimethylated form is predominantly excreted [69]. The monomethylated Se in urine is now characterized to be a selenosugar (Se-methyl-N-acetylgalactosamine) [70]. Overall, Se is regulated at physiological levels by urinary excretion as opposed to other major trace elements like iron that are regulated by absorption.

Physiological Functions of Selenium

Gpx

Selenium, an essential trace element for humans, forms selenoproteins that have a variety of beneficial effects for the body [6]. These selenoproteins include four different glutathione peroxidases (Gpx 1, 2, 3 and 4), which catalyze the reduction of peroxides that can cause cellular damage [6]. Gpx was described, in 1973, as the first selenoprotein with clear metabolic functions [3]. These enzymes are usually classified in three different forms: cytosolic, phospholipd and extracellular glutathione peroxidases (c-Gpx, p-Gpx and e-Gpx) and have differences in structural, kinetic, immunological and electrophoretic properties [71, 72]. Gpx enzymes play a major role in protecting cells and tissues from damage by free radicals and hydroperoxides by reducing them into their less reactive forms [73]. Both intracellular and extracellular Gpx are effective in reducing hydrogen peroxide and other organic hydroperoxides to prevent injury to cell membranes [3]. Gpx has been used as a major indicator of Se status at physiological doses. The justification for using Gpx as a marker for Se is related to the linear relationship between whole blood Gpx and plasma Se when the concentration of Se is below 100 µg/L [74]. When Se is depleted at experimental and clinical levels, the plasma Gpx activity is reduced in humans and

small animals. In addition, both experimental and clinical Se depletion have been shown to reduce tissue, blood, and plasma GPx activity in both humans and rats [75, 76].

Gpx1: Gpx1 is the classic form of selenium in the body. It is believed to account for about half of the body's total selenium and is found in all tissues, primarily in liver, kidney, and RBCs. SeCys is the active moiety of the Gpx enzyme [77] and Gpx1 has a primarily defensive role as it scavenges reactive oxygen species (ROS) generated from oxidative damage to the body [78]. A knockout model resulted in mice that showed no apparent adverse health effects [79], with the exception of higher susceptibility to a Coxsackie virus [80], and to acute paraquat toxicity [7]. The cyclic oxidation and reduction of paraquat in cells, with the resulting in production of free radicals of oxygen, leads to lung injury and eventual death [81]. Gpx1 is also purported to protect bone and cartilage from oxidative damage by possibly preventing the accumulation of H_2O_2 in the cell [67].

Gpx2: This enzyme was first identified from human liver DNA and is enriched in epithelium especially in intestine and lung [82]. The primary GPx species found in rat intestine is glutathione peroxidase-2, and it is postulated to be active as a peroxide scavenger [82]. Knockout-mice models of either Gpx1 or Gpx2 show redundancy under healthy conditions. However, ileocolitis has been shown to develop with mice that have both Gpx1 and Gpx2 knocked out [83, 84].

Gpx3: Gpx3 is predominantly secreted by the kidney and is the main form of selenium found in breast milk [85, 86]. It is most abundantly found in the plasma [87]. The main activity of Gpx3 is hypothesized to be protection against oxidative damage in the intracellular spaces (mostly in the kidneys) [88].

Gpx4: Glutathione Peroxidase-4 is found in high quantities in sperm and testis [89]. The reason may be due to the need to reduce the high level of hydroperoxides being generated during spermatogenesis as a substitute for glutathione in case of shortage [89]. Possibly Gpx4 may also function as a structural protein of sperm [90]. Gpx4 differs from the previous proteins as the

ROS species that it scavenges are comparatively larger [89]. Gpx4 is also lipophilic possibly destroying peroxides along membranes [89]. Deficiency in Gpx4 was found to be associated with increased breakage of sperm mid-piece leading to male infertility [90]. Gpx4 KO is embryonically lethal [90]. Additional glutathione peroxidases, such as Gpx5, Gpx6, and Gpx7 have also been identified, but their functions have not yet been fully clarified.

Gpx5: Glutathione peroxidase-5, also known as epididymal secretory glutathione peroxidase is encoded in humans by the GPX5 gene. It is specifically expressed in the epididymis in the mammalian male reproductive tract, and is androgen-regulated. The mRNA for Gpx uniquely does not contain a selenocysteine (UGA) codon. Thus, the encoded protein is selenium independent, and has been proposed to play a role in protecting the membranes of spermatozoa from the damaging effects of lipid peroxidation and/or preventing premature acrosome reaction [91].

Gpx6: This enzyme is not well studied and is described as an odorant-metabolizing protein, with about 40% amino acid sequence identity to Gpx1. It is expressed in the Bowman's gland of the rodent olfactory system [92].

Gpx7: The function of Gpx7 is not well known. To date it has only been found as a Cys homolog which has not yet been well characterized [6].

Iodothyronine Deiodinases

Iodothyronine deiodinases consist of three selenoenzymes and are required for metabolism of thyroid hormones [93]. Thyroxine 5'-deiodinase-1 (DIO-1), or Type 1, is abundantly present in liver and converts thyroxin (T_4) to triiodothyronine (T_3) that circulates in plasma [93]. When Se is deficient, the activity of DIO-1 decreases and results in lower circulating T_3 [93]. Deiodinases Type II and Type III (DIO-2 and DIO-3) are present in different types of tissues of the body [94]. DIO-2 and DIO-3 produce T_3 and are present in brain, pituitary, brown adipose tissue, placenta and skin [94]. DIO-3 is important in the deiodination of T_4 and T_3 into inactive forms playing a role in maintaining optimum levels of T_4 and T_3 in the body [95].

Thioredoxin Reductase (TrxR)

Mammalian TrxRs are selenoenzymes which catalyze reduction of small intracellular molecules that regulate intracellular redox state contributing to antioxidant defense systems in the cells [96]. TrxR1 is located in the cystosol and nucleus, while TrxR2 is present in the mitochondria [67]. When Se is deficient in rats, the TrxR activity is less affected than Gpx1 activity but more affected than selenoprotein P [67]. Loss of TrxR activity may be important in the development of the signs and symptoms of selenium deficiency [97]. The discovery that made clear the role of TrxR in reducing vitamin E and dehydroascorbate to the semihydroascorbate radical further substantiated selenium's antioxidant role and suggested a potential anticarcinogen function as well [96, 97].

Selenophosphate Synthetase

Certain enzymes are postulated to be responsible for liberating Se from its conjugates. Selenophosphate synthetase-1 (SPS1) may be involved in recycling selenium from selenocysteine, while selenophosphate synthetase-2 (SPS2) might use selenite-reduced selenium [98]. SPS is a selenocysteine-containing selenoprotein that plays a role in providing active Se for the synthesis of SeCys in mammals. SPS1 is essential for selenoprotein biosynthesis [99].

Plasma Selenoprotein P

Other selenoproteins include selenoprotein P, which accounts for about 40% of plasma selenium and is the main plasma selenoprotein in the body [88]. It was first recognized in the plasma of rats and constituted about 50-60% of plasma Se [100]. It is secreted by the liver [100]. Adverse liver conditions in hospital patients have been shown to result in decreased plasma selenoprotein P [101]. Se deficiency leads to a 5-10% reduction in selenoprotein P when compared to control, signifying the importance of dietary Se in regulating selenoprotein P [102]. Synthesis of selenoprotein P is given a higher priority compared to other selenoproteins and levels of the protein decline less rapidly than Gpx when exogenous Se supply is limited [102]. There is also the potential of using selenoprotein P as a marker for Se in individuals with

adequate Se intake as the level of selenoprotein P correlates with plasma Se level [103]. It is also a transport protein in the blood [104]. Decreased level of Se in testes and brain and increased level of urinary Se excretion have been shown in selenoprotein P knockout mice suggesting the important role of selenoprotein P in transport [88].

Selenoprotein W

Selenoprotein W is largely found in muscle and is smaller in size than selenoprotein P [105]. Its role is postulated to be as an antioxidant because it has been shown to bind to glutathione [36]. The discovery of selenoprotein W came from the investigation of the factor involved as the cause of white muscle disease in Se deficient sheep [106].

Other selenoenzymes important in coping with oxidative stress includes selenoprotein R or methionine-R-sulfoxide reductase as it is produced during oxidative burden [107]. Selenoprotein K and Selenoprotein S are unique, being the only identified selenium-containing membrane proteins [92].

The Relationship of Se with Selenoproteins

The selenoproteins represent the largest portion of Se in the body and are regulated by the SeCys pool [67]. The effect of Se levels on selenoprotein function was studied in rats and showed differential expression of selenoproteins based on the Se status of the body [108]. Severity of Se deficiency leads to significantly lower levels of mRNA for GPX1 activity in Se deficient male rats which showed a decrease of 1% to 7% compared to Se adequate animals [108]. Severe Se deficiency leads to significantly lower levels of mRNA for Gpx1 and of GPX1 protein [109].

When weanling rats fed Se deficient diet were supplemented with graded dietary Se, the liver GPX1 and its mRNA showed a sigmoid response with increased response with increased level of dietary Se intake [110]. The study showed, when Se intake is higher than 0.1 µg Se/g diet, the Se status fails to regulate both GPX1 activity and its mRNA. In contrast, the liver GPX4 activity decreased only to 40% of the Se adequate level and reached a plateau at 0.05 µg Se/g diet

while the mRNA for liver GPX4 remained not significantly affected by Se intake [110]. The activity of plasma GPX3 was also reduced in these deficient rats to 7-8% of the level in Se adequate rats and reached a plateau at 0.07 µg Se/g diet [110]. Other studies also demonstrated that liver TrxR, DIO-1 and selenoprotein P activities in Se deficiency decreased to 5-10% of the Se adequate level [111].

In conclusion, these studies show obvious differences in level of selenoproteins by Se status. When Se is deficient, there will be reduced levels of selenoproteins [67]. Factors other than Se deficiency need to be considered when Se status is evaluated using selenoproteins [110]. Considering the progress in the sequencing of the human genome and the current scientific advancement, mRNA evaluation of selenoproteins might need to be the preferred approach in evaluating Se status in the future.

Food Sources of Selenium

Selenium concentration in grains and seeds is associated with the variable selenium content of the soil used to grow the plants [1]. Food grown in areas where Se is deficient has much lower levels of Se/g compared to food grown in seleniferous areas [30]. Therefore, cereals and grains range from <0.1 to >0.8 µg Se/g while fruits and vegetables usually have less than 0.1 µg Se/g [30]. Se content of livestock also depends on Se content of the food they consume. Concentration of Se in organ meats and sea foods ranges from 0.4 to 1.5 µg Se/g. Muscle meats contain 0.1 to 0.4 µg Se/g and dairy products contain 0.1 to 0.3 µg Se/g [30]. In the United States most livestock are supplemented with inorganic Se and animal foods here have levels of Se as selenoproteins closer to 1.5 µg Se/g [30]. Organ and muscle meats are, therefore, good sources of selenium. Pork, beef, chicken, and eggs are the major sources of selenium intake in the U.S. diet [112]. Generally, drinking water has insignificant amounts of Se, but well water in seleniferous areas may contain higher Se content.

Human Requirements for Selenium

The upper limit and RDA set by the Food and Nutrition Board (FNB) of the Institute of Medicine is still disputed [6], because factors such as the type of Se compound, exposure time, physiological status, an unregulated SeMet pool, and interactions with other metals are important [88]. The expression of selenoenzymes, on the other hand, is regulated by Se status so a biochemical approach (instead of dietary intake or tissue concentration or balance study) was used to determine the RDA for Se intake. In 1980, an initial estimated safe and adequate daily dietary intake was extrapolated for humans (50 to 200 µg Se/d) from animal experiments that assessed Se status using the activity of GPX [113].

In 2000, however, the FNB of the Institute of Medicine evaluated the level of Se that plateaued the plasma Gpx3 for Chinese men and adjusted the requirement for North American males to 55 µg Se/d (**Table. 2.1**). The data from New Zealand was evaluated by the FNB and the plasma Gpx activity increase between the group who consumed 38 µg Se/d was found to be not different from the group who consumed 68 µg Se/d and the Estimated Average Intake (EAR) was suggested to be 38 µg Se/d. The Adequate Intake for infants for Se varies according to age. Based on level of Se concentration in breast milk, 15 and 20 µg Se/d is calculated for under six months and 6-12 months old infants respectively. The RDA during pregnancy is 60 µg Se/d based on fetal transfer and Se excretion in milk (**Table 2.1**).

Table 2.1: Selenium intake recommendations for healthy US and Canadian populations [113]

	AGE (years)								
	0-6 months	7 - 12 months	1 - 3	4 - 8	9 - 13	14 - 50	>70	Pregnancy	Lactation
AI µg/day	15	20	-	-	-	-	-	-	-
RDA µg/day	-	-	20	30	40	55	55	60	70

AI: Adequate Intake; RDA: Recommended Dietary Allowance

The recommendations for Se intake in the rest of the world are lower than the United States of America which recommends 55 µg Se/d for all persons 14 years and above **(Table 2.1)**. The World Health Organization (WHO) recommended Se intake based on Se needed to achieve two-thirds of maximum achievable Gpx3 activity [114]. With adjustment for inter-individual variations taken into account, 40 µg/d and 30 µg/d were proposed for adult males and females respectively which is in line with typical Se consumption worldwide **(Table.2.2)** [114]. The New Zealand study used 67% of maximum Gpx3 activity and calculated a Se intake recommendation of 39 µg/d, which was similar to what the value WHO recommended.

Table 2.2: Recommended nutrient intake (RNI) for selenium (µg/day) [115]

Age Group	Assumed Weight	Average normative requirement $Se_R^{normative}$ (kg/day)	$Se_R^{normative}$ (total/day)	RNI, µg/day
Infants and children				
0–6 months	6	0.85	5.1	6
7–12 months	9	0.91	8.2	10
1–3 years	12	1.13	13.6	17
4–6 years	19	0.92	17.5	22
7–9 years	25	0.68	17.0	21
Adolescents				
Female,10–18 years	49	0.42	20.6	26
Male, 10–18 years	51	0.50	22.5	32
Adults				
Female,19–65 years	55	0.37	20.4	26
Male, 19–65 years	65	0.42	27.3	34
Female, 65+ years	54	0.37	20.2	25
Male, 65+ years	64	0.41	26.2	33
Pregnancy				
2nd trimester				28
3rd trimester				30
Lactation				
0–6 months post-partum				35
7–12 months post-partum				42

Recommended nutrient intake (RNI)

<u>Selenium Interaction with other Nutrients</u>

Se interacts with several other nutrients that affect the antioxidant system. Copper and zinc are part of superoxide dismutase (SOD), and iron is a component of catalase [43]. Se also

interacts with vitamin E in minimizing lipid peroxidation [116] and with vitamin C as TrxR catalyzes regeneration of the reduced form of vitamin C from its oxidized form, dehydroascorbic acid [117]. The role of Se in iodine metabolism makes it an important nutrient in thyroid hormone synthesis [118]. The effect of iodine deficiency is exacerbated with concomitant Se deficiency. Se dependent enzymes iodothyronine deiodinases are important for conversion of T_4 to its biologically active form of T_3 [63].

<u>**Techniques of Determining Selenium Status**</u>

The common techniques for assessing selenium status include measurement of selenium concentration in blood, tissues, and excreta.

Plasma Se

Protein bound Se is associated to α and β - globulins of lipoproteins. Plasma and serum Se concentrations are comparable and both reflect short term changes in Se intake, mainly of SeMet compared to inorganic forms of Se [119]. SeMet is not subject to homeostatic control as this form of Se incorporates into tissue proteins in place of methionine [120]. Plasma Se values less than 0.1 μmol/L are associated with depletion and with clinical features of deficiency [121]. There are no universally agreed upon cut-off values for plasma Se [122]. Cut-off points suggested by Thomson are only for assessment of the adequacy of Se [123]. Plasma or serum Se are measured more accurately using inductively coupled plasma mass spectrometry (ICP-MS) [122]. Plasma Se is said to be affected by Se intake, age, puberty, pregnancy and lactation, prematurity, smoking and chronic diseases in humans [122]. Due consideration must be given to these factors while interpreting results.

