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Whole Body Vibration as a Treatment to Counter Bone Detriment and Obesity Caused by Poor Nutritional Intake

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Abstract of Thesis

Whole Body Vibration as a Treatment to Counter Bone Detriment and Obesity Caused by Poor Nutritional Intake

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Consumption of diets high in fat content or carbohydrate content is known to cause obesity and diabetes. In fact the causes and effects of diabetes and obesity are inter-related and are known to have associations with the onset of osteoporosis. Despite the availability of pharmacological treatments, an effective cure for osteoporosis has not yet been developed highlighting the need to ensure optimal, maximum bone growth during the growing years of the skeleton. It is therefore important to identify factors which can be detrimental or beneficial to healthy bone deposition or increase rate of bone resorption. It is known that nutrition plays a major role in bone development. The first part of this project documented the hypothesized detrimental effects of a high-fat diet and high-fructose diet consumption on the morphology, density, mass and architecture of load-bearing bones in mice over a short (6 week) and long (15 week) study period. We also proposed that changes in bone tissue will be accompanied by and will be related to increases in bodily fat content and disturbances in glycemic balance. Results showed that the high-fructose diet consumption led to significant decreases in bone quantity and density with deterioration of trabecular micro-architecture over a 6 week period. The

fructose diet also caused severe decreases in insulin and hyperglycemia accompanied with loss of body mass. Both the increased levels of glucose and decreased weight bearing were found to have negative implications on bone volume and density. The high-fat diet was detrimental to bone quantity, density and structure over a longer (15 week) period and attrition of bone tissue was accompanied by large increases in bodily fat content.

Exercise is recommended as being the most effective non-pharmacological therapy to increase bone formation, decrease adipogenesis and prevent obesity related complications such as diabetes. But compliance to regular exercise is low and strenuous or regular exercise regimens are not suited to some populations of individuals such as the elderly. Recently it has been proposed that high frequency low magnitude mechanical signals which mimic the low level forces generated by muscular contractions during daily activity can elicit an anabolic bone response and decrease adipogenesis. However, the response of the skeleton to WBV treatment has varied with skeletal site, species of animal, sex, genetic background and age which are all factors that may modulate the sensitivity of the skeleton to mechanical signals. We proposed that composition of daily nutritional intake is another such factor which can alter the body's response to WBV. The second part of this project aimed at testing the efficacy of whole body vibration therapy in increasing bone formation in animals suffering from the catabolic skeletal effects of daily high-fat and high-fructose consumption over 6 weeks or 15 weeks. We found that response of load-bearing bones to WBV varied from being catabolic to no-response to anabolic depending on diet-type and duration of treatment. In this research, the efficacy of WBV in eliciting an anabolic response from bone tissue was not strongly supported except in the high-fat diet group which saw an anti-resorptive effect. Application of WBV for 6 weeks was able to attenuate the severe glucose-insulin imbalances caused by high-fructose diet consumption. Finally, WBV did not have any effects on the fat deposition in any diet group.

The results presented in this thesis emphasize the damaging effects of high-fat and high-fructose diets on load-bearing bones, metabolic health and body composition. Further, the data suggests the potential of WBV as a tool to ameliorate not only the bone loss caused

by poor nutrition and also to regulate metabolic balance. Finally, our findings indicate that the sensitivity of the skeleton to WBV can be different in populations that have different dietary lifestyles, thus emphasizing the necessity of taking diet into account as an important factor that will affect efficacy while developing WBV as a treatment for osteoporosis and obesity.

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CHAPTER ONE

Introduction

Obesity and Osteoporosis: Bone tissue is an important connective tissue of the body, providing structure, protecting vital organs, coordinating with muscle to carry out movements, housing stem cells and acting as a mineral reserve. Bone biology is complex and dynamically changing, involving cells that are actively remodeling their surroundings. One of these cell types are osteoblasts – the basic "building" units of bone that are responsible for formation or laying down of new bone. The other cell type, responsible for undoing and balancing the bone formation of osteoblasts, are osteoclasts – cells responsible for carrying out resorption of bone. Osteoblasts are mononucleate cells that produce a collagen based osteoid matrix and then mineralize this matrix(1). Osteoblasts that consequently get trapped in this matrix are known as osteocytes(1). Osteocytes participate in bone formation by acting on osteoclasts via signaling(2). Osteoclasts are large multinucleated cells that remove bone by digesting the mineralized matrix. Osteoclasts take on a ruffled border appearance during resorption which mainly takes place by endocytosis of calcium and phosphate ions which are then released into extra-cellular matrix and the bloodstream(3). Thus these two basic functional units of bone, osteoblasts and osteoclasts, maintain the resorption - formation balance by responding to signals from each other and from various other sources such as hormonal, nutritional or loading signals. Bone tissue is capable of modulating its architecture, not only in response to internal cues such as hypothalamic signals, but also to external environmental cues, such as increase or decrease in loading(4). In a healthy individual, a perfect tandem is maintained between bone formation and breakdown, leading to bone that has optimal volume and strength, appropriately maintained such that it is capable of meeting the load demands imposed on it. However, when the balance shifts in favor of increased resorption, it leads to excessive breakdown which is not compensated by an equal rate of formation, leading to weakening of overall material properties and compromised strength, load bearing ability. As the severity of this condition increases, it