Whole Blood Se

Whole blood Se is stable and is used as an index of long term Se intake [122]. The whole blood Se changes after a period of depletion (months), which makes the relationship of current Se intake with whole blood concentration somewhat difficult to associate [122]. As a result, criteria for interpretation of the values of whole blood Se have not yet been established. Whole blood Se

could also be assessed using AAS and ICP-MS, though the analysis is said to be difficult. Factors affecting plasma Se affect concentration of Se in whole blood as well [122].

Erythrocyte and Platelet Se

Erythrocyte Se is mostly associated with the hemoglobin, while only 15% is associated with its glutathione peroxidase. This too reflects long term Se status. For people consuming stable intakes of Se, positive correlation was seen between erythrocytes, plasma and dietary intake [124]. Erythrocyte Se is lower in disease conditions that affect absorption of Se, and it responds slowly to Se supplementation compared to plasma Se. The longer period required for the synthesis of the erythrocyte and the limited transferability of hemoglobin-bound Se contributed to slow response of erythrocytes to Se supplementation [125]. The type of Se used for supplementation determines the rate of response by erythrocytes. The erythrocyte response to supplementation with inorganic Se is slower than with SeMet, even though SeMet is not subject to homeostatic regulation [126]. Determination of erythrocyte Se is not highly recommended due to problems with measurements. Information on factors affecting erythrocyte Se concentration is lacking but associations exist between chronic diseases affecting Se absorption, long term low Se intake, genetic diseases such as sickle cell anemia and Down's syndrome [127].

Urinary Se

Se excretion in urine helps to regulate homeostasis of Se in the body and it is the major excretory pathway for Se (50 - 60%), while the remainder gets excreted via feces [128]. Urinary excretion correlates well with dietary intake and plasma Se such that dietary Se intake can be roughly estimated to equal twice as much as urinary Se [129]. Urinary Se excretion is lower in females, pregnant women and aged people [122] and reduction of Se in the aged population is associated with reduction in muscle mass [130]. Urinary Se is used as an index of toxicity and the allowable maximum concentration is at 1.3 µmol/L [131]. Fasting urine samples are preferred for measurement of urinary Se at the population level [122]. A fluorometric method is commonly used to measure urinary Se but the AAS method can also be used [122].

Gpx

Glutathione peroxidase activity in cells and plasma is used for the assessment of long and short-term selenium deficiency, respectively [30, 132]. When Se intake is below threshold (1.15 μmol/L), erythrocyte Gpx1 activity is used to assess Se status and it correlates with whole blood or erythrocyte Se level [133]. The correlation no longer exists when Se intake is beyond the threshold value making it a difficult indicator of Se status [134]. Gpx1 activity in platelets is also a sensitive indicator as platelets contain significantly higher concentration of Se than any other tissues and have a high turnover. On the other hand, platelet separation is difficult [134]. Gpx3 is measured more accurately than other glutathione peroxidases and it contains 12% of the Se in plasma [135]. A strong correlation has been identified between plasma Se and Gpx3 activity [126], and plasma Se and Gpx3 are said to be good measures of Se status [120]. Plasma Gpx3 activity increases following supplementation and this is not dependent on the type of Se used for supplementation [122]. Gpx3 is also used in population studies where Se status is low [124]. Gpx3 is more stable at -80°C than Gpx1 activity. Enzyme-linked immunosorbant assay (ELISA) kits are also used [122].

Plasma selenoprotein P

Selenoprotein P is said to be more sensitive to Se deficiency than glutathione peroxidase activity [133]. Response to Se supplementation by selenoprotein P is higher than Gpx3 [136] and selenoprotein P and plasma Se correlate positively with Se status [122]. Optimal level for plasma selenoprotein P has yet to be defined [122]. Selenoprotein P could be measured by competitive radioimmunoassay using ^{75}Se labeled human selenoprotein P [135].

Molecular biomarkers

In rodents mRNA levels used to determine Se requirements have revealed a hierarchy in the transcriptome level of selenoprotein expression [137]. Gpx1 is less labeled than other selenoproteins in Se deficiency [137], while Sepp1 (gene that encodes selenoprotein P) and DIOl mRNA levels decrease less than Gpx1 mRNA levels [138]. Sunde and colleagues reported that

blood Gpx1 mRNA can be used as a molecular biomarker to verify dietary Se requirements in rats [139]. Barnes and co-workers characterized the Se regulation of rat molecular biomarkers in liver, kidney, and muscle in Se depleted (<0.01 µg Se/g diet) male weanling rats and rats with graded levels of Se (0 – 0.8 µg Se/g diet). Most selenoprotein mRNA was found not to be significantly regulated by Se status, with the exception of selenophosphate sythetase-2, which was up-regulated in Se deficiency. No biomarkers for Se status determination were characterized for high (> 0.8 µg Se/g diet) Se intake [140].

There is inadequate data on the biomarkers of Se status in humans [123]. Sunde and colleagues conducted a longitudinal study in the U.K. measuring the efficacy of molecular biology markers for assessing Se status in humans [141]. In addition to biochemical markers like levels of plasma Se, selenoprotein mRNA levels from ~40 participants with an average plasma Se concentration of 1.13 ± 0.16 µmol/L (35.13 ± 5.44 mg/L) were taken as molecular biomarkers. No significant change was seen over time in mRNA levels and levels did not correlate with plasma Se, indicating that the subjects were on the plateau of the Se response curves. Although the molecular biomarkers were readily detectable, they did not distinguish differences in Se levels, particularily when plasma levels were this low [141].

Hair and Toe Nail

Hair and toenails can be used as indicators of short-term and long-term selenium intake, respectively [122]. There are 4 main analytical methods used to assess selenium status [142-145]. Selenium status determination through fluorometry requires very precise, minute measurements of Se with the main risk being Se loss during dissolution of a given sample [142]. Neutron activation analysis is also used for Se measurement in biological samples, and is a relatively faster method. However, it requires the use of research reactors [143]. Routine analysis is more commonly performed by atomic spectroscopy using either hydride generation [144] or graphite furnace [146]. Inductively coupled plasma-mass spectroscopy can also be used to measure selenium in combination with analysis of other elements [145].

Multiple Indices

For individuals with low Se status, the measurement of total Se and Gpx3 in plasma is recommended [122]. For those having adequate Se status, Se status could be assessed by total Se in plasma and erythrocytes as a marker of current and longer term status respectively [122]. When blood collection is limiting, analysis of toenail Se is recommended as a marker of long term Se status. When Se status is studied as a risk factor for disease, interaction of Se with other antioxidant nutrients, polyunsaturated fats, heavy metals and iodine status must be investigated to rule out any confounding effects of these nutrients [122].

Selenium Deficiency

Manifestations of Se deficiency are species specific. In mice, deficiency causes degeneration of muscle and organs such as liver and pancreas and reproductive failure in male rodents due to defects in sperm production [147]. Rats fed on a diet that was deficient in Se, vitamin E and sulfur amino acids developed liver necrosis, which caused reproductive failure and death within three to four weeks. Diets deficient in Se but not in vitamin E and sulfur amino acids fed to rats and chickens showed that Se was still an essential nutrient [148]. Se deficiency in mice results in multiple necrotic degeneration of skeletal muscle, heart, kidney, liver, and pancreas and reproductive failure. [148]. Knockout mice models have been used to show Se as key in neurological function and gastrointestinal disease [100, 149].

Various species of food animals have different responses to chronic Se deficiencies [6]. Mulberry heart, a cardiac condition, is present in swine low on Se [67]. Lambs present with muscular dystrophy known as white muscle disease. Turkeys develop gizzard myopathy and cattle develop myopathy of skeletal and heart muscle [67]. In cattle, Se deficiency resulted in muscle myopathy and reproductive system problems, manifested as reproductive failure in bulls and retention of placenta in cows [147]. Chickens with severe Se deficiency manifested with symptoms related to exudative diathesis secondary to degeneration of capillary beds [147] . The reasons for these species specific manifestations of Se deficiency are not clear.

In humans, selenium deficiency is associated with Keshan disease, which is an endemic juvenile cardiomyopathy due to low Se intake [150]. It is reported mainly in areas of China and Eastern Siberia where low Se content in the soil is common (mean Se content 0.125 μg/g) [151]. A comprehensive supplementation of Se in the 1970s was used to combat Keshan disease in the peasant population of certain hilly and mountainous regions in China with soil low in Se [66]. Several studies contributed to the decisions for the governmental supplementation program [152].

More recently, selenium supplementation for children in Kashin Beck Disease-affected areas of China has been investigated by Chinese scientists [153]. Bai and colleagues orally supplemented children ages 3-13 years with sodium selenite in Jingcuan County of the Ganshu Province for 2 years. In children, selenium content increased from 56.5 ng/g to 251.7 ng/g and metaphyseal damage detected by finger X-ray decreased from 79.2% to 34.4%. In children without supplementation, however, the researchers saw an increase in the detected metaphyseal damage of finger X-ray from 57.6% to 65.5% showing that selenium supplementation promoted lesion repair [154]. In Guide County of the Qinghai Province, Li and colleagues administered three different types of oral selenium supplementation to children. The supplementation period lasted for a year and showed a therapeutic effect on metaphyseal joints. Additional data on participants were not available [155].

A report from New Zealand of patients given total parenteral nutrition (TPN) without Se supplementation showed a tendency towards Se deficiency [156]. After surgery and TPN, patients exhibited symptoms including dry flaky skin, bilateral muscular myalgia and pain with a great drop of plasma Se from 25 μg/L to 9 μg/L after surgery and TPN [156].

Selenium and iodine deficiency interact and lead to the development of endemic myxedematous cretinism manifested by goiter and lowered intelligence and neurological disorders [147]. Se supplementation alone without a concurrent iodine supplementation leads to aggravation of the condition due to activation of deiodinases, which increased synthesis of T_3 exacerbating iodine deficiency [157].

Selenium Toxicity

Acute and chronic selenium toxicity were first reportedly seen in livestock due to high soil levels resulting in the accumulation of selenium in plants [158]. Inorganic selenium and selenoaminoacids have increased bioavailability and could be toxic, if consumed in excess, as opposed to methylated forms (trimethylselenonium chloride, dimethylselenide) which are less toxic. Hydrogen selenide is in comparison the most toxic form. The biomechanical mechanism underlying selenium toxicity is not known. The human body lacks homeostatic mechanisms to control or reduce Se absorption even under chronic toxic intake [36]. Safer and colleagues reported selenite inactivating eukaryotic initiation factor 2-α [159] but more detail is not well known. To examine the mechanism of toxicity in mice, Hasegawa and colleagues investigated the liver of Imprint Control Region (ICR) male mice treated with selenocystine [160].

In humans, modest intakes of selenium (<800 μg Se/d) are not evidenced to be toxic[161]. A study in South Dakota and Wyoming on Se in the water supply showed no signs of selenium toxicity in 142 subjects who consumed as much as 724 μg Se/d [162]. Increased levels (50x higher than the standard 10 μg/L) of inorganic Se in well water resulted in increased Se in urine in humans but not in blood [162]. Blood concentration in this study did not reflect the exposure to increased Se intake [162].

More common than acute selenotoxicity is human chronic selenium toxicity which results in hair loss and brittle nails [163]. Exacerbated selenosis presents with skin lesions, gastrointestinal issues, nervous system disturbances and mottling of the teeth [161]. Abnormal endocrine function, reduced synthesis of thyroid and growth hormones, and reduced metabolism of insulin-like growth factor have also been reported. Effects on the immune system have also been documented with low natural killer cell production and hepatotoxicity [164]. Excess Se has also been postulated to inhibit protein synthesis and increase the risk of cancer by catalyzing hydrosulfide oxidation [164]. In China, nail morphology was used as an endpoint to calculate the no-observed adverse-effect level (NOAEL) for selenium [165]. The participants with nail

problems exhibited lower glutathione concentrations and slightly longer prothrombin times [165]. Yang et al calculated 853 µg Se/d as the NOAEL value [165] and this was used to set the UL of Se at 400 µg Se/d [163].

Fatal toxicity from supplements has also been reported. Helzlouer and colleagues reported 13 people who took dietary selenium with 27.3 mg Se/tablet, which was 182 times higher than indicated on the label [166].

McConnell and Portman reported in 1952 the median lethal dose for mice at 1.3 g of Se as dimethyl selenide per kg of body weight (1.8 g of dimethyl selenide total injected through intraperitoneal injection), and 1.6 g of Se per kg of body weight (2.2 g dimehtyl selenide) for rats [167]. In rats, 0.1 µg Se/g diet is the minimum dietary requirement, while intake over 2 µg Se/g diet produces toxicity [67] resulting in a 20 fold factor difference between the requirement and the onset of toxicity. Wilber reported the toxicity in rats of different selenium compounds injected via the intraperitoneal gland, and found sodium selenate to be 5.5 – 5.8 mg/kg body weight [168]. Raines and Sunde measured Se regulation of the liver transcriptome (all RNA in the cell) in mice and rats with three microarray experiments. The weanling mice and rats were fed Se-deficient diets supplemented with up to 5 µg Se/g diet. They found no toxicity effect in mice at 0.2 µg Se/g diet and in rats at 2.0 µg Se/g diet. Rats fed 5 µg Se/g diet showed 23% reduced growth when compared to Se-adequate rats, and significantly altered expression of over 1000 liver transcripts. High but non-toxic Se intake for both mice and rats (less than 2 µg Se/g diet) had fewer than 10 transcripts altered [169].

Effects of Selenium on Bone

Antioxidant protection can be observed from physiological doses of selenium. As an essential cofactor of glutathione peroxidase, selenium is important in the reduction of hydrogen peroxide: a product of oxidized species [170]. Dreher and colleagues showed antioxidative defense for human fetal osteoblasts that was mediated by expressed glutathione peroxidase. Their evidence suggested that glutathione peroxidase expression is essential for osteoblast function and

could be involved in metabolic bone diseases [171]. A model of heparin-induced osteoporosis in New Zealand white rabbits showed sodium selenite to restore structural alterations in femur when taken in combination with vitamin E and C [172]. Rats supplemented with selenium (0.15 mg/kg diet) showed less necrosis in the chondrocytes of the growth plates of tibia when compared to rats fed a diet from Kashin-Beck disease endemic areas. They also showed better bone volume/tissue volume ratio (BV/TV), trabecular thickness, and trabecular number, and reduced trabecular separation [173].

Impact of Selenium Deficiency on Bone Health

Selenium deficiency is associated with Kashin-Beck disease, a severe type of osteoarthritis that affects the bone and joints [174]. It is a degenerative, disabling endemic osteoarticular condition that affects the bone and joints of its sufferers, with a typical onset in the first or second decade of life.

Kashin-Beck disease (KBD) was first identified in 1849 by a Russian doctor, Nikolai Ivanonvich Kashin, but its cause is still unknown. In Tibet, the risk factors seem to include selenium deficiency in the soil [10], fungal contamination of barley (the staple grain) [175], organic matter (fluvic acid) in the water [10], and iodine deficiency [176]. Kashin-Beck disease has been reported in certain areas of Tibet, northern China, Mongolia, Siberia, and North Korea [10, 177]. Thirty million people are reported to live in areas of China where the disease is endemic, and about 2-3 million of this population are estimated to be affected [10].

Early symptoms of KBD in pre-adolescents and adolescents include stiffness, swelling, and pain in the interphalangeal joints of the finger; symptoms that are reported as been reversible [9]. Disease progression into the third decade of life presents with generalized osteoarthritis in the elbows, knees, and ankles, and with joint locking [9, 178]. Impaired bone development as a result of degeneration and necrosis of the bone's epiphyseal growth plate has been suggested by Ge and Yang [179]. While selenium deficiency is accepted as a cause of the disease, all selenium-deficient areas do not exhibit the disease, implicating other factors as necessary for full

development of true Kashin-Beck disease. Kashin-Beck disease has been suggested by Suetens and co-workers to result from oxidative damage to cartilage and bone cells when associated which decreased antioxidant defense [180].