leads to osteopenia and consequently to a condition known as osteoporosis. Osteoporosis is a major health concern today, as a disease which can severely debilitate quality of life. Both age induced (Type 2) or menopause induced (Type 1) osteoporosis occur among the middle aged-elderly population. As the disease progresses along with overall aging of the body and reduction in daily activity, bone loss can severely reduce load bearing capacity, leading to fractures. According to estimates, in 2005, there were 2 million osteoporotic fractures in the United States(5). It is estimated that the cost of osteoporotic fractures was as high as 17 billion USD per year in 2005(5). Besides being a serious disease condition by itself, there is now conclusive research which indicates that osteoporosis is also influenced by another increasing health risk namely obesity.

Obesity refers to a condition where an individual has a body weight which is much greater than that which is considered healthy. A body mass index (BMI) of between 25 kg/m^2 and 30 kg/m^2 is a sign of being overweight and a BMI exceeding 30 kg/m^2 characterizes obesity. The National Health and Nutrition Examination Survey estimated that in 2004, 32.2% of adults in the United States were obese(6). In a majority of cases, obesity is a lifestyle related disease caused by a combination of unhealthy diet and inactivity. The condition often results from consumption of foods and drinks that are high in fat or sugar content. Obesity and its increasing prevalence are concerning because of its links with cardiovascular disease, diabetes and an increased risk for some types of cancer(10). While adipose tissue is a reserve for energy, excess accumulation of adiposity beyond that which is needed by the body can prove to be very harmful for many reasons, one of which is a possible influence on the skeletal system. Adipocytes (fat cells) are derived from mesenchymal stem cells – a progenitor cell type that can differentiate into many lineages of tissues such as chondrocytes (cartilage), adipocytes (fat), smooth muscle cells (epithelium) or osteoblasts (bone cells) (11). There is now increasing evidence to suggest that there might be an antagonistic relationship between fat accumulation and bone mass – adipocytes and osteoblasts(12-13). Thus, an increase in adipogenic differentiation of stem cells may be one of the causes of reduced osteogenesis and bone formation. A lack of an effective, permanent cure is another feature that both diseases have in common. The current recommendation for treating obesity is exercise and restrictions on caloric intake. In extreme cases, where obesity has progressed to the

extent that it severely hampers quality of life and poses a health risk, other surgical treatment techniques such as gastric bypass are employed. It is accepted that the best recourse is to prevent obesity by regular exercise and a healthy, balanced diet.

Relationship between Adiposity and Bone Health: Dietary composition is an important determinant of bone health because of the nutrition it provides and because diet is the main factor affecting BMI. It has been suggested that obese individuals have the advantage of increased weight bearing which according to Wolff's law should lead to an increase in bone strength(4). Although this claim, intuitively, makes sense and also seems logical from an evolutionary perspective (heavier individuals will tend to acquire stronger bones), studies which have been carried out with the sole purpose of establishing a relationship between bone mineral content/density and fat mass, have often come up with opposing results. For example Hla et al. performed a study with postmenopausal women and found that both fat mass and lean mass are positively correlated with bone mineral content at important fracture sites such as hip and spine (14). On the other hand, research carried out by Pollock et al. showed the opposite – that in adolescent women, percentage body fat is inversely related to cortical bone mineral content and cortical bone area (15). Coming to the young adult age, Wang et al. carried out studies in 20-25 year old women and found that both amount of fat mass and lean tissue mass are positively correlated with bone mineral density and content (16). Li et al. carried out similar research with perimenopausal women and found that there is no relationship between fat mass and bone mineral density (17). It seems that differences in results can be accounted to the different age groups studied and the relationship between fat deposits and bone health must change with changes in age. However other studies in perimenopausal women have shown that for a given body weight, fat mass and levels of the hormone leptin are indeed inversely proportional to bone mineral density (18). Another study done by Hsu et al. which uses Chinese subjects found that in both men and women, across all age groups, increasing fat mass was linked to an increased risk of osteoporosis as indicated by lowered bone mineral density (19). Hence we see that under similar experimental methods and using similar outcome variables (Dual energy X-ray absorptiometry measurements, whole body mineral content and BMD) the fat-bone relationship cannot be easily identified and conclusively defined. It might vary with age and ethnicity among other factors. Fat tissue

is a source of androgen derived estrogen hormone and hence an improvement in bone mass with increased deposits of fat (and consequent increase in levels of estrogen the lack of which causes osteoporosis) has an understandable mechanism behind it(20). Hence, an increase in fat deposits, especially after menopause, has been considered important in osteoporotic women for reasons other than increased load bearing. Load bearing itself, as mentioned before, is a more evident and direct cause for increased bone strength with increase in fat mass. However, it must be remembered that fat tissue is also a source of the hormone leptin, which has been shown to negatively affect bone deposition(21). Whether the positive effects of increased fat deposits and load bearing and increased estrogen production sufficiently offset the negative effects of increased leptin levels in influencing bone mass is unclear. In many studies, examining the relationship between load bearing and bone mass, lean tissue mass was found to have a stronger relationship with bone mass than fat tissue mass and hence was a better predictor of risk of osteoporosis (16). Hence, it is of significance to examine whether the advantages of increased load-bearing can be allowed to exist in form of more adipose tissue deposits or must be in the form of lean tissue to confer a protective effect on bone. A widespread problem in studies with human subjects is the difficulty in obtaining a sample set that is both large enough and minimizes variability. Nevertheless, such studies have played an important role in establishing the fat-bone relationship at-least in the context of certain age groups. Conclusions from such studies are often based exclusively on DXA measurements, which give a more systemic idea of mineral content but are not effective in estimating other outcome measures that are more region specific or tissue specific, such as material properties or micro-architecture. Further insight can be gleaned by separating bone into its two compartments namely trabecular and cortical and examining them individually. It is now known from animal studies that trabecular bone and cortical bone can have very different formation/resorption responses to external or internal stimuli(22-23). This can perhaps be attributed to the fact that trabecular bone has a high turnover rate and is more metabolically active – hence more prone to show effects.

High Fat Diet Consumption and Bone Health: As mentioned earlier, the cause of obesity can, in many cases, be the consumption of a high-fat diet and the direct effect of such a diet on bone must be considered. It is possible, that in cases where the increased

weight bearing is a result of consumption of a diet high in fat content, the diet by itself may interfere with bone formation. Much research has been done on how the mechanical strength, mineral content and material properties of bone change in response to high fat diet consumption. For example, Krey et al. have shown that fatty acids found in high-fat diet directly interact with peroxisome proliferator-activated receptors to modulate gene expression (24). Both adipocytes which constitute fat tissue and osteoblasts which are essential to bone formation are derived from the same progenitor cell in the bone marrow and genes such as PPAR-γ are involved in maintaining the inverse relationship of adipogenesis-osteogenesis in the bone marrow environment (12, 25). Other studies have also shown how the genes PPAR-γ and Bmd-8 interact to form a relationship between diet intake and bone mineral density (26). Thus, fatty acids may be initiating adipogenesis at the expense of reduced bone formation and decreased bone volume. This effect may be exacerbated by the reduced absorption and increased urinary excretion of calcium. Coming back to the different responses of different parts of bone tissue, a study done on roosters found that consumption of a high fat diet for 20 weeks decreased cancellous bone mineral content, bone strength as seen through mechanical testing and impaired bone material properties while having no effect on cortical bone (27). In mice, it has been shown that consumption of a high-fat diet for 19 weeks can reduce the bending stiffness and fracture toughness of cortical bone while, at the same time, increasing the physical dimensions of the bone samples. Hence, although seemingly "more" bone is present probably due to attempts of the skeleton to compensate for increased weight bearing, it can be of poorer quality (28). Similar decreases in mechanical properties of cancellous bone were found in roosters, fed on a high fat diet for 20 weeks, while there was no effect on cortical bone (27). Kyung et al. showed similar results that the bone mineral density of the femur is reduced, when mice are fed a high fat diet for 19 weeks, and also showed that this deterioration of bone quality is accompanied by increased osteoclastogenesis in the bone marrow of these mice (29). Cao et al. have used micro computed tomography technology to examine the effects of high fat diet consumption on the micro-architecture of bone tissue. In agreement with other studies, it was found that trabecular bone volume decreased and trabecular separation increased in the high fat diet group of mice. However, the body weight of the mice in the high fat group was 31