Reactive oxygen species are produced during the process of bone resorption. Active osteoclasts produce superoxide, $NO^{\cdot}$, and H_2O_2. H_2O_2 is believed to be the principal stimulator of bone resorption. Modulation of H_2O_2 may be an important way by which bone metabolism is regulated [181]. It has been found that $NO^{\cdot}$ is produced by both the osteoblast and osteoclast and that it has major effects in producing osteoclast detachment and exerting a toxic inhibition of bone resorption [182]. Key and colleagues conducted a study that suggested that superoxide generated at the osteoclast-bone interface is involved in bone matrix degradation [183]. The researchers localized superoxide formed along the osteoclast-bone interface by demonstrating the electron-dense deformazan granules between the osteoclastic membrane and the bone surface. The formation of this reaction product was inhibited by a superoxide scavenger, the deferoxamine mesylate-manganese complex, confirming the specificity of the reaction product. The scavenger also inhibited bone resorption. High concentrations of superoxide generated in vitro at neutral pH degraded osteocalcin into numerous peptide fragments, demonstrating the ability of superoxide to break peptide bonds [183].

The deficiency of selenium played a role in the etiology of Kashin-Beck disease in selenium-deficient male Wistar rats [184]. The rats were fed the Se depleted diets for 3 – 11 months, after which they were killed. Their articular cartilages were studied both with light and electron microscope but no clear changes in the articular chondrocytes were observed from light microscopy. The electron microscope, however, showed degeneration in the deeper layers of the chondrocytes. Sasaki and colleagues also measured the bone mineral density (BMD) of femur using the microdensitometry method and ash weight. Rats in the Se-deficient group sacrificed from the 5th month onward had lower BMD (ash weight). Serum Se concentrations, alkaline phosphatase activity, and urine Se concentrations were decreased as well. [184]. Mice fed a Se

deficient diet and supplemented with fulvic acid had decreased cartilage in the knee joints and developed fibrocartilage at the articular surface, similar to early stages of osteoarthritis [185]. There was also underdevelopment of the articular space and meniscus, while the subchondral bone was poorly formed, and early differentiation was not seen during endochondral ossification [185]. Similarly, Yao and colleagues investigated the effects of supplemental selenium and selenium with iodine on bone and growth plate cartilage histology on 96 Wistar rats of both sexes that were randomly given either a control diet, a depleted diet, a selenium supplemented diet, or a diet with both iodine and selenium [153]. After 4, 8, and 12 weeks, rats were randomly sacrificed and the left knee including the distal femur and the proximal tibia was harvested and fixed in 4% (w/v) formaldehyde. The static parameters analyzed consisted of the bone volume/tissue volume ratio (BV/TV), the trabecular thickness (TbTh), the trabecular separation (TbSp), and the trabecular number (TbN). The rats on the depleted diet had comparatively reduced BV/TV, TbTh, and TbN while TbSp was increased [153].

Selenium deficient and fulvic acid supplemented mice, considered by Yang and colleagues [161] to be an animal model of Kashin Beck-disease, had irregular bone formation and substantial reduction in the number of lysine residues in type I collagen from bone and type II collagen from cartilage. A lower melting point of type I collagen from bone, and lower breaking force of bone were also found in the animals. In a study aiming to understand the role of selenium deficiency in the etiology of Kashin-Beck disease, Sasaki and colleagues [159] observed decreased femur ash weight, and a decrease in the sulfotranferase activity (involved in glucosaminoglycan synthesis) in 3 to 11 month selenium-deficient rats.

Suetens and colleagues [155] also suggested a second mechanism whereby normal stimulation of bone remodeling by thyroid hormones may be blocked by certain mycotoxins in the fungal contaminated grain. Chasseur and colleagues [149] did not observe a decrease in the prevalence of Kashin-Beck disease by iodine supplementation when a fungal species (*Alternaria sp.*) was present, and thus suggested a competitive binding of a mycotoxin to a thyroid hormone

receptor in bone cells. Fulvic acid, an environmental contaminant involved in the etiology of Kashin-Beck disease, has been shown to covalently bind with iodine [162], suggesting that fluvic acid may interfere with iodine bioavailability.

Ren and colleagues studied the role of combined selenium and iodine deficiency in bone development as a possible experimental model for Kashin-Beck osteoarthropathy in 48 Sprague-Dawley rats and showed that combined selenium and iodine deficiency impaired the growth of bone and cartilage [163]. The rats were randomly divided into four different diet groups: Selenium and iodine deficient, selenium sufficient and iodine deficient, iodine sufficient and selenium deficient, and selenium and iodine sufficient diets. They found that mean tibial length of rats fed the depleted diet was significantly shorter. The group that consumed the selenium sufficient and iodine deficient diet had smaller proliferative zones and thinner growth plate cartilage [163].

Role of Pro-Inflammatory Cytokines and Free Radicals in Bone Loss

Normal growth and development in young growing mammals (humans and rodents) depends on many factors including growth hormone (GH), thyroid hormones and nutritional status. The growth promoting effect of GH is believed to be mediated in part by insulin-like growth factor-1 (IGF-1) [164]. Growth hormone causes the liver (and to a lesser extent other tissues) to produce several small peptides called somatomedins (at least four somatomedins have been isolated), that in turn have potent effects of increasing all aspects of bone growth [165]. The most important of these is somatomedin C, also called IGF-1, which in turn regulates GH secretion through a feedback stimulation of somatostatin [164]. Both GH and IGF-1 influence bone growth and increase bone mineral content and bone mineral density [166]. The bioavailability and bioactivity of IGF-1 are modulated by IGF-binding protein-3 (IGFBP-3), a GH dependent glycoprotein and the main carrier for IGF-1 in blood [164].

Yanavski and coworkers [166] investigated bone status and the levels of IGF-1 and IGF binding protein in white and African American girls and found BMC, BMD and free IGF-1 to be

higher in African American than white American girls, while IGF-binding protein-3 was similar or lower in African American girls. In the study, free IGF-1 was positively correlated with BMC and BMD in both groups.

A study by Basu and colleagues examined the role of free radicals in bone resorption by looking at the effect of oxidative stress on bone mineral density (BMD) in 48 women and 53 men from a population-based study. The specific biomarkers they examined included 8-iso-$PGF_2\alpha$ (a major F_2-isoprostane and a biomarker of oxidative stress) and a control, 15-keto-dihydro-$PGF_2\alpha$ (a biomarker of inflammatory response) through analysis of urine samples and quantitative ultrasound (QUS) measurements. Through multivariate linear regression analyses, 8-iso-$PGF_2\alpha$ were negatively associated with bone BMD and QUS, while no association was found for 15-keto-dihydro-$PGF_2\alpha$. Their findings established a biochemical link between increased oxidative stress and reduced bone density [167].

Bone turnover is regulated by a balance between osteoblast (bone-forming) activity and osteoclast (bone-resporption) activity [5, 156] . Smith and colleagues reported the use of 90-day time-release lipopolysaccharide (LPS) pellets to study effects of inflammation on bone [168]. To develop an in vivo model of bone loss induced by chronic systemic inflammation, twenty-four 3-month-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) were randomly assigned to one of three groups: Low dose LPS (3.3 μg/day), high dose LPS (33.3 μg/day), or placebo. The rats were anesthetized with an intramuscular injection of a ketamine/acepromazine/atropine cocktail (35 mg, 1.5 mg, and 0.04 mg per kg body weight, respectively) for implantation of the pellet. Tail blood samples were collected at one month, two month, and three month points of the study period for a neutrophil count. At the end of the study, day 90, rats were anesthetized, DEXA scanned, and bled via cardiac puncture. To assess the alterations in local regulators of bone metabolism and inflammation-related proteins in the proximal metaphysis of the tibia, cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β expression were evaluated by immunohistochemistry. Neutrophil counts were elevated ($P< 0.05$) in the low

(971×10^3 cells/mL) and high (1049×10^3 cells/mL) dose LPS groups at 30 days compared to placebo (405×10^3 cells/mL). Neutrophils remained elevated in the High dose group at 60 (912×10^3 cells/mL) days and at the end of the study (860×10^3 cells/mL) indicating that inflammation was still present and the animals had not yet developed a tolerance at the tissue level. Immunohistochemistry revealed LPS induced a dose-dependent increase in the expression of COX-2 – the rate limiting enzyme in PGE_2 production – in the proximal tibial growth plate region and metaphysis. An increase was also seen in TNF-α and IL-1β ($P<0.05$) in the tibial metaphysis. In the epiphyseal region of the proximal tibia, the only inflammatory mediator to have this behavior was COX-2. TNF-α and IL-1β showed increase in this region of the tibia only in the high dose group. In general, osteoclast and monocyte-like cells had stronger intensity of staining for IL-1β and TNF-α in the proximal tibia metaphysis than in the epiphyseal plate. Bone loss was confirmed through DEXA and μCT analysis of excised femur and tibia [168].

Indicators of Bone Quality

The quality of bone is defined through measures of bone strength such as material properties (collagen and mineral), which are affected by turnover and structural properties (geometry and microarchitecture) [169]. Bone quality may be determined by several factors, including its properties that affect its strength. The geometry of bone consists of the size and shape of bone. The size of bone is a determinant of bone strength. Reduced bone mineral content and a smaller vertebral bone were seen in women with fractures of the spine [170]. Silva and Gibson developed a 2-dimensional model of human vertebral trabecular bone from four women (ages 47, 55, 85, and 86 years) and investigated its mechanical behavior using finite element analysis [170]. The structural arrangement of bone (microarchitecture) is also strongly related to bone strength [171]. Turan and colleagues studied the microarchitecture in rats fed diets with graded quantities of selenium and vitamin E. The stiffness (modulus of elasticity) of bones (femur and tibia) was measured by a tensile test, and biomechanical strength of both the deficient and excess groups were decreased when compared to control [171].

Collagen content and structure also affect bone quality. There is a reduced concentration of cross-links in bones from patients with osteoporosis [169]. Collagen has smaller influence on the stiffness of bone, but improves bone toughness through intramolecular cross-links [172]. Collagen fiber orientation explained 71% of variation in bone tensile strength in a linear regression analysis [173]. Bone is formed by the production of a protein framework that hardens when calcium and phosphorus are deposited on it. Bone strength partly depends on this mineral deposition [173]. Apart from bone mineral content, the perfection and the maturity of mineral crystals are also important determinants of bone strength [173].

Bone Mineral Density

Bone mineral density refers to the amount of minerals in a three-dimensional volume of bone. However, bone mineral density is also estimated by dual energy X-ray absorptiometry (DEXA) based on a two-dimensional area. There is a strong correlation between fracture risk and low bone mass. The WHO has developed diagnostic categories that compare a person's bone density with the peak value for a healthy young adult using a T-score [174]. A normal bone is indicated when bone mineral density or bone mineral content is within 1 standard deviation (SD) (+1 SD or -1 SD) of the young adult mean value. A low bone density (osteopenia) is indicated by a bone mineral density or bone mineral content of 1 to 2.5 SD below the young adult mean (-1 to -2.5 SD). Osteoporosis is defined by a bone density or bone mineral content of 2.5 SD or more below the young adult mean (>-2.5 SD). Severe osteoporosis is said to exist when bone mineral density or bone mineral content is more than 2.5 SD below the young adult mean and there have been one or more fractures due to osteoporosis [174].

Bone Microarchitecture

Bone mass is not the only property that affects bone strength. The microarchitecture of bone is also an important factor in strength, and is considered in measuring bone mechanical properties. Bone microarchitectural parameters of trabecular bone like trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp), connectivity density, and parameters

of cortical bone such as width and porosity indicate bone fragility independent of bone density [175]. An aged model of human vertebral trabecular bone was developed by Silva and Gibson by concurrently reducing the trabecular thickness and trabecular number of a young model with intact values. Trabecular number and thickness decrease in aging. Increase of trabecular thickness alone in the model saw bone mass rebounding as strength increased by 60%, but this was only 37% of its original value. Therefore, increase of trabecular number seems to be essential for full recovery of bone loss [170].

The structural model index (SMI) is a 3-D bone structural parameter that quantifies the plate versus rod characteristics of trabecular bone [176, 177]. An SMI of zero (0) pertains to a purely plate-shaped bone, and a value of 3 indicates a purely cylindrical rod-like structure, and values between designate mixtures of plate and rod forms [177]. Human tibial cancellous bone changes with aging from plate-like to rod-like, indicating a deterioration of the structure of bone with aging [176].

Microarchitectural deterioration such as decreased trabecular connectivity has been related to increased possibility of fracture and one of the positive effects of parathyroid hormone (PTH) on bone is the restoration of moderate lost trabecular connectivity [178]. Even though connectivity is believed to be important in the biomechanics of bone in osteoporosis [178], there is not much evidence to support this hypothesis in healthy bone. Kabel and coworkers observed an inverse association of connectivity with bone stiffness [179]. Connectivity seems to be inversely associated with elastic properties of cancellous bone of people with no known bone disorders.

Degree of anisotropy (DA) refers to the extent to which a material has different properties in different directions [180, 181]. Poor bones seem to have higher DA values. An analysis of porous hydroxyapatites with an anisotropic characteristic (higher DA) intended for the bone-graft market found the specimens to possess lower compressive moduli than isotropic specimens with the same apparent densities [182]. Similarily, Chappard and colleagues [181] found higher DA

values in the bone of subjects with vertebral fracture than in control subjects. Furthermore, an improvement in the structural properties of the vertebra (L_1 and L_2) of dogs following alendronate treatment was accompanied by a decreased degree of anisotropy in the bone specimens [183].

Bone Biomechanical Properties

Biomechanical properties of bone are those properties of bone that are associated with elastic and inelastic reactions when a force is applied. They also involve the relationship between stress and strain [184]. Biomechanical properties range from elastic abilities to stress responses of bone, and include stiffness, hardness, strain, fatigue life (fracture of bone under repetitive stress), and strength [185]. Bone strength depends on bone matrix volume, bone microarchitecture and the degree of mineralization of bone [186]. The more cancellous bone is mineralized, the higher its stiffness. Young human bone is less mineralized than mature bone [186]. Ciarelli and colleagues suggest that both low and high mineralization may be deterimental to bone mechanical properties, with low mineralization level causing reduced stiffness and strength and high mineralization leading to reduced fracture toughness due to increased brittleness [187].

Bone mechanical properties can be determined using three or four point bending techniques and fatigue tests for long bones [180, 185, 188]. The compressive tests are more appropriate for small and cubic samples or trabecular bone [186].

There is not much information about the effects of iodine and selenium on the biomechanical properties of bone in growing individuals. However, retarded growth and lower breaking force of the tibia have been observed in selenium-depleted mice compared to controls [161]. Growth retardation and osteopenia were seen in second generation selenium-deficient male rats [189]. Methamizol-induced hypothyroidism during postnatal development leads to decreased bone length and biomechanical competence (measured as Vickers microhardness) of the femora and humeri in birds [190].

<u>Biomarkers of Bone Metabolism</u>

Bone density determination is valuable for evaluation of patients at risk for osteoporosis, but it does not give any information about the rate of bone turnover, therefore, supplementing bone density information with measurement of markers of bone turnover may enhance the prediction of fracture risk. Bone markers indirectly measure bone cell activities [177]. Biochemical markers of bone metabolism are byproducts that are released into the blood stream and urine during the process of bone remodeling, which involves bone resorption and bone formation [191].

Serum and urine tests can detect these markers and provide information about the rate of bone resorption and formation. Bone formation can be evaluated using serum non-specific alkaline phosphatase (ALP), bone-specific alkaline phosphatase (B-ALP), osteocalcin, carboxyterminal propeptide of type I collagen (PICP), and aminoterminal propeptide of type I collagen (PINP) [191]. Indicators of bone resorption such as cross-linked C-telopeptide of type I collage, tartrate resistant acid phosphatase (TRAP), N-telopeptide of collagen cross-links (NTx), and C-telopeptide of collagen cross-links (CTx) can be determined in serum. Other bone resorption markers such as hydroxyproline, free and total pyridinoline, free and total deoxipyridinoline as well as NTx and CTx can be assessed in urine [191].