percent greater than the control group perhaps accounting for the differences in micro-CT data (23). This highlights an important problem, commonly found in diet studies, that in cases where experimental and control groups vary in terms of body weight, it is difficult to separate the effects of diet, effects of increased fat deposits and the effects of increased weight bearing. One way of doing this is to correlate body weight to parameters of bone quality as a covariate. However, as discussed before, weight consists of both fat tissue and lean tissue and further confounding factors may arise if such an approach is used. All in all, majority of the evidence suggests that although the protective effect of diet induced increased adiposity in form of increased weight bearing is real and significant it is often overshadowed by other negative effects of adiposity and the high-fat diet which causes it.

Carbohydrate Diet Consumption and Bone Health: While a cure for osteoporosis remains to be found, one way of combating the disease is to isolate factors which interfere with the deposition and build up of bone in early adulthood. The effects of consumption of a high-fat diet have been established, to some definite degree. Another type of nutritional intake which should be considered is the consumption of high quantities of carbohydrates such as sucrose, fructose, glucose and high fructose corn syrup. Carbohydrates are an integral part of the daily diet, found for example in the form of starch. Carbohydrates are the primary energy source of the body and insufficient consumption of sugars leads to malnutrition in form of inadequate caloric availability. On the other hand, excessive consumption of sugars, especially of the refined type can lead to obesity, in most cases. Lately, sucrose has been replaced by high fructose corn syrup (HFCS) in many food products. HFCS is also a main ingredient of aerated beverages, the consumption of which, on a daily basis, has been found to deteriorate bone health (30). Sugars such as fructose and sucrose may alter the mineral balance of the body, besides decreasing calcium and phosphorous retention. For example, a study done in a cohort of young men found that high dietary fructose disrupts the magnesium balance, increases the urinary content of phosphorous and reduces the calcium balance, thus disrupting the homeostasis of minerals besides increasing serum alkaline phosphatase levels (31). Similar findings have been reported in rats where urinary calcium is increased in response to consumption of water sweetened with fructose/glucose/high fructose corn syrup (32). It has also been suggested that sugars such as fructose cause damage to bone

because they replace milk in the daily diet and there is not any significant direct bearing of sugar-consumption on bone health (33). In any case, since identification of measures which help in prevention of osteoporosis are considered important in absence of a cure, it is essential to study how carbohydrates which are a major part of fast-foods, beverages and the overall daily diet, affect bone health. Fewer studies have been done in this research area than in regards to high-fat diet consumption. However, findings in literature suggest a definite link between high-carbohydrate consumption and bone quantity and quality. For example, a study done in a group of high-school teenage girls found that fracture risk and consumption of cola beverages are significantly related (34). Tsanzi et al. showed that administering sugar sweetened drinking water to rat's altered urinary calcium and Ca retention. While there were no significant differences in femoral BMD or BMC in any of the groups compared to controls, the bone mineral density and bone mineral content seemed to be lowest in the group administered glucose compared to fructose (32). Another group examined the effects of a high-fat sucrose (HFS) diet on the material properties of bones in rats. It was found that vertebrae of animals in the HFS group had significantly lower mechanical loads and compressional rigidity, compared to animals on a low carbohydrate diet. Vertebral mineral content was also found to be reduced in HFS animals. A sucrose diet seemed to decrease the strain energy, which the vertebral body could absorb before breaking. However, the study did not have a control group of animals fed only a standard chow diet (35). Similarly, Tjardane et al. showed that increased sucrose ingestion can reduce the breaking strength of the tibia and femur and also reduce the calcium content of tibia and femur, in Wistar rats (36). Other groups have indicated that this deterioration of bone quality might be attributable to increased osteoclastogenesis. A study with BL6 female mice showed that consumption of a high fat sucrose diet for 10 weeks increases the serum TRAP staining levels but does not affect osteocalcin levels. Further, RANKL was found to be up-regulated in mice on the sucrose diet. Using micro-computed tomography the study demonstrated that the sucrose diet decreased cortical bone area, thickness, in addition to decreased peak loads (37). It must be noted however, that the body weight of the mice on the HFS diet was significantly greater than control. Li et al. showed that a high-fat sucrose diet decreased the maximum load and failure energy and tensile stress of tibias (38). The high fat sucrose diet seems to