Bone specific alkaline phosphatase is an osteoblast product that is believed to be an essential enzyme for bone mineralization [191]. Both bone specific and tissue non-specific alkaline phosphatase can promote mineralization by hydrolyzing a variety of phosphate compounds to make inorganic phosphate available for bone mineralization [192]. It has been suggested that alkaline phosphatase may destroy inhibitors of mineral crystal growth and behave like a calcium binding protein [193].

Osteocalcin (bone gla-protein) is a peptide synthesized and secreted by osteoblasts during bone formation. It is mostly incorporated into bone matrix with some escaping into the blood; therefore, osteocalcin is accepted as a marker of bone formation. However, osteocalcin is also

released from bone to the circulation during bone resorption. Therefore, osteocalcin is more a marker of bone turnover than of bone formation [191].

Aminoterminal and carboxyterminal propeptide of type I collagen direct the assembly of the collagen triple helix and are separated from the newly formed collagen molecules and released into circulation [191]. Therefore, their concentration in serum may be an index of bone formation. However, these byproducts of collagen synthases are also produced by other type I collagen and are less useful than alkaline phosphatase (ALP) and osteocalcin (OC) as indicators of bone formation [191].

TRAP (tartrate resistant acid phosphatase, also known as type-5 acid phosphatase) is an iron-containing protein produced in different tissues with acid phosphatase activity and is one of the most abundant enzyme in osteoclasts [194]. Serum TRAP is used as a biochemical marker of osteoclastic activity and bone resorption [195]. However, it lacks specificity because other cells that are not related to bone such as erythrocytes and platelets also release TRAP into serum [195].

NTx and CTx are degradation products of type I collagen, mainly produced by cathepsin K. Pyridinoline, deoxypyridinoline, and cross-linked C-telopeptide of type I collagen (ICTP) are also degradation products produced by matrix metalloproteases [196]. Pyridinoline and deoxypyridinoline are the two cross-links present in the mature form of type I collagen. Urine levels of pyridinoline and deoxypyridinoline correlate with the breakdown of collagen released from bone matrix by the osteoclasts [197]. This cross-linking structure, which is unique to collagen and elastin molecules, creates bonds between polypepetide chains in collagen fibrils to enhance stability. Pyridinoline and deoxypyridinoline cross-links can be excreted free or still bound to the peptide chains and either form can be measured. Deoxpyridinoline is the more abundant cross-link in bone collagen and is generally the one measured [197].

Studies on the Effects of Selenium on Bone

Selenium is required for T_3 synthesis and thyroid hormone homeostasis [198]. Therefore it may indirectly protect bone through thyroid hormones. Physiological doses of selenium may

also directly protect bone through its antioxidative properties. Selenium is an essential component of the enzyme glutathione peroxidase as well as thioredoxin reductases as the active center of which selenium catalyzes reduction of hydroperoxides produced from oxidized species such as superoxide and lipoperoxides [143]. Thus, it may protect bone and cartilage cells against oxidative damage [144]. Dreher and colleagues [144] demonstrated a selenite-dependent Gpx mediated antioxidative defense of fetal human osteoblasts against hydrogen peroxide and reactive oxygen species. In the study it was shown that osteoblasts express an antioxidative system to protect themselves against H_2O_2 after bone resorption is mediated by osteoclasts. Therefore, lack of Gpx may lead to impaired osteoblast function and could be involved in metabolic bone disease.

Selenium in Cell Culture Studies

Using a cell culture model, Lean and colleagues suggest that estrogen provides a protective effect for bone through the lowering of reactive oxygen species (ROS) concentrations via better antioxidant activity. High concentrations of ROS not only damage cell constituents, but could also affect signaling proteins like TNF-α and NF-kβ, which, at appropriate levels, are essential for osteoclast development. Cytokines such as IL-1 upregulate their own activity by inducing the production of oxidants that may inhibit cytosolic enzymes. Selenium dependent glutathione peroxidases and thioredoxin reductases help keep cytosolic enzymes in their reduced form, therefore adequate selenium nutrition may protect bone by down-regulating cytokine signaling [199].

Selenium in Epidemiological Studies

An epidemiological study on patients with rheumatoid arthritis (RA) showed healthy controls having significantly (<0.001) higher plasma selenium concentrations than the patients [200]. Likewise, Kamanli and colleagues found lower concentrations of plasma Gpx, catalase, glutathione, β-carotene, and vitamin E in patients with RA as compared with the controls, In the same study, significantly higher concentrations of C-reactive protein, lipid peroxidation markers, and rheumatoid factor were found in patients with RA than in controls [201]. A similar study on

children and juveniles with RA conducted by Araujo et coworkers reported oxidant/anti-oxidant levels imbalanced where levels of lipid peroxidation products were elevated in the synovial fluid, while plasma antioxidant levels dropped [202]. In the study by Arajuo and colleagues, the extent of lipid peroxidation was estimated by measurement of peripheral plasma lipid hydro-peroxides that are the major initial molecular products of lipid peroxidation, and thiobarbituric acid reactive substances (TBARS), mostly malondialdehyde (MDA), a secondary product of lipoperoxidation [202].

Selenium Studies in Animal Models

Ren and colleagues investigated the roles of combined selenium and iodine deficiency in bone development as a model of Kashin-Beck osteoarthropathy [163]. They randomly divided Sprague-Dawley rats (n = 48) into selenium deficient diet (-SE+I), iodine deficient diet (+Se-I), combined selenium and iodine deficienct (-Se-I) diet, and selenium and iodine sufficient diet (+Se+I) groups. Within two generations of rats (F_0 and F_1), they measured the growth of bone and cartilage, and the expression of type X collagen and parathyroid hormone-related peptide. They found that tibial length in –Se-I rats was significantly shorter in F_1 generation rats. In +Se-I fed F_1 rats, the thickness of the growth plate cartilage, and the proliferative zone was smaller, while in –Se–I rats the growth plate, and the proliferative and hypertrophic zones were also thinner in the F_1 generation. In articular cartilage, type X collagen expression was increased in the deep zone in –Se–I rats of the F_0 generation, and in –Se+I, +Se–I and –Se–I rats of the F_1 generation. Parathyroid hormone-related peptide expression was increased in the middle zone of –Se+I, +Se–I and –Se–I rats of both F_0 and F_1 generations. In the growth plate cartilage, type X collagen and parathyroid hormone-related peptide were expressed in the hypertrophic zone. Type X collagen expression was significantly reduced in –Se+I and –Se–I rats in both F_0 and F_1 generations, while parathyroid hormone-related peptide expression was stronger in –Se+I, +Se–I and –Se–I rats in both F_0 and F_1 animals. The researchers concluded that combined selenium and iodine deficiency impaired the growth of bone and cartilage. The changes in the expression of

type X collagen and parathyroid hormone-related peptide induced by combined selenium and iodine deficiency were comparable to measurements of type X collagen and parathyroid hormone-related peptide in Kashin-Beck osteoarthropathy [163].

Turan and colleagues conducted a study to investigate the effects of dietary selenium on the biomechanical properties of bone noting the correlation between catalytic activity of selenium compounds and toxicity in past literature. Newborn Wistar rats of both sexes were fed for 12-14 weeks with either a control diet (225 µg Se/kg), or selenium (9.8 µg Se/kg diet) and vitamin E deficient, or a selenium-excess (4.2 mg Se/kg) diet and vitamin E –adequate diet. The animals were housed individually in wire-bottomed cages and given deionized water with negligible amounts of Se (< 1 µg/L). All groups were evaluated for the stiffness (modulus of elasticity) of the femur and tibia by tensile test. The tensile strength of the bones was machine tested after the proximal and distal ends were fixed with adhesive. The tensile tests were performed on all specimens with a constant speed of 2 mm/min and the loading was of a displacement-controlled type using a 500-N load cell. The force versus deflection curves obtained from the tests were transformed into stress versus strain diagrams. Finally, the modulus of elasticity of each bone was calculated from the slope of the linear region of the stress-strain curve. The researchers found that, compared to the control, the deficient and the excess groups had decreased biomechanical strength. To support the biomechanical results for both experimental groups, X-ray diffraction analysis and a Fourier transform infrared spectroscopy (FTIR) study were performed on the femurs and tibias. The X-ray diffraction test on the femora and the tibia of both experimental groups showed possible alterations in crystallinity or a poor crystalline substance. The FTIR spectra of femora and tibiae from both experimental groups show a decrease in intensity of carbonate bands in the spectral region of 2900-3000 cm^{-1}, and at 1750 cm^{-1} with respect to those of the controls. The researchers concluded that the bones of both the excess and deficient group showed a decrease in crystallinity which was more profound for the excess group

in both tibia and femur. Both the X-ray diffraction and FTIR analyses correlated very well with the biomechanical data [171].

Moreno-Reyes and colleagues investigated whether growth inhibition cause by selenium deficiency in rats is associated with changes in bone metabolism. Female Wistar rats on a selenium-deficient diet (0.005 mg selenium/kg) were mated with selenium-adequate male rats in house and housed individually after pregnancy was confirmed. The pregnant dams were continuously fed the deficient diet through delivery and weaning. Male pups (n = 24) were weaned at 21 days of age and remained on the diet until the end of the experiment (day 74). Control male mice were obtained in the same way but the parental generation was fed diets supplemented with 0.19 mg of selenium/kg. Control and experimental mice were pair-fed. Rats were sacrificed on day 74 and femurs and tibias were collected while pituitaries were dissected and frozen.

To determine selenium status, plasma selenium was measured by atomic absorption spectrometry using the Zeeman background correction. The researchers found reduced plasma selenium concentration in rats fed low selenium diet. Rats fed low selenium diet also had reduced glutathione peroxidase activity by 99% (3 ± 0.3 U/mg protein vs. 574 ± 27 U/mg protein; $p < 0.001$). To determine growth parameters, tail length and body weight of F_1 generation weaning male rats were measured once a week starting on day 21 until the end of experiment. Body weight and tail length were significantly lower in the selenium-deficient rats two weeks after weaning, and the difference increased with age. At the end of the experiment, body weight was reduced by 31% and tail length was reduced by 13% in the selenium-deficient rats. The lengths of the dissected bones were also significantly reduced.

Concentrations of plasma proteins and albumin were slightly lower in selenium-deficient animals ($p = 0.02$). Plasma thyroid hormones and alkaline phosphatase were not significantly different between groups, but there was a trend ($p = 0.06$) toward lower T_3 and lower alkaline phosphatase in the selenium-deficient rats. Furthermore, the researchers found a 68% reduction

in pituitary GH concentrations and a 50% reduction in insulin-like growth factor I (IGF-I) in selenium-depleted rats. Several markers were used by Moreno-Reyes and co-workers to gauge bone metabolism. Plasma calcium concentration was lower in selenium-deficient rats while urinary calcium concentration from 24-hour collections was 2-fold greater compared to controls. Plasma and urinary phosphate did not show significant differences between groups. Plasma IGF-I was significantly correlated with plasma calcium in the selenium-deficient group ($p = 0.025$). Although plasma 25(OH)D_3 concentrations were not different in both groups, selenium-deficient rats showed a 25% reduction in plasma ostecalcin concentrations and 57% reduction in urinary deoxypyridinoline concentrations. Dual-energy X-ray absorptiometry was used to measure the BMC and BMD of the distal end of the femur and the proximal end of the tibia. Morphometric measurements of left femur were taken by slicing longitudinally in the coronal plane of the distal end. Trabecular bone volume and surface were measured on a SAMBA 2005 image analyzer in the metaphysis of the left femur. Femur and tibia BMC and BMD were significantly reduced by the selenium-depleted diet and remaining significantly lower even after controlling for body weight in a multiple regression analysis. Trabecular bone volume, trabecular surfaces, and diameter were also reduced in the selenium-deficient rats. The osteoblast number was increased by 3-fold and the osteoclast number was increased by 2-fold. The trabecular bone architecture was clearly deteriorated in selenium-deficient rats with fewer and thinner trabeculae. No specific measures of oxidative stress or lipid peroxidation were included [189].

CHAPTER III

METHODOLOGY

This section recounts the experimental design of the study including the diet given to mice, and the methods of assessing Se status and bone quality.

Animal Experiment and Study Design

This study has a 4 x 2 factorial design (four diet groups with an LPS group and a placebo group) and randomization occurred as shown in **Fig 3.1**. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University (OSU). Second generation selenium-deficient animals were used to demonstrate the effects of Se in a relatively short time.

Animal Feeding and Handling

Forty-one timed-pregnant C57BL6 mice (Harlan, Indianapolis, IN) (60-80g) were fed a commercially purchased Torula yeast selenium-deficient diet (modified AIN-93G; Teklad Diets, Harlan Laboratories, Madison, WI) for the last 5 to 7 days of pregnancy and through lactation. Animals were housed in an environmentally controlled animal care facility and delivered their litters approximately 5-6 days after arrival. Male pups were weaned at 23 or 24 days of age and randomly assigned to the depletion diet or to diets supplemented with 0.2, 2 or 4 mg/kg diet of Se, added as sodium selenate, for 14 weeks. Feeding occurred on a daily basis *ad libitum* at about 5 g diet/day/mouse. Clean water was also provided every 2-3 days and bedding was changed weekly.

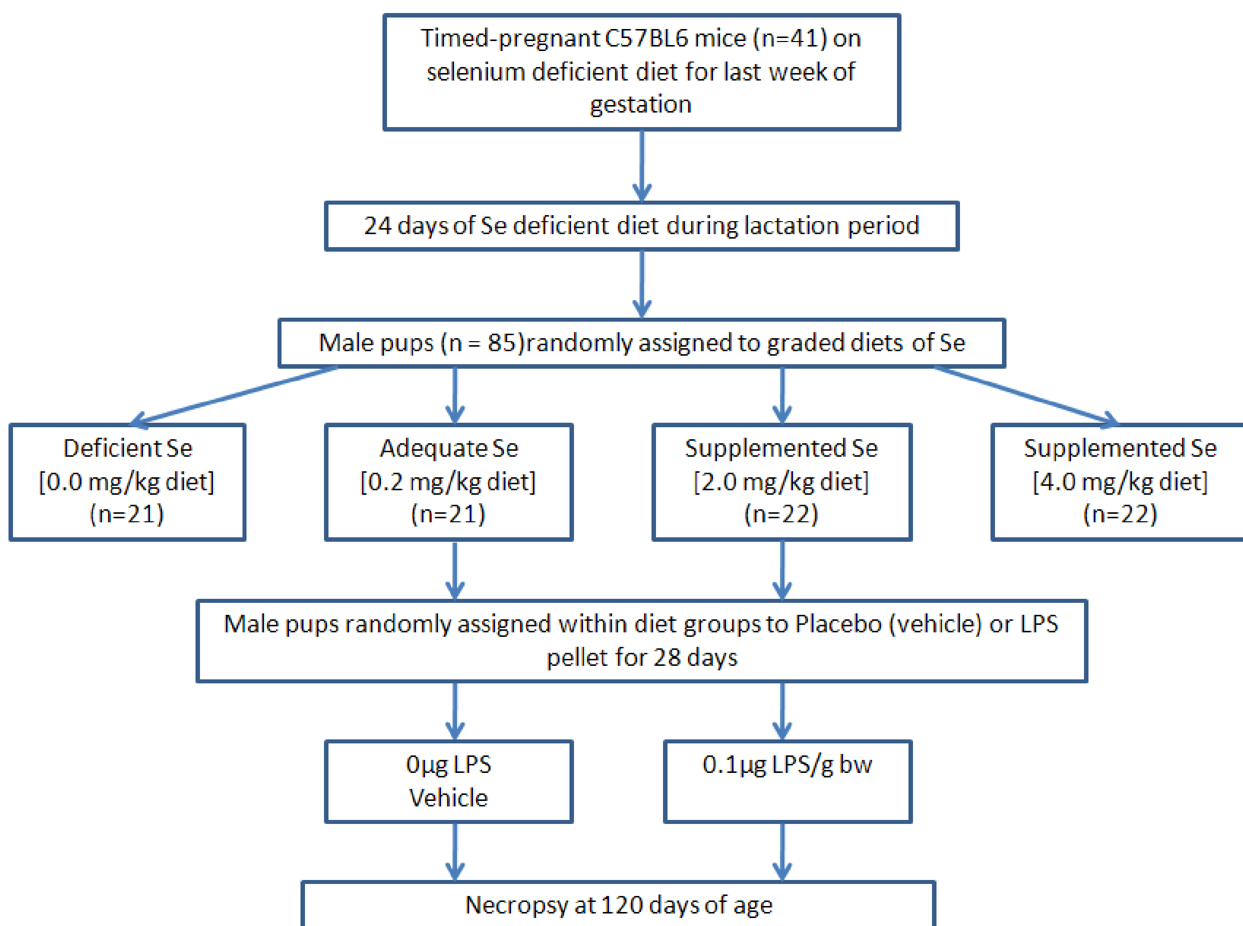


Figure 3.1: Study design randomization flowchart

Preparation of the Experimental Diets

The experimental diets followed a modification of the recommendations of the American Institute of Nutrition (AIN-93) for growing rodents and were isocaloric and isonitrogenous [1]. Minerals and vitamins were equivalent for the four different diets, except for selenium, which was added or omitted according to the experimental design. The basal Se depletion diet (Torula yeast-based, approximately 0.02mg Se/kg diet) was produced commercially, while the supplemented diets were prepared either commercially or in-house from a basal mix at the Nutritional Sciences laboratory at Oklahoma State University in 5 kg batches in a commercial mixer. Selenium was added in the form of sodium selenate at 0.2, 2.0 or 4.0 mg Se/kg. Diets

were refrigerated until being partitioned for feeding. The composition of the experimental diets is presented in **Table 3.1**.

Table 3.1. Composition of the experimental diets

	Added Se (mg/kg diet)			
Ingredients (g/kg)	**0 Se**	**0.2 Se**	**2.0 Se**	**4.0 Se**
Torula yeast	340	340	340	340
L-cysteine	3	3	3	3
Dextrose, monohydrate	399.02	399.02	399.02	399.02
Sucrose	100	100	100	100
Soybean oil	60	60	60	60
Cellulose	50	50	50	50
Mineral mix	35	35	35	35
Vitamin mix AIN-93-VX	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5

Chronic Inflammation

At 96-98 days of age mice were randomly assigned within diet groups to placebo or to lipopolysaccharide (LPS) (*E. coli* Serotype 0127:B6) treatment to produce inflammatory stress. Time release pellets (0 or 0.1 µg/g body weight/d) were implanted subcutaneously after anesthetization with intraperitoneal injections mixed ketamine (10 mg/mL) and xylazine (1 mg/mL) at a concentration of 0.006 mL/10 g body weight. Body weight was taken on the day of implantation as well as at 14-days after implantation.

Necropsy of Pups

All necessary surgical instruments were autoclaved and preparations for tissue harvests and collection of blood were organized before the day of necropsy. The day before the necropsy, the pups were fasted overnight for 12 hours and their body weight was recorded. On the day of necropsy, each mouse was anesthetized with injections of mixed ketamine (10 mg/mL) and xylazine (1 mg/mL) at a concentration of 0.006 mL/10 g body weight. PIXImus whole body scans were taken. Blood was drawn with syringes pre-coated with EDTA from the carotid artery before dissection for harvesting of organs and tissues. The blood was held on ice for up to 2-3

hours until the end of necropsy. Plasma was obtained from whole blood by centrifugation at 4,000 rpm (Eppendorf #5415R) for 20 min and stored at -80°C. Liver was placed in liquid nitrogen and stored at -20°C. The right tibia and spine were excised and stored at -20°C.

Determination of Weight Change, Organ Weight, and Body Lean and Fat Mass

Weight fluctuations were assessed by calculating weight data gathered during the 30 days of LPS treatment. Harvested organs were weighed immediately before storage. Body lean mass and fat weights were assessed using PIXImus.

Laboratory Analyses

Plasma Gpx

Se status was measured by plasma glutathione peroxidase 3 activity using a kinetic enzyme assay (Product No. FR17, Oxford Biomedical Research, Inc. Oxford, MI). The assay was conducted at room temperature (20 - 25°C) and spectrophotometric readings were done at 340 nm. The spectrophotometer was zeroed at 340 nm with deionized water. The plasma samples from mice fed 0.2, 2 and 4 mg/kg diet were diluted 1:10 using assay buffer provided in the kit. The plasma samples from mice fed the selenium-depleted diet were not diluted with assay buffer. An appropriate volume of assay buffer, pre-diluted NADH reagent and sample were pipetted into the cuvette and placed in the spectrophotometer followed by addition of tert-butyl hydroperoxide and mixed by pipetting. The GPX coupled reduction of tert-butyl hydroperoxide from the oxidation of NADPH by glutathione reductase and concomitant oxidation was monitored for three minutes in a spectrophotometer by the decrease in absorbance at 340 nm. In each reading, the rate of decrease in A340/minute was calculated and the net rate for the sample was calculated by subtracting the rate from the water blank. The net A340/min for each sample was then converted to NADPH consumed. One unit of GPX is expressed as the amount of GPX needed to oxidize 1 μmol of NADPH per min. The value for each sample was corrected for dilution factors and expressed as GPX μM/mL.

Bone Analyses

Tibia and vertebral columns were stored at -20°C. Individual bones were thawed for 15 min at room temperature before analysis.

Bone Measurement by Microcomputed Tomography (µCT)

After bones were properly thawed and defleshed they were placed in 12 mm or 16 mm µCT tubes with either water or 10% ethanol and pre-scanned in the µCT-40 (Scanco Medical AG, Zurich, Switzerland) to confirm repositioning. The microarchitecture of the trabeculae of vertebra (L_4) and proximal tibia were scanned using microcomputed tomography (µCT). The vertebral body was scanned for a region VOI distally from the proximal growth plate at medium resolution (16 µm per slice), while the proximal tibial was scanned distally at high resolution for a minimum of 150 slices at 12 µm per slice. For cortical bone analysis, the midshaft region calculated precisely at the middle of the tibia was scanned at 12 µm per slice for a minimum of 45 slices.

Bone Densitometry by PIXImus

Two-dimensional analysis of tibial and L_4 bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) were assessed by the PIXImus (Lunar Corp. Madison, WI). BMC is an estimate of the amount of mineral present in the bone, BMA estimates the two-dimensional area occupied by bone, and BMD is equal to BMC divided by BMA.

Bone Structure Analysis

The microarchitecture of the trabeculae of the L_4 vertebra, and the proximal tibia were analyzed using µCT. Contours were placed on L_4 vertebrae to identify a VOI beginning at 10 slices (16 µm per slice) away from the growth plate in order to include in the VOI only the secondary spongiosa within the two growth plates. µCT analysis for vertebra was performed at a threshold of 315, a sigma of 0.7, and a gauss support of 1.0.

Contours were placed on a total of 100 consecutive tomographic slices (12 µm per slice) of tibia beginning at 10 slices from the growth plate (sigma = 0.7, threshold = 360, gauss support

= 1.0). Contours were also placed on 30 slices scanned from the midshaft region of the tibia to analyze cortical bone volume, thickness, and porosity.

Bone morphometric parameters including cortical porosity, trabecular bone relative volume (BV/TV), trabecular number (TbN), trabecular separation (TbSp), and trabecular thickness (TbTh), as well as the structural model index (SMI), connectivity density (ConnD) of the trabeculae, and the degree of anisotropy (DA) were obtained for L_4 and tibia.

Bone Biomechanical Tests

Bone Strength Simulation Using Finite Element Analysis by Micro-CT

The finite element analysis (FEA) simulates compression of a region of interest (ROI) of bone to determine the response to pressure. The FEA simulation software (v. 1.16), is used in combination with the micro-computed tomography (μCT) histomorphometric data. Biomechanical properties of tibia and the L_4 vertebra were modeled using a VOI composed of trabecular bone, which was subjected to a high friction compression test in the z direction. This allowed determination of mechanical properties such as average strain, total force and physiological force, stiffness, size independent stiffness, and von Mises stress of the trabecular cores.

Liver Ash Weight and Mineral Content Using Inductively Coupled Plasma-mass Spectroscopy

Liver samples were ashed following a modified protocol from Hill and colleague [2]. Liver tissue was ashed in duplicate to determine the Se content with inductively coupled plasma mass spectrometry (ICP-MS). Excised liver previously stored at -80°C was kept on ice to remove approximately 0.1 g of tissue. The liver sample was oven dried overnight at 100°C in acid-washed, glass borosilicate tubes. Dry weights of the samples were taken, and acid digestion was conducted with 1 part concentrated nitric acid (HNO_3) and 5-6 parts hydrogen peroxide H_2O_2 added every 30-60 minutes while the samples were being heated continuously at 95°C. Acid

digestion continued until the tissue samples turned a solid white. Se concentrations were determined in triplicate using ICP-MS (ELAN 9000, PerkinElmer SCIEX, Waltham, MA).

Statistical Analyses

Data were analyzed using SAS (Statistical Analysis System) version 9.3 (SAS Institute Inc., Cary, NC). Two-way ANOVA was performed using PROC GLM followed by post hoc analysis with Fisher's least significant differences test for means separation when F values were significant. Data are represented as means ± SEM and $p < 0.05$ was considered significant. When interaction terms were significant, data are presented graphically.

CHAPTER IV

RESULTS AND DISCUSSION

This section recounts the results of the study and discusses the implications of the results with respect to the existing literature.

Results

Body Weight, inflammation, and diet indicator

Body Weight and Bone Density

There were no statistically significant differences in body weight by dietary Se intake at d 120 ($p > 0.05$, **Table 4.1**). Some weight fluctuations were seen in both the placebo and LPS group at 14 days after LPS implantation ($p = 0.002$), but by necropsy (d 120) the weight differences were no longer significant but showed a trend for lower weight in the LPS group ($p = 0.07$). This indicates that LPS treatment and/or Se deficiency and supplementation did markedly affect growth of the mice. BMD and BMC measurements were reduced by LSP treatment when compared to control ($p = 0.02$). Mice in the Se adequate diet had lower BMD ($p = 0.004$) and BMC ($p < 0.0001$) compared to mice in the Se deficient and supplemented groups.

Organs Weights

Kidney and thymus weight showed no significant differences by dietary Se intake or by LPS at 120 days of age at necropsy ($p > 0.05$, Table 4.2). Thymus weight, however, tended to be higher for LPS (0.050 g) compared to placebo group (0.043 g) ($p = 0.06$). Liver weight was significantly affected by diet ($p = 0.04$) with mice in the 4.0 mg Se/kg diet group having the smallest liver indicating a possible toxicity effect.

Table 4.1 Body weight and bone density of mice fed supplemental Se with and without LPS (mean ±SEM)

Treatment means							
Added Se (mg/kg diet)	**Pellet**	**n**	**Body Weight at LPS(g)**	**Body Weight at 14d post LPS (g)**	**Body Weight at necropsy (g)**	**BMD (g/cm^2)**	**BMC (g/cm^2)**
0	placebo	10	25.2 ± 0.3	25.6 ± 0.2	25.7 ± 0.2	0.052 ± 0.001	0.58 ± 0.01
	LPS	10	25.0 ± 0.6	24.7 ± 0.4	24.1 ± 0.5	0.050 ± 0.001	0.55 ± 0.02
0.2	placebo	11	25.5 ± 0.4	25.7 ± 0.5	25.5 ± 0.5	0.049 ± 0.000	0.53 ± 0.01
	LPS	10	24.5 ± 0.3	24.2 ± 0.2	25.1 ± 0.2	0.047 ± 0.000	0.50 ± 0.01
2	placebo	11	25.8 ± 0.5	26.2 ± 0.5	25.7 ± 0.6	0.050 ± 0.001	0.55 ± 0.02
	LPS	10	26.3 ± 0.6	25.5 ± 0.6	26.0 ± 0.7	0.050 ± 0.001	0.55 ± 0.02
4	placebo	10	25.8 ± 0.4	25.9 ± 0.5	25.2 ± 0.4	0.050 ± 0.001	0.56 ± 0.01
	LPS	10	25.4 ± 0.4	25.1 ± 0.3	24.8 ± 0.4	0.050 ± 0.001	0.57 ± 0.02
Diet means							
Added Se (mg/kg diet)	**N**		**Body Weight at LPS(g)**	**Body Weight at 14d post LPS (g)**	**Body Weight at necropsy (g)**	**BMD (g/cm^2)**	**BMC (g/cm^2)**
0	20		25.1 ± 0.3	25.1 ± 0.2	24.9 ± 0.3	0.051 ± 0.001[a]	0.56 ± 0.01[a]
0.2	21		25.0 ± 0.3	25.0 ± 0.3	25.5 ± 0.3	0.048 ± 0.000[b]	0.51 ± 0.01[b]
2	21		26.0 ± 0.4	25.9 ± 0.4	25.9 ± 0.4	0.049 ± 0.001[a]	0.55 ± 0.01[a]
4	20		25.6 ± 0.3	25.5 ± 0.3	25.0 ± 0.3	0.050 ± 0.001[a]	0.56 ± 0.01[a]
LPS means							
Placebo	42		25.6 ± 0.2	25.9 ± 0.2[a]	25.6 ± 0.2	0.051 ± 0.001[a]	0.55 ± 0.01[a]
LPS	40		25.3 ± 0.3	24.9 ± 0.2[b]	25.0 ± 0.3	0.049 ± 0.001[b]	0.54 ± 0.01[b]
***p*-values**							
Se			0.09	0.17	0.16	0.004	<0.0001
LPS			0.39	0.002	0.07	0.02	0.02
Se x LPS			0.48	0.80	0.27	0.55	0.33

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

Table 4.2: Selected organ weights of mice fed supplemental Se with and without LPS (mean ±SEM)

Treatment means							
Added Se (mg/kg diet)	**Pellet**	**n**	**Liver (g)**	**n**	**Kidney (g)**	**n**	**Thymus (g)**
0	placebo	9	1.31 ± 0.02	10	0.17 ± 0.00	10	0.04 ± 0.00
	LPS	10	1.36 ± 0.02	10	0.17 ± 0.00	10	0.05 ± 0.00
0.2	placebo	11	1.39 ± 0.05	11	0.16 ± 0.00	11	0.04 ± 0.00
	LPS	10	1.39 ± 0.03	10	0.16 ± 0.00	10	0.05 ± 0.00
2	placebo	11	1.30 ± 0.04	11	0.17 ± 0.01	11	0.05 ± 0.00
	LPS	9	1.38 ± 0.04	9	0.16 ± 0.01	10	0.05 ± 0.00
4	placebo	10	1.30 ± 0.04	9	0.17 ± 0.01	10	0.05 ± 0.00
	LPS	11	1.32 ± 0.05	11	0.17 ± 0.01	11	0.05 ± 0.00
Diet means							
Added Se (mg/kg diet)	**N**		**Liver (g)**	**n**	**Kidney (g)**	**n**	**Thymus (g)**
0	19		1.35 ± 0.00[a]	20	0.17 ± 0.00	20	0.05 ± 0.00
0.2	21		1.39 ± 0.03[a]	21	0.16 ± 0.00	21	0.05 ± 0.00
2	20		1.33 ± 0.03[ab]	20	0.17 ± 0.00	21	0.05 ± 0.00
4	21		1.27 ± 0.03[b]	20	0.17 ± 0.00	21	0.05 ± 0.00
LPS means							
Placebo	41		1.31 ± 0.02	41	0.17 ± 0.00	42	0.04 ± 0.00
LPS	40		1.36 ± 0.20	40	0.16 ± 0.00	41	0.05 ± 0.00
***p*-values**							
Se			0.04		0.17		0.89
LPS			0.12		0.22		0.06
Se x LPS			0.55		0.87		0.40

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

Plasma Se and Gpx3 Activity

Plasma Se of mice from different experimental diets at necropsy showed significant differences by diets supplemented with Se (0.0; 0.2; 2.0 and 4.0 mg Se/kg diet) (p = 0.02) **(Table 4.3)**. Mice fed the Torula yeast Se-deficient diet with no added Se had significantly lower plasma Se compared with other groups **(Table 4.3)**. However, plasma Se was not significantly affected by LPS.