exert a measureable and significant deleterious effect on the material properties of bone. While it may be a little out of the present context, another study of interest was performed by Terada et al., which showed that increased concentration of glucose directly affects bone marrow stromal cell line in-vitro, in terms of decreased proliferation (39). Thus, previous studies give a clue towards understanding the effects of sucrose/high-fat-sucrose/glucose diets on bone strength. None of these studies, however, have focused exclusively on fructose, the most important ingredient of regularly consumed beverages. Focus has been on assessing changes in bone material properties and strength, but the effects on trabecular/cortical bone have not been separately studied. Few of the studies have closely monitored or taken into consideration body weight, fat deposition while interpreting results. There is a need to examine the effects of regular fructose consumption, on both, the micro-architecture and density of trabecular and cortical bone separately. High sugar consumption also causes major changes in glucose and insulin levels and often leads to a diabetic state. It is entirely possible that the changes seen in the skeletal system may be correlated with such metabolic changes.

Insulin and Glucose – Interactions with Bone: Impaired glycemic control and insulin imbalance may be predictors of bone health and are often the result of high-fat-fructose consumption. It has been shown that diabetic individuals have an increased fracture risk (40). Although the relationship between diabetes and bone mineral density has been studied, it is unclear how BMD and BMC are affected by insulin levels. In a majority of studies, insulin levels are found to be positively related to BMD and BMC. While obesity, dietary intake and insulin/glucose imbalances or a diabetic state are not always found together or dependent on each other, they are definitely not unrelated. Obesity, for example, is often associated with increased insulin levels and insulin resistance(41). Poor glycemic control or disturbance in insulin levels can be part of the chain of events which explain the effect of obesity on bone. It is interesting then to introduce two additional factors, namely insulin levels and glucose levels, along with fat mass in any experiment which aims to document the effects of dietary intake on bone health. Consumption of a high fat and a high-fructose diet has been shown to induce hyperglycemia, either due to insulinopenia, or insulin-resistance(42). Insulin has been shown to affect the proliferation and function of osteoblasts via actions similar to IGF-1. Insulin receptors are also found

on osteoclast like cells(43). Abnormally high levels of insulin and glucose eventually lead to diabetes, which has been associated with osteopenia and poor bone material properties (44-46). Diabetes also leads to hyperglycemia, and increased circulating levels of glucose have been shown to have an association with reduced bone mass and quality. Hou et al. showed that when diabetes is artificially induced in rats, the increased glucose levels have a negative correlation with normal stresses of the femoral neck. Diabetic rats (increased glucose levels) also saw a reduction in femur mass and length, bone area and structural strength (47). In a study done with Type 2 diabetic men, Kanazawa et al. showed that, the levels of hemoglobin H1ac were negatively correlated with levels of osteocalcin (48). Surprisingly another study found that glycosylated blood glucose levels were inversely correlated with trabecular bone density in the tibia and whole body bone mineral content in adolescent Type-1 diabetes patients (49). There is also data suggesting that, besides decreasing BMD, glucose imbalance can increase resorption of bone (44). In contrast, some reports suggest that bone mineral density is in-fact increased in diabetic patients though it comes with a higher fracture risk (50). Either ways, hyperglycemia seems to affect the quality more than the quantity of bone. The relationship between glucose levels and bone material properties may be explained by non-enzymatic glycosylation (NEG). Glucose intolerance and increased levels of glucose in blood can lead to an increase in the deposition of glycation end products in the extra-cellular matrix. These end products, may interfere with collagen deposition, or even cause damage to existing collagen fibers. Advanced glycation end products or AGE's, can interfere with collagen network cross-linkage, interaction of collagen fibers with other cells/matrix components and flexibility of collagen leading to a stiffer bone that is more prone to brittle fractures (51). Vashishth et al. provided some in-vitro proof of this by culturing cortical bovine bone samples in ribose. Increased non enzymatic glycation was responsible for stiffening of the collagen network in-vitro (52). Glucose may also interfere with calcium uptake. In-vitro studies have shown that, when MC3T3 osteoblastic cell line is cultured in presence of high glucose levels, calcium uptake by bone nodules and calcium deposition into culture in significantly reduced (53). It is possible then, that insulin has an indirect protective effect on bone quality, because it checks glucose levels. Whether or not insulin is also considered to be directly responsible

for modulating bone parameters is unclear. Claims in favor of a direct insulin-bone relationship are based on the fact that insulin may have similar functions as insulin like growth factor (IGF-1), albeit on a smaller level and also that receptors for insulin have been found on osteoclasts (43). IGF-1, in its part, seems to play a prominent role in bone development (54). There is data to show that insulin levels are inversely proportional to bone mineral content in certain populations (55). In the study by Hou et al. administration of insulin after inducing diabetes partially overcame the detrimental effects of increased glucose levels on femur properties (47). In another study, Type-2 diabetes patients (hyperinsulinemia) had a higher BMD although they also had an increased fracture risk (50). However, in a normal glycemic state, hyperinsulinemia leads to reduced bone turnover, affecting both resorption and formation of bone (45). Then, there are also studies which have shown that after adjusting for factors such as age and BMI, there are no tangible associations between insulin levels and BMC (56). A study by Irwin et al. offers direct evidence that insulin may not have any role to play in bone health maintenance. The study uses genetically reconstituted insulin receptor knockout mice that do not express insulin receptors in the bone but have a normal blood glucose level. It was found that in the absence of insulin receptors, there were no differences in bone density and cortical or trabecular bone volume fraction between the knockout and wildtype mice (57). In any case, it is known that there is a link between insulin and urinary excretion of calcium and this could be a factor having implications on bone strength (58). Thus, it can be seen that while increased glucose levels seem to be predictors of deteriorating bone health, it is difficult in the case of insulin to establish an unambiguous association with bone. It is also difficult to tease apart the individual effects of insulin and glucose since an up-regulation or down-regulation in one leads to unavoidable changes in the other. Despite these difficulties, it is obvious that diet induced changes in insulin and glucose levels must be included in any study that examines the relationship between diet, fat and bone. Especially since, increased fat tissue accumulation is sometimes accompanied by reduced glucose tolerance, it is important to see if changes in density or micro-architecture of bone are accompanied by changes in fat tissue accumulation and further changes in insulin/glucose levels. Diet induced alterations in BMI can work both for and against weight-bearing. While a high-fat diet or a high-carbohydrate diet may cause

weight gain, if it also causes diabetes and diabetes related weight-loss, it may cause unloading of bone. Glucose balance is essential to numerous bodily functions and increased levels of glucose in the body and glucose intolerance can cause many systemic harmful effects such as muscle wastage, artherosclerosis, interference with proper skeletal muscle contraction and attrition of endothelial function (59-60). Skeletal muscle contractions are very important in maintaining bone formation because they act as an anabolic stimulus load which induces bone formation. Hence, there are many pathways via which the sequence of events which start with diet induced glucose/insulin imbalance can affect bone health. Most studies in this regard have focused solely on bone mineral density, whole body mineral content or fracture risk as representative outcome parameters of bone health. While fracture risk is undoubtedly important because it has direct clinical relevance, other parameters, such as those related to the micro-architecture of bone, have not been observed in sufficient detail. Other confounding factors such as body weight, fat tissue content have also not been given their due importance. Finally, there are the problems associated with obtaining a variability-free population of human subjects that do not differ in activity levels, diets, lifestyles, diabetes treatments, age etcetera. There is hence, a need to study glucose and insulin levels, fat content, body weight, food intake, bone micro-architecture and density in parallel in a controlled animal study where variations due to genetics or activity levels are minimal.

Mechanical Signals to Regulate Bone Formation: As mentioned before, mesenchymal stem cells in the bone marrow give rise to both adipocytes and osteoblasts and hence the etiology of both conditions is related to a shift in the balance of osteogenesis vs. adipogenesis (25). A controlling influence which would be able to bias lineage selection towards increased osteoblastogenesis would then prove to be a potential tool for curing both obesity and osteoporosis. As mentioned before exercise is a commonly recommended prevention tactic for both diseases. There is evidence to prove that exercise reduces obesity not only because it “burns fat” but also because mechanical signals of a regular pattern and frequency can be perceived by stem cells as an indication that increased bone formation and reduced fat formation is required (25, 61). However regular exercise that generates high levels of strains is not a feasible option for certain affected population segments such as overly obese, elderly, paralyzed or bedridden individuals.