When mice in different dietary Se groups were compared, the group that consumed no added dietary Se showed significantly lower plasma Gpx activity than the other three dietary Se groups containing 0.2, 2.0 and 4.0 mg Se /kg of diet ($p < 0.0001$, Table 4.2). The diet with 0.2 mg Se added/kg diet represented the control diet and additional selenium supplementation did not increase Gpx activity. Also, LPS had no significant effect of Gpx activity

Liver Se content

To examine the concentrations of tissue Se in mice fed different dietary groups, ~0.1 g of wet liver was ashed and measured for Se content by ICP-MS. The Se content of liver **(Table 4.4)** in Se deficient mice was significantly lower than that of other dietary groups (p <0.0001). The Se content in two experimental diet groups (2.0 mg Se/kg and 4.0 mg Se/kg) are very similar, while the Se content of mice in the Se-adequate diet group (0.2 mg Se/kg) was < 40% less than the two Se supplemented groups though this repression did not reach the level of statistical significance.

Table 4.3: Plasma Se content and Gpx3 activity in mice fed supplemental Se with and without LPS (mean ± SEM)

Treatment means					
Added Se (mg/kg diet)	**Pellet**	**n**	**Plasma Se (mg/L)**	**n**	**Gpx (mU/L)**
0	placebo	5	0.095 ± 0.03	5	32.5 ± 11.7
	LPS	5	0.103 ± 0.05	5	35.6 ± 5.4
0.2	placebo	4	0.260 ± 0.04	5	977.5 ± 148.4
	LPS	5	0.192 ± 0.05	5	725.4 ± 208.2
2	placebo	4	0.168 ± 0.06	5	937.4 ± 100.5
	LPS	5	0.247 ± 0.04	5	880.8 ± 144.0
4	placebo	5	0.188 ± 0.03	5	1039.2 ± 161.9
	LPS	5	0.206 ± 0.03	5	1010.4 ± 65.9

Diet means				
Added Se (mg/kg diet)	**n**	**Plasma Se (mg/L)**	**n**	**Gpx (mU/L)**
0	10	0.099 ± 0.03^{b}	10	34.1 ± 6.1^{b}
0.2	9	0.222 ± 0.03^{a}	10	851.5 ± 127.6^{a}
2	9	0.212 ± 0.03^{a}	10	909.1 ± 83.3^{a}
4	10	0.197 ± 0.02^{a}	10	1024.8 ± 82.5^{a}

LPS means				
Placebo	38	0.187 ± 0.02	40	746.7 ± 110.0
LPS	40	0.173 ± 0.02	40	663.1 ± 105.1

***p*-values**		
	Plasma Se	**Gpx**
Se	0.02	<0.0001
LPS	0.75	0.36
Se x LPS	0.4	0.74

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

Table 4.4: Liver Se concentration in mice fed supplemental Se with and without LPS (mean ± SEM)

Treatment means			
Added Se (mg/kg diet)	**Pellet**	**n**	**Se in Liver (ng/g wet wt)**
0	placebo	7	114.3 ± 94.6
	LPS	3	48.2 ± 15.1
0.2	placebo	4	803.6 ± 164.8
	LPS	5	850.1 ± 105.7
2	placebo	5	1673.1 ± 509.0
	LPS	5	1242.0 ± 80.2
4	placebo	5	1399.4 ± 167.2
	LPS	5	1497.8 ± 80.5
Diet means			
Added Se (mg/kg diet)		**n**	**Se in Liver (ng/g wet wt)**
0		10	94.5 ± 65.6[b]
0.2		9	829.4 ± 87.7[a]
2		10	1457.6 ± 253.3[a]
4		10	1448.9 ± 89.0[a]
LPS means			
Placebo		18	922.7 ± 189.1
LPS		21	1005.2 ± 125.8
***p*-Values**			
Se			< 0.0001
LPS			0.57
Se x LPS			0.60

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

Bone Microarchitecture

To examine alterations in trabecular bone and/or cortical bone, tibia and fourth lumbar vertebrae (L_4) were analyzed using µCT. As a measure of trabecular bone microarchitecture, SMI quantifies the organization of trabecular bone where a value of 0 signifies completely plate like trabeculae, and a value of 3 represents all rod-like trabeculae. Connectivity density measures the number of connected trabeculae in a specific volume. In cortical bone of tibia, both BV/TV ($p = 0.07$) and cortical thickness ($p = 0.06$) tended to be reduced by LPS treatment.

Cortical porosity tended to be increased by LPS ($p = 0.07$). The Se-adequate diet also tended to have a negative effect on cortical thickness ($p = 0.06$) **(Table 4.5)** and mice in the Se deficient group had slightly thicker trabeculae than control animals.

In trabecular bone of tibia, BV/TV ($p = 0.004$), and trabecular number ($p < 0.0001$) were both significantly reduced by LPS treatment **(Table 4.6)**. LPS also significantly increased trabecular separation ($p < 0.0001$). There was an interaction effect of Se and LPS on connectivity density ($p = 0.01$) and SMI ($p = 0.02$) (Figure 4.1). LPS significantly lowered connectivity density in the selenium-depleted group, but not in the other diet groups. In the deficient diet group, SMI was significantly increased by LPS indicating more rod-like structure, while other diet groups were not significantly affected **(Table 4.6)**.

In trabecular tissue of the L_4 section of spine, BV/TV ($p = 0.01$), connectivity density ($p = 0.0001$), and trabecular number ($p = 0.001$) were significantly reduced by LPS treatment **(Table 4.7)**. SMI ($p < 0.0001$) and trabecular separation ($p < 0.0001$) were significantly increased by LPS. Mice fed the Se depleted diet had significantly higher BV/TV, connectivity density, and trabecular number, and had significantly lower SMI ($p = 0.03$), and trabecular separation ($p = 0.002$) than mice on the other dietary treatments. Taken together, these data show that LPS treatment primarily has a negative effect on trabecular microarchitecture similar to the trend seen in cortical bone. The effect of diet can primarily be seen only in the Se deficient mice. These mice had significantly better microarchitecture when compared to bones of mice in the other dietary treatments.

Table 4.5: Effects of LPS and diet on µCT measurements on tibia cortical bone volume fraction, cortical porosity and cortical thickness (means ± SEM)

Treatment means					
Added Se (mg/kg diet)	**Pellet**	**n**	**BV/TV (%)**	**Cortical Porosity (%)**	**Cortical Thickness (mm)**
0	placebo	10	95.4 ± 0.2	4.6 ± 0.2	0.213 ± 0.004
	LPS	10	92.4 ± 24.3	7.6 ± 2.4	0.200 ± 0.004
0.2	placebo	9	95.1 ± 0.1	4.9 ± 0.1	0.199 ± 0.004
	LPS	9	94.8 ± 0.2	5.2 ± 0.2	0.192 ± 0.004
2	placebo	10	94.8 ± 0.1	5.2 ± 0.1	0.203 ± 0.005
	LPS	10	92.1 ± 2.6	7.9 ± 2.6	0.196 ± 0.004
4	placebo	10	94.7 ± 0.2	5.3 ± 0.2	0.198 ± 0.004
	LPS	10	93.7 ± 1.3	6.3 ± 1.3	0.202 ± 0.004
Diet means					
Added Se (mg/kg diet)		**n**	**BV/TV (%)**	**Cortical Porosity (%)**	**Cortical Thickness (mm)**
0		20	93.9 ± 1.2	6.1 ± 1.2	0.207 ± 0.003
0.2		18	95.0 ± 0.1	5.0 ± 0.1	0.196 ± 0.003
2		20	93.4 ± 1.3	5.8 ± 0.6	0.199 ± 0.003
4		20	94.2 ± 0.6	5.8 ± 0.6	0.200 ± 0.003
LPS means					
Placebo		39	95.0 ± 0.1	5.0 ± 0.1	0.203 ± 0.002
LPS		39	93.2 ± 0.9	6.8 ± 1.0	0.198 ± 0.002
***p*-values**					
Se			0.74	0.74	0.06
LPS			0.07	0.07	0.06
Se x LPS			0.72	0.45	0.2

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

Table 4.6: Effects of LPS and diet on µCT measurements of tibia trabecular bone parameters in mice (means ± SEM)

Treatment means								
Added Se (mg/kg diet)	**Pellet**	**N**	**BV/TV (%)**	**Connectivity Density ($1/mm^3$)**	**SMI**	**Trabecular Number ($1/mm^3$)**	**Trabecular Thickness (mm)**	**Trabecular Separation (mm)**
0	placebo	10	18.8 ± 0.8	292.65 ± 30.15[a]	1.50 ± 0.11[d]	5.986 ± 0.159	0.0441 ± 0.0009	0.159 ± 0.005
	LPS	10	14.0 ± 1.2	180.63 ± 14.03[b]	2.03 ± 0.11[ab]	5.183 ± 0.106	0.0448 ± 0.0017	0.185 ± 0.004
0.2	placebo	9	15.5 ± 1.2	155.53 ± 15.60[bc]	1.87 ± 0.09[abc]	5.039 ± 0.154	0.0049 ± 0.0016	0.190 ± 0.006
	LPS	10	11.6 ± 1.2	113.91 ± 11.50[c]	2.15 ± 0.11[a]	4.556 ± 0.117	0.0457 ± 0.0021	0.213 ± 0.006
2	placebo	10	15.4 ± 1.4	150.92 ± 15.50[bc]	1.86 ± 0.13[abc]	4.942 ± 0.111	0.0494 ± 0.0019	0.193 ± 0.005
	LPS	10	14.2 ± 1.3	157.49 ± 16.99[bc]	1.84 ± 0.09[abc]	4.726 ± 0.160	0.0474 ± 0.0021	0.206 ± 0.008
4	placebo	10	16.2 ± 4.1	191.25 ± 21.29[b]	1.77 ± 0.13[bcd]	5.240 ± 0.133	0.0467 ± 0.0015	0.181 ± 0.005
	LPS	9	15.7 ± 1.2	173.07 ± 16.60[b]	1.64 ± 0.14[cd]	4.788 ± 0.163	0.0488 ± 0.0017	0.203 ± 0.007
Diet means								
Added Se (mg/kg diet)	**N**		**BV/TV (%)**	**Connectivity Density ($1/mm^3$)**	**SMI**	**Trabecular Number ($1/mm^3$)**	**Trabecular Thickness (mm)**	**Trabecular Separation (mm)**
0	20		16.4 ± 0.9	236.64 ± 20.66	1.76 ± 0.10	5.584 ± 0.131[a]	0.0444 ± 0.0009	0.172 ± 0.004[b]
0.2	19		13.4 ± 0.9	133.63 ± 10.49	2.02 ± 0.08	4.785 ± 0.109[b]	0.0472 ± 0.0013	0.202 ± 0.005[a]
2	20		14.8 ± 0.9	154.21 ± 11.22	1.85 ± 0.08	4.834 ± 0.098[b]	0.0484 ± 0.0014	0.200 ± 0.005[a]
4	19		16.0 ± 0.9	182.64 ± 13.49	1.71 ± 0.10	5.026 ± 0.114[b]	0.0477 ± 0.0011	0.192 ± 0.005[a]
LPS means								
Placebo	39		16.5 ± 0.6 [a]	198.67 ± 13.99	1.75 ± 0.06	5.308 ± 0.095[a]	0.0472 ± 0.0008	0.181 ± 0.003[b]
LPS	39		13.8 ± 0.6[b]	155.85 ± 8.29	1.92 ± 0.06	5.059 ± 0.046[b]	0.0466 ± 0.0010	0.202 ± 0.025[a]
***p*-Values**								
Se			0.098	<0.0001	0.06	<0.0001	0.11	<0.0001
LPS			0.004	0.003	0.05	<0.0001	0.64	<0.0001
Se x LPS			0.24	0.01	0.02	0.21	0.43	0.69

Means in a column with superscripts not sharing a common letter are significantly different at $p < 0.05$.

Table 4.7: Effects of LPS and diet on µCT measurements of L_4 trabecular bone parameters in mice (means ± SEM)

Treatment means								
Added Se (mg/kg diet)	Pellet	N	BV/TV (%)	Connectivity Density ($1/mm^3$)	SMI	Trabecular Number ($1/mm^3$)	Trabecular Thickness (mm)	Trabecular Separation (mm)
0	placebo	10	21.4 ± 0.8	291.67 ± 15.36	1.331 ± 0.076	5.638 ± 0.078	0.046 ± 0.0009	0.172 ± 0.003
0	LPS	10	17.9 ± 1.1	226.87 ± 10.17	1.704 ± 0.071	5.277 ± 0.101	0.044 ± 0.001	0.187 ± 0.001
0.2	placebo	11	18.7 ± 0.8	241.31 ± 11.86	1.528 ± 0.057	5.353 ± 0.105	0.043 ± 0.001	0.183 ± 0.004
0.2	LPS	11	14.7 ± 0.9	176.02 ± 11.00	1.965 ± 0.068	4.887 ± 0.077	0.042 ± 0.001	0.203 ± 0.003
2	placebo	10	17.9 ± 1.2	219.19 ± 12.24	1.633 ± 0.112	5.230 ± 0.104	0.044 ± 0.001	0.188 ± 0.004
2	LPS	10	16.7 ± 1.1	204.97 ± 11.36	1.782 ± 0.085	5.054 ± 0.085	0.044 ± 0.002	0.196 ± 0.004
4	placebo	10	18.1 ± 0.9	239.30 ± 14.49	1.558 ± 0.087	5.290 ± 0.100	0.043 ± 0.0009	0.186 ± 0.005
4	LPS	10	17.3 ± 0.9	190.49 ± 6.45	1.808 ± 0.053	5.035 ± 0.068	0.046 ± 0.002	0.195 ± 0.003
Diet means								
Added Se (mg/kg diet)		n	BV/TV (%)	Connectivity Density ($1/mm^3$)	SMI	Trabecular Number ($1/mm^3$)	Trabecular Thickness (mm)	Trabecular Separation (mm)
0		20	19.8 ± 0.7[a]	259.27 ± 8.57[a]	1.517 ± 0.056[b]	5.458 ± 0.066[a]	0.045 ± 0.0009	0.179 ± 0.003[a]
0.2		22	16.7 ± 0.6[b]	208.66 ± 8.17[b]	1.746 ± 0.054[a]	5.120 ± 0.063[b]	0.043 ± 0.0009	0.193 ± 0.003[b]
2		10	17.3 ± 0.6[b]	212.08 ± 8.37[b]	1.707 ± 0.055[a]	5.142 ± 0.064[b]	0.044 ± 0.0009	0.192 ± 0.003[b]
4		10	17.7 ± 0.7[b]	214.90 ± 8.57[b]	1.683 ± 0.056[a]	5.163 ± 0.066[b]	0.044 ± 0.0009	0.190 ± 0.003[b]
LPS means								
Placebo		41	19.1 ± 0.5[a]	247.03 ± 7.68[a]	1.516 ± 0.045[b]	5.374 ± 0.053[a]	0.044 ± 0.0006	0.182 ± 0.002[b]
LPS		41	16.6 ± 0.5[b]	199.01 ± 5.67[b]	1.818 ± 0.037[a]	5.059 ± 0.046[b]	0.044 ± 0.0008	0.195 ± 0.002[a]
***p*-Values**								
Se			0.01	0.0001	0.03	0.001	0.35	0.002
LPS			0.0005	<0.0001	<0.0001	<0.0001	0.96	<0.0001
Se x LPS			0.21	0.11	0.26	0.4	0.17	0.44

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

Bone Biomechanics

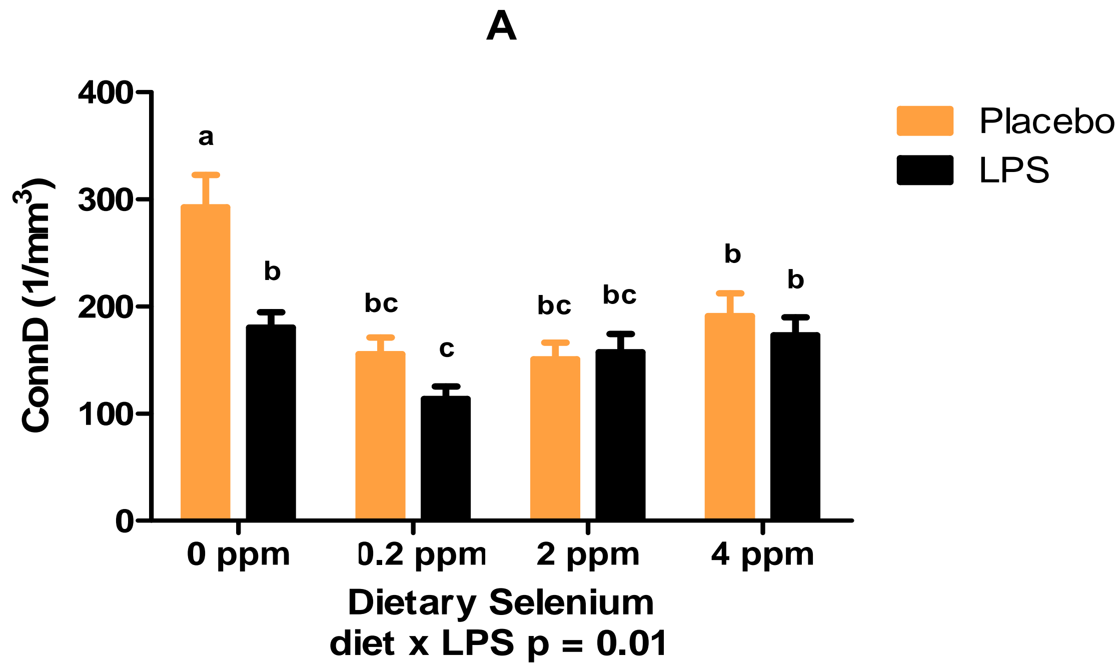
The influences of chronic inflammation and graded levels of Se in the diet on trabecular bone biomechanical properties were evaluated using finite element analysis (FEA) of µCT images of the proximal tibia metaphysis and L_4 of spine. Diet had a significant effect on tibia biomechanical properties (**Table 4.8**). Compressive strength of the trabecular bone from tibia was significantly lower in mice fed adequate diet (0.2 Se mg/kg) compared to the other diet groups ($p = 0.04$). Bone stiffness was also reduced in mice fed adequate diet ($p = 0.04$). The average von Mises stress was increased in animals fed the adequate Se diet ($p = 0.0001$).

In L_4, an interaction effect was present **(Figure 4.2)** for most of the biomechanical properties (**Table 4.9**). Size independent stiffness was significantly reduced by LPS ($p = 0.03$). In strain, an interaction effect can be seen between diet and LPS. In the adequate diet group only, LPS significantly reduced average strain and average apparent strain. In bone from mice in the deficient diet group, LPS resulted in significantly reduced physiological force. The simulated compression tests demonstrated that diet had a more significant effect than LPS on tibia strength and stiffness, where mice on the sufficient diet had lower quality bone and Se supplementation did not provide additional benefit. LPS treatment resulted in compromised trabecular strain in spine mostly in mice fed adequate Se diet (0.2 Se mg/kg). The von Mises measurements in the LPS treated group of the 0.2 Se mg/kg diet group were the highest.

Table 4.8: Effects of LPS and diet on biomechanical properties of tibia- physiological force, stiffness and von Mises stresses in mice (means ± SEM)

Treatment means					
Added Se (mg/kg diet)	**Pellet**	**n**	**Physiological Force (N)**	**Stiffness (N/mm)**	**von Mises Stress (MPa)**
0	placebo	10	0.0487 ± 0.0044	2704.30 ± 242.32	34.13 ± 7.95
	LPS	10	0.0364 ± 0.0046	2025.10 ± 256.88	46.44 ± 11.24
0.2	placebo	9	0.0371 ± 0.0059	2059.78 ± 329.89	75.16 ± 9.26
	LPS	9	0.0243 ± 0.0042	1349.67 ± 230.68	119.18 ± 20.04
2	placebo	9	0.0429 ± 0.0073	2436.22 ± 404.93	39..09 ± 9.52
	LPS	11	0.0470 ± 0.0073	2608.64 ± 406.02	51.037 ± 13.00
4	placebo	10	0.0477 ± 0.0076	2651.40 ± 421.77	42.66 ± 14.64
	LPS	10	0.0487 ± 0.0061	2706.90 ± 336.64	36.12 ± 8.04
Diet means					
Added Se (mg/kg diet)		**n**	**Physiological Force (N)**	**Stiffness (N/mm)**	**von Mises Stress (MPa)**
0		20	0.0426 ± 0.0034[a]	2364.70 ± 188.69[a]	40.38 ± 6.85[b]
0.2		18	0.0307 ± 0.0038[b]	1704.72 ± 213.41[b]	97.17 ± 11.96[a]
2		20	0.0456 ± 0.0051[a]	2531.05 ± 281.28[a]	45.66 ± 8.24[b]
4		20	0.0482 ± 0.0047[a]	2679.15 ± 262.70[a]	39.39 ± 7.16[b]
LPS means					
Placebo		38	0.0445 ± 0.0032	2474.24 ± 175.57	47.27 ± 5.42
LPS		40	0.0397 ± 0.0032	2204.05 ± 177.81	61.49 ± 8.19
***p*-values**					
Se			0.04	0.04	0.0001
LPS			0.23	0.23	0.07
Se x LPS			0.42	0.42	0.22

Means in a column with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.



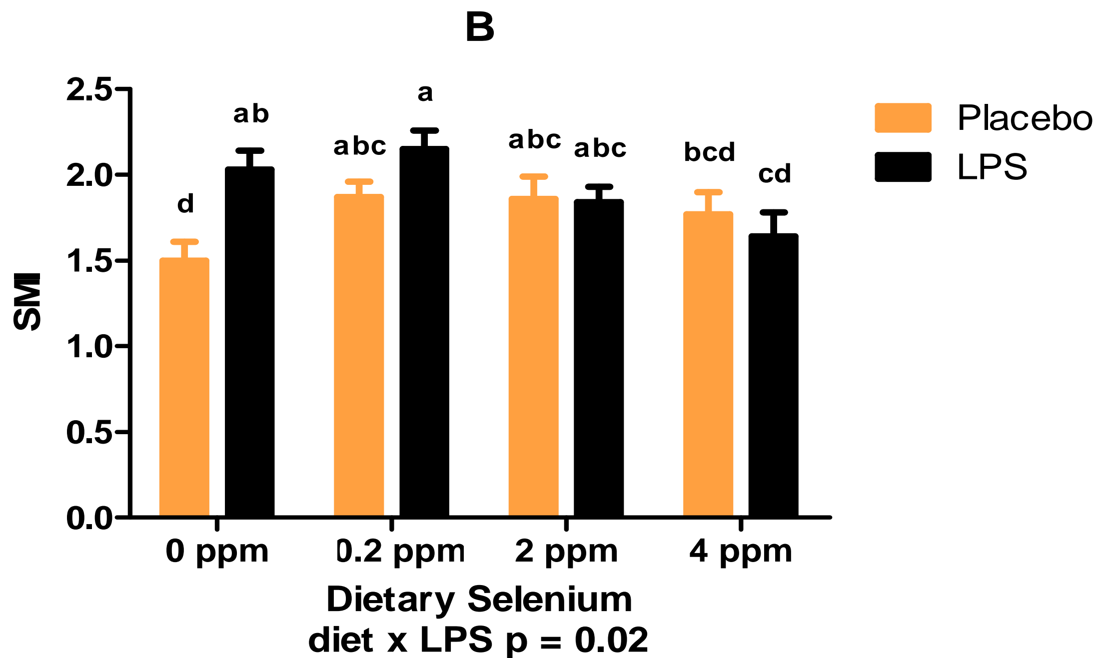


Figure 4.1: Diet and LPS interaction for proximal tibia connectivity density (A) and SMI (B). The four dietary treatment groups (0.0 mg Se/kg diet, 0.2 mg Se/kg diet, 2.0 mg Se/kg diet, 4.0 mg Se/kg diet) are separated by LPS and placebo groups to better show the interactive effect between diet and LPS. Bars not sharing a common letter are significantly different from each other at $p < 0.05$.

Table 4.9: Effects of LPS and diet on biomechanical properties of spine - average apparent strain, size independent stiffness, physical force, von Mises stress in mice (means ± SEM)

Treatment means						
Added Se (mg/kg diet)	**Pellet**	**n**	**Average Apparent Strain**	**Size Independent Stiffness**	**Physiological Force (N)**	**von Mises Stress**
0	placebo	10	0.277 ± 0.014^{a}	6.424 ± 0.449	0.0179 ± 0.0012^{a}	132.36 ± 4.91^{c}
	LPS	10	0.223 ± 0.106^{a}	4.263 ± 0.647	0.0116 ± 0.0019^{bc}	189.63 ± 17.58^{ab}
0.2	placebo	10	0.492 ± 0.132^{a}	4.416 ± 0.662	0.0115 ± 0.0016^{bc}	183.97 ± 14.22^{bc}
	LPS	10	0.183 ± 0.015^{b}	2.642 ± 0.262	0.0071 ± 0.00070^{c}	244.73 ± 15.93^{a}
2	placebo	12	0.217 ± 0.019^{a}	4.223 ± 0.544	0.0114 ± 0.0015^{bc}	194.31 ± 26.02^{ab}
	LPS	10	0.221 ± 0.019^{a}	3.749 ± 0.606	0.0103 ± 0.0017^{bc}	208.09 ± 17.20^{ab}
4	placebo	10	0.218 ± 0.024^{a}	4.108 ± 0.685	0.0111 ± 0.0018^{bc}	206.74 ± 31.51^{ab}
	LPS	11	0.261 ± 0.022^{a}	4.890 ± 0.615	0.0140 ± 0.0018^{ab}	169.37 ± 13.41^{bc}
Diet means						
Added Se (mg/kg diet)		**n**	**Average Apparent**	**Size Independent Stiffness**	**Physiological Force (N)**	**von Mises Stress**
0		20	0.250 ± 0.014	5.343 ± 0.457^{a}	0.0148 ± 0.0013	160.00 ± 11.05
0.2		20	0.338 ± 0.074	3.529 ± 0.402^{b}	0.0093 ± 0.0010	208.83 ± 15.50
2		22	0.219 ± 0.013	4.008 ± 0.399^{b}	0.0109 ± 0.0011	200.57 ± 15.92
4		21	0.241 ± 0.017	4.518 ± 0.455^{ab}	0.0126 ± 0.0013	187.17 ± 16.65
LPS						
Placebo		42	0.297 ± 0.036	4.766 ± 0.319^{a}	0.0129 ± 0.0009	177.43 ± 12.20
LPS		41	0.223 ± 0.011	3.910 ± 0.299^{b}	0.0108 ± 0.0009	202.13 ± 8.87
***p*-values**						
Se		0.08		0.02	0.008	0.11
LPS		0.02		0.03	0.05	0.07
Se x LPS		0.002		0.05	0.03	0.05

Means with superscripts not sharing a common letter are significantly different from each other at $p < 0.05$.

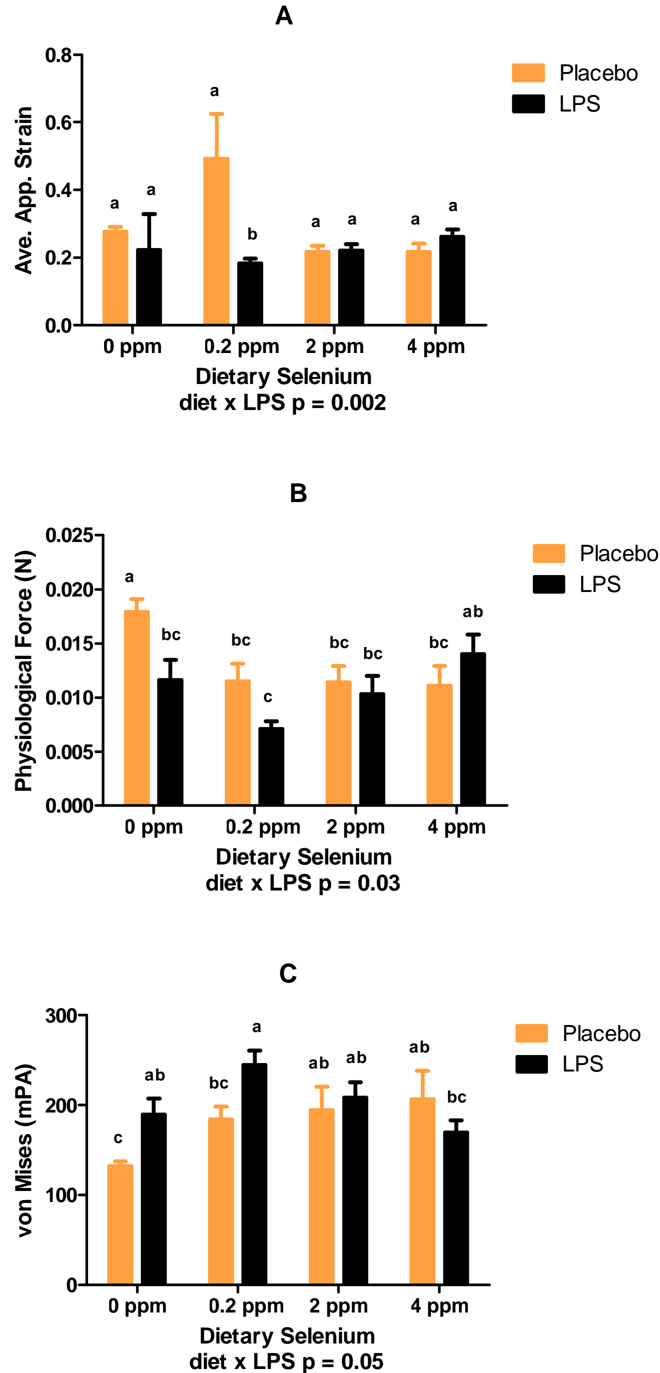


Figure 4.2: Diet and LPS interaction for L_4 average apparent strain (A) physiological force (B) and von Mises stress (C). The four dietary treatment groups (0.0 mg Se/kg diet, 0.2 mg Se/kg diet, 2.0 mg Se/kg diet, 4.0 mg Se/kg diet) are separated by LPS and placebo groups to better show the interactive effect between diet and LPS. Bars not sharing a common letter are significantly different from each other at $p < 0.05$.

Discussion

Se is considered an essential element for antioxidant enzymes such as glutathione peroxidase that catalyze the reduction of peroxides that can cause cellular damage [1]. Peroxides and other reactive oxygen species (ROS) generated through inflammation lead to damage of bone cellular matrix [2]. Smith and colleagues reported systemic bone loss due to 90-day time-release pellets in male Sprague-Dawley rats [3]. Ishihara and coworker showed that LPS induced bone resorption in a *BALB/c* mouse calvaria organ culture [4]. The scavenging ability of Gpx has been shown to have a protective effect on cells against oxidative damage [5]. Dreher and colleagues reported antioxidative defense for human fetal osteoblasts that was mediated by expressed glutathione peroxidase [5].