Compliance to exercise is poor and physical activity is declining in both young children and elderly individuals. Recently it has been proposed that low magnitude high frequency mechanical vibrations also known as whole body vibrations (WBV) can also act as anabolic mechanical signal to bone(62). Bone tissue is highly dynamic and regulated, constantly altering bone formation and resorption rates to maintain a bone structure that is suited to the demands imposed on it. In tennis players for example there is notably increased bone mass in the dominant arm used for playing(63). Bones of astronauts on the other hand adapt to their low-gravity environment by gradually losing ~2% BMD every month(64). In an average individual, loads on the skeleton consist mainly of daily activities such as walking, standing etc which generate continuous, very low strain and high frequency muscle contractions(65). Therefore it makes sense that osteoblasts and osteoclasts are activated in response to such low level physiologic strain application instead of very high-loads which are rarely experienced(65). These strains can be transduced from the outside environment to the bone marrow cavity via muscular contractions which are known to stimulate bone formation. However these mechanical loads can also cause fluid flow which can be sensed by cells in the marrow(66). It is possible that whole body vibrations of low magnitude mimic muscular contractions and if applied on a regular basis at certain constant frequency and duration can generate an anabolic response. Results from testing and development of whole body vibration regimens have estimated that WBV of 0.2g magnitude at high frequencies generate strains of less than 10 microstrain when transmitted to the load bearing bones(67). Thus WBV treatment has been found to be safe when applied daily to the fragile skeletons of elderly individuals(68) and developing bone in early stages of growth(62). Besides having an anabolic effect on the skeleton such treatment also improves muscle strength and overall balance in elderly individuals, reducing risk of falling(68). The efficacy of these vibrations in causing a population shift in the bone marrow stem cell niche by decreasing the number of hematopoetic stem cells and increasing the number of mesenchymal stem cells has been shown(25). Furthermore Luu et al. showed that these signals are capable of simultaneously increasing bone volume fraction and reducing visceral adiposity in mice. The encouragement of osteoblastic differentiation and suppression of adipocytic differentiation was evident by the increases in RUNX2

transcription and decreases in PPARγ (25). Xie et al. have shown that 15 minutes of daily exposure to WBV can increase trabecular bone volume, cortical bone area and mineralized bone surface area(62). Similarly, WBV also increases bone mineral content and trabecular number while reducing trabecular separation, promotes plate shaped trabeculae and an improvement in material properties of femurs in sheep (69). Hwang et al. showed that this osteogenic effect was noticeable in non-load bearing bones such as the cranium as well(70). Besides being anabolic, there is evidence that WBV has an anti-catabolic effect inhibiting osteoclastic activity in the tibia(71). Studies have demonstrated that whole body vibration therapy is an effective anabolic signal to bone not only in young subjects(72) but also in a relatively elderly postmenopausal group of women where any therapy aimed at bone regeneration has to override conflicting hormonal deficiencies(73).

There are many factors such as age, sex and nutrition which may influence the response of the skeleton to WBV treatment (74-75). Genetic differences for example can cause differences in the magnitude of response(76). A study by Judex et al. demonstrated this by testing WBV in three strains of mice and found that the anabolic response was largest in strains which are genetically programmed to have low baseline bone mass(77). Others have found that ovareictomized rats are less sensitive to WBV compared to normal rats suggesting that perhaps hormonal balance plays a role in WBV induced bone tissue changes (78). If low bone mass caused by genetic makeup increases the sensitivity of the skeleton to mechanical signals then perhaps it is possible that low bone mass that is caused by a decrease in bone formation or an erosion of bone tissue will also increase the sensitivity of the skeleton to mechanical signals. Studies have shown that with age the skeleton becomes relatively insensitive to externally applied loads(79) probably because of age weakened ability to form new bone. But it is not clear if bone loss due to nutritional factors can enhance/dampen the ability of bone cells to initiate repair mechanisms in response to WBV treatment. Nutritional intake is a strong modulator of bone health, adipose tissue accumulation and enzymatic imbalances. It is not known if the response to WBV affected by regular consumption of certain kinds of diets. WBV has been shown to successfully inhibit adipogenesis and promote osteogenesis in dietary induced obesity (25). Similarly can WBV inhibit adiposity and promote increased bone

mass and quality in cases of high sugar consumption? As mentioned before, a high sugar diet is a proven cause of low BMD and poor material quality of bone either directly or via insulin-glucose mediated pathways. Metabolic imbalances are linked to decreased BMD and bone mass. Fatty acids present in a high-fat diet can directly control gene expression in the bone marrow cavity(24). High-sugar diets can sometimes cause weight loss and muscle wastage as is often seen in diabetic individuals(80). Loss of body mass can cause unloading of the skeleton leading to reduced bone formation. Thus there are many inter-related pathways via which nutritional intake has a very strong controlling influence on the rates of bone formation and resorption. It is not known whether signals generated by WBV are large enough to induce bone cells to increase bone formation in the presence of all these other biochemical influences. Or else it is possible that WBV may influence metabolic, hormonal status to bring about changes in bone mass. For example studies have tried to examine the effects of WBV on insulin, glucose levels without any conclusive findings(81). The second part of this research project aimed at generating preliminary data to look for interactions between type of dietary intake and effects of WBV on load bearing bones. A second goal was to test the efficacy of WBV in improving bone mass and morphology in bone tissue that faces developmental challenges due to poor nutrition.