Se deficiency is associated with Kashin-Beck disease, a severe type of osteoarthritis [6]. Rats supplemented with selenium showed less necrosis in the chondrocytes of the growth plates of tibia when compared to rats fed diets from Kashin-Beck disease endemic areas. They also showed better BV/TV, trabecular thickness, and trabecular number, while trabecular separation was reduced [7]. Most of the literature examines the effect on bone of Se deficiency/supplementation and LPS inflammation separately. To our knowledge, there is little research that investigates the effect of both Se status and chronic inflammation on bone loss, and the potential benefit of selenium supplementation.

In this study second generation Se deficient mice were fed graded Se concentrations of 0.02 (deficient), 0.2 (control/adequate), 2.0 and 4.0 mg Se/kg diet for 3 months and randomly assigned LPS treatment on 96-98 days of age to produce inflammatory stress. The deficient diet (0.02 mg Se/kg) was well below daily requirements for rodents (0.1 mg Se/kg) [8] and the Se sufficient diet (0.2 mg Se/kg) was adequate [1]. Raines and Sunde reported no toxicity effect in

mice fed Se at 0.2 μg Se/g diet, but growth retardation (23%) was seen in rats fed 5 μg Se/g [9]. High (relative to 0.1 mg Se/kg diet) but non-toxic Se intake for both mice and rats (less than 2 μg Se/g diet) showed little to no toxic effect [9].

Chronic inflammation due to LPS implantation (0.1 μg/g body weight/d) did not cause major changes to body or organ weights. Body weight and organ weight data suggested that LPS treatment did not cause major undue stress and illness to the mice. Body weight tended to decrease due to LPS treatment by the end of the study (d 120, p = 0.07). A previous low-dose LPS model also showed non-significant body weight changes between LPS-treated and placebo groups [3]. Thymus weight, tended to be reduced by chronic inflammation ($p = 0.06$).

Although changes were not seen in body parameters due to LPS, measurements of bone mineral showed that LPS had induced bone loss. Bone densitometry in LPS-treated mice was significantly lowered when compared to placebo. LPS-induced catabolic state in bone microarchitecture was primarily observed in trabecular bone as opposed to cortical bone indicating that trabecular bone is primarily affected by LPS-induced inflammation. This result supports the notion that the detrimental effects observed with chronic LPS administration in the study were not a result of compromised animal health. Cortical thinning in the lumbar vertebra has been observed in adjuvant models [10], but the slower rate of cortical bone turnover and the relatively low grade inflammation induced in this study may require a longer study duration to observe such changes. The results of this study did indeed show a trend towards cortical bone damage in tibia under a condition of chronic inflammation.

The bone loss seen through bone densitometry only reduced bone strength in spine, while the same was not seen in tibia. LPS-induced inflammation did not show significant effects on

tibia biomechanics, but did in spine. A higher turnover in vertebral bone compared to tibia could explain a significant LPS effect being only seen in spine [11].

Taken separately, the dietary treatments at 0.0 mg/Se kg diet, 0.2 mg/Se kg diet, and 2.0 mg/Se kg diet did not have negative health consequences. A potential toxicity effect, however, was detected in the mice supplemented with 4.0 mg Se/kg diet. Toxicity levels in rats have been reported at higher than 5.0 mg Se/kg diet [9] with LD_{50} of sodium selenate at 5.8 mg Se/kg diet [12]. Chronic Se levels in rats of 4-5 mg Se/kg have been reported to cause growth inhibition and tissue damage [13]. A 30 month dietary treatment of 0.5 – 2 mg Se/kg have shown liver toxicity in rats [14]. No severe health effects were recorded in the mice fed the 4.0 mg Se/kg diet, but some behavior oddities like sluggish response to feeding time were noticed. The effects of added Se at 3.0 mg/Se kg diet could mitigate any health effects and dietary levels could still be still be high enough to see the effects of supplemented Se on a chronic inflammation model.

Se adequate diets had mice with less dense bone compared to the other dietary treatments. Bone mineral density (BMD) and bone mineral concentration (BMC) were higher in the Se deficient group (0.0 mg Se/kg diet) compared to the Se adequate group (0.2 mg Se/kg diet). Se deficiency has been associated with reduced growth in animals [15]. Although the body parameters data do not show weight reduction due to dietary treatment, changes in bone might have occurred. Relatively slow growth rate of mice in the Se deficient group could lead to increased bone mass and account for the higher bone densitometry when compared to Se adequate mice. The mice in the selenium supplemented group (2.0 mg Se/kg diet and 4.0 mg Se/kg diet) had higher bone densitometry compared to the Se adequate mice. These supplementary dietary treatments were 10 to 20 times higher than adequate for rodents [9] and are defined as being high levels that have been associated with less than optimal health in rats

[13]. The slowed growth seen though chronic dietary Se supplementation at ~4mg Se/kg diet [13] could have a similar effect to that of Se deficiency [15]. In our study, the dietary treatments were given to growing animals, and were not chronic as in previous literature, so any significant health damage may not yet have been visible.

Se status was confirmed through measurement of Se concentration in liver tissue. As expected, liver Se content in mice fed the deficient diet was significantly lower than mice in the Se adequate group. Se supplementation did not significantly increase liver Se content compared to control. Organs like kidney and liver have very high levels of Se as a component of glutathione [16], levels of which have been shown to not be affected by Se supplementation [17]. The Se concentration in liver was consistent with Burke and colleagues who reported liver selenium levels at 0.1 µg/g in rats fed a low-selenium diet and at 0.7 µg/g in rats fed a selenium-adequate diet [18]. Liver Se concentration in the Se adequate diet did not differ significantly from the Se supplemented groups most likely due to high variability within the diet group. An increase in sample size might separate and better evaluate the differences.

Bone microarchitecture parameters were relatively better in mice fed the Se deficient diet. A different result was seen in rats supplemented with selenium compared to rats on a Se deficient diet [7]. Yao and coworkers compared the femur microarchitecture of Wistar rats on graded Se diets and found Se deficient diets to have lower quality bones [19]. Increased Se in the diet, however, did not show beneficial effects on trabecular bone.

The biomechanical strength of tibia was significantly lower in mice fed Se adequate diet compared to the other dietary treatments as evidenced by stiffness being relatively low and von Mises measurements being comparatively high. This is different from previously published work where Wistar rats fed selenium deficient or selenium-excess diets had decreased

biomechanical strength when compared to control [20]. In an induced diabetic animal model where Se was used as an antioxidant, rat mandible strength was weaker in the selenium supplemented control group when compared to the diabetic group supplemented with the same amount of Se [21]. Dellibasi and colleagues reported this finding as unexpected and recommended more studies into the mechanisms of how Se affects organisms [21].

Interactive effects between LPS and diet in measurements of tibia microarchitecture showed that dietary Se at adequate levels or above protected bone from the damage caused by LPS. Connectivity density showed that the LPS effect of reducing trabecular bone was only significant in the Se deficient group. No other dietary treatment group showed a significant difference between LPS-treated and placebo mice. This supports that Se does provide anti-oxidant protection from bone through glutathione peroxidase [1]. SMI similarly also showed significant difference only in the Se deficient group where LPS reduced the quality of bone.

Interactive effects in biomechanical measurements of L_4 spine, however, only show LPS effect in the Se adequate group. Interactive effects of diet and LPS treatment did show increased strain and von Mises stress in the LPS treated Se adequate mice compared to placebo mice in the same dietary group. This could be explained by the remodeling process that is more active in spine than in peripheral bones [11], and vertebra has more chancellors bone and is thus more prone to bone alterations than in tibia. More biomechanical testing such as bone-bending would be useful to have better understanding of this unexpected result.

In conclusion, mice in the Se deficient group had higher quality bone microarchitecture when compared to the Se adequate mice, but the bone biomechanical testing showed that Se deficiency did not weaken bone as much as the recommended Se intake amount for mice. Protective effects from excess Se were also not observed.

CHAPTER V

SUMMARY, CONCLUSIONS, AND SUGGESTIONS FOR FURTHER STUDY

Summary

In this study we investigated the effects of selenium supplementation and chronic inflammation on bone microarchitecture and strength in mice. To ascertain the Se status of the mice we assessed plasma and liver Se concentrations, and Gpx activity. We assessed bone densitometry by PIXImus scans and microarchitecture and strength by microcomputed tomography.

Se status

Significant difference in Se status was only observed between the mice fed the basal diet and the Se supplemented groups as evidenced by Gpx activity and plasma and liver Se concentrations.

Body parameters

Chronic inflammation did not have a significant effect on body or organ weights, but bone density was reduced. Mice on 0.0 mg, 0.2, and 2.0 mg added Se/kg diet did not show negative health effects as evidenced by organ weights ($p > 0.05$), but mice on 4.0 mg added Se/kg diet had reduced liver weight ($p = 0.04$) indicating possible toxicity. Thymus weight tended to be increased by LSP ($p = 0.06$).

Microarchitecture

Overall, LPS induced chronic-inflammation weakened trabecular bone quality but not cortical bone. LPS did not significantly increase cortical porosity or significantly reduced cortical thickness in tibia ($p > 0.05$). Tibia and spine trabecular bone was reduced as shown by trabecular number and separation. Interactive effects between diet and LPS in tibia trabecular measurements of SMI and connectivity density only showed the catabolic effect of LPS only in the mice fed the basal diet, indicating that Se supplementation beyond what is considered deficient does promote Gpx activity and provided protection against bone loss.

Bone Biomechanics

Bone weakness due to LPS was seen in spine, but not in tibia. The mice fed the Se adequate diet had comparatively weaker tibia than the mice in the other dietary treatments. Spine strength measurements had several interactive effects, but stiffness was reduced by LPS treatment. Interaction between LPS and diet showed the LPS effect of reducing bone strength was seen in mice fed the basal diet as evidenced by von mises and physiological force measurements. The von Mises sress and strain measurements were also affected by LPS in the mice fed the Se adequate diet.

Conclusions

For this study we hypothesized:

1. The inflammatory treatment would reduce bone quality/microarchitecture and strength. Overall this effect of reducing bone microarchitecture was more pronounced in trabecular bone than cortical. LPS reduced many trabecular bone parameters significantly, but only tended to reduced cortical bone. Strength was not affected in tibia by LPS, and catabolic effects from inflammation were only seen in mice fed the basal and Se adequate diet.
2. Higher dietary selenium will result in better microarchitecture parameters. Our results did not support this hypothesis. Overall, mice in the Se deficient diet had better microarchitecture compared to mice in the other dietary treatments.
3. Higher dietary selenium will result in bone that is stronger. Our results did not support this hypothesis. Overall, mice in the Se adequate diet group had comparatively weaker tibia than mice in the other dietary treatments. Spine strength measurements showed multiple interaction effects between LPS and diet. LPS reduced bone strength measurements in the Se adequate and Se deficient mice only.

Suggestions for Further Studies

- Since our dietary treatment of 4.0 mg Se/kg diet reduced liver weight, a measurement of liver tissue damage makers like alanine amino transferase (ALT) would help determine the toxicity of

this dietary treatment. If 4.0 mg Se/kg diet was potentially toxic, it would be interesting to see what effects a lower concentration of Se would have (e.g. 3.0 mg Se/kg diet).

- Even though chronic inflammation did show bone damage through measurements of bone microarchitecture, lipid peroxidation assessment could have also established inflammatory status. Liver thiobarbituric acid reactive substances (TBARS) assessment is a common method used to determine lipid peroxidation. In addition, antioxidant status could be determined by ferric reducing ability plasma (FRAP). Bone metabolism could be determined by assessing biochemical markers such as alkaline phosphate (ALP), and serum tartrate resistant acid phosphatase (TRAP). Osteocalcin could also be analyzed to determine bone formation. This could help better determine exactly how LPS reduced bone quality: through increase of bone loss, or disruption of bone formation, or both.
- To better understand the effects of dietary treatments on bone strength, bone biomechanical testing using 3-point bending could provide more information to better interpret the results we had.
- To determine if the dietary treatments did cause growth retardation, it would be useful to measure femur or tibia length, and ash the bones to look at weight.

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VITA

Abiy Girma Melaku

Candidate for the Degree of

Master of Science

Thesis: EFEFCTS OF SELENIUM SUPPLEMENTATION AND CHRONIC INFLAMMATION ON BONE MICROARCHITECTURE AND STRENGTH IN MICE

Major Field: Nutritional Sciences

Biographical:

Education: Received an International General Certificate of Secondary Education at Sandford International School, Addis Ababa, Ethiopia in June 2002. Earned an International Baccalaureate from Sandford International School in June 2004. Received a Bachelor of Arts degree in Biochemistry and Molecular Biology at Cornell College in Mt. Vernon, Iowa in May 2008. Completed the requirements for the Master of Science degree with a major in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2012.

Experience: Program Manager Assistant at USAID, Addis Ababa, Ethiopia in 2007. Student researcher in the Biochemistry laboratory at Cornell College, Mt. Vernon, Iowa in 2008. Research associate in virology department of Genvec, Inc, Germantown, Maryland from 2008 to 2010. Graduate research and teaching assistant in the Department of Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma from 2010 to 2012.

Professional Memberships: Ethiopian Public Health Association

Name: Abiy Girma Melaku Date of Degree: July, 2012

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF SELENIUM SUPPLEMENTATION AND CHRONIC INFLAMMATION ON BONE MICROARCHITECTURE AND STRENGTH IN MICE

Pages in Study: 98 Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study: The effects of dietary Se and lipopolysaccharide (LPS)-induced inflammation on microarchitecture and strength of bone were investigated in C57BL/6 mice. Timed-pregnant mice were fed a Torula yeast selenium-depletion diet from the final days of gestation through lactation. At 23 days of age, pups were weaned and randomly assigned to the depletion diet or to diets supplemented with a 0.2, 2 or 4 mg/kg diet of Se added as sodium selenate for 14 weeks. At 96 – 98 days of age mice were randomly assigned to placebo or to lipopolysaccharide (E. coli Serotype 0127:B8) treatment to produce chronic inflammation. Time release pellets (0 or 0.1 µg/g body weight/d) were implanted subcutaneously. Mice were killed at 120 days of age after an overnight fast. Bone densitometry and organ and body weights were used to determine health status. Se status was assessed through Gpx3 activity, and plasma and liver Se concentration. Micro-CT was used to assess trabecular bone of the fourth lumbar vertebra (L_{4}) and trabecular and cortical bone of tibia. Using finite element analysis a simulated compression test in the z direction was used to assess strength of trabecular cores.

Findings and Conclusions: Low dose inflammation did not cause significant loss of body weight by necropsy, but LPS tended to increase thymus weight ($p = 0.06$). Reduced BMD and BMC showed a catabolic state due to LPS ($p = 0.02$). LPS-treatment tended ($p = 0.06$) to reduced tibia cortical thickness and increase cortical porosity ($p = 0.07$), while significantly reducing many parameters of trabecular bone. Inflammation tended ($p = 0.07$) to increase von Mises stress in tibia trabecular bone, and significantly reduced stiffness in L_4. GPx3 activity as well as plasma and liver Se concentration were significantly higher in the Se adequate group than in the Se deficient group, but didn't differ significantly from mice in the highly supplemented groups. Liver weight was significantly reduced in the 4 mg Se/kg group indicating possible toxicity. Contrary to our hypothesis, tibia and L_4 trabecular bone parameters in Se deficient mice were significantly improved compared to Se adequate mice and compared with the Se supplemented groups. Tibia biomechanical measurements showed physiological force ($p = 0.04$) and stiffness ($p = 0.04$) to be higher, and von Mises stress (0.0001) to be lower in the Se deficient, 2.0 mg/kg and 2.0 mg/kg compared to the Se adequate mice. Interactive effects between LPS and diet in tibial connectivity density and SMI showed LPS to reduce bone strength only in the Se deficient group. Interactions between diet and LPS in average apparent strain, physiological force and von Mises stress of L_4 also showed catabolic LPS effects only in the Se deficient and Se adequate groups. Further study in the mechanism of Se action in growing animals needs to be conducted to better explain these results.

ADVISER'S APPROVAL: Dr. Barbara J. Stoecker