CHAPTER TWO

Hypothesis and Specific aims

Nutritional imbalances, increased intake of sugar and fat are very common lifestyle characteristics of the average individual today. The aim of this research is to document in a controlled in-vivo study the detrimental effects of increased high-fat and high-fructose consumption on diet induced obesity, complications related to DIO and on the skeleton. While genetic and hormonal predispositions to osteoporosis and obesity are preset and cannot be altered, identification of nutritional factors that increase the risk of obesity and bone loss and an understanding of the relationship between fat and bone will add to the existing knowledge of how to entirely prevent, delay onset or attenuate the severity of these diseases that currently don't have a cure. Many studies have proven the efficacy of whole body vibration therapy on increasing bone mass and reducing adiposity. The consumption of high-fructose drinks is ever increasing and is negatively associated with BMD. This research aimed at documenting the efficacy of vibrations in treating bone loss caused by a high fructose diet and a high fat diet.

The overall hypothesis of this study was that diets high in fat content and diets high in sugar content cause accumulation of adipose tissue, deterioration of bone mass and structure which is related to imbalances in the enzymatic activity of glucose and insulin. 15 minutes of daily whole body vibration treatment at 0.2 g and 90 Hz can restore deteriorated bone quality and decrease the risk of obesity and diabetes.

Hypothesis 1: Compared to control, both high fat and high-fructose diets have a catabolic effect on the load-bearing bones. Bone density and volume negatively correlates to increases in adiposity and negatively correlates to glucose levels. Detrimental effects of the increased fat and fructose consumption on the bone phenotype is evident as early as 6 weeks and sustained, magnified over the longer time period of 15 weeks.

Specific aim 1: To document and compare the effects of a high fat diet and a high fructose diet on onset of obesity as seen by adipose tissue accumulation, onset of a diabetic state as seen by enzymatic imbalances and an associated increase in bone

degeneration as seen by reduction in BMD values, cortical and trabecular bone volume and deterioration of trabecular micro-architecture at 6 weeks and 15 weeks.

Specific aim 1(a): To compare the severity of the obese and diabetic condition caused by consumption of high-fat and high-fructose diets over a short (6 weeks) and long (15 weeks) time-period as seen by body weight, primary fat pad weights, abdominal fat content, plasma glucose and insulin concentrations.

Specific aim 1(b): To use micro-computed tomography to quantify diet induced alterations in cortical and trabecular bone morphology, bone volume and BMD in the tibia, femur over a short (6 weeks) and long (15 weeks) time-period.

Specific aim 1(c): To use regression analysis at each time-point to quantify the relationship between extent of obesity as seen by quantity of abdominal fat and values of tissue mineral in the tibia and femur. Further use regressions to quantify associations between plasma levels of glucose, insulin and bone volume, density, descriptors of trabecular structure such as trabecular thickness.

Hypothesis 2: WBV inhibits the increase in deposition of adipose tissue due to a high-fat and high-fructose diet and decreases the risk of obesity while simultaneously counteracting the deleterious effects of high-fat and fructose diets on bone as seen in form of an increase in, or restoration of tissue mineral density values, bone volume and an improvement of trabecular micro-architecture.

Specific aim 2: To test the efficacy of low magnitude high frequency mechanical signals (whole body vibrations, WBV) on preventing diet induced obesity, reducing adipose tissue deposition, promoting an increase in tissue mineral density values, bone volume and structural integrity of trabecular micro-architecture and acting as a restorative treatment to counteract the damaging effects of high-fat, fructose diet on the aforementioned parameters of bone health.

Specific aim 2(a): To use in-vivo micro-CT and fat pad mass measurements to ascertain the efficacy of WBV in suppressing obesity as seen by decreases in abdominal fat volume in vibrated control, high-fat and fructose diet groups compared to untreated.

Specific aim 2(b): To use micro-computed tomography to evaluate the effectiveness of WBV in ameliorating bone loss induced by high fat and fructose diets as seen by a recovery/increase of tissue mineral density, bone volume and restoration of trabecular micro-architecture in tibia and femur of vibrated groups as compared to untreated.

Specific aim 2(c): To quantify the effect of WBV on restoring glycemic control by examining differences in insulin, glucose levels of vibrated and untreated groups and to check if increases in bone quantity and density and decreases in adipogenesis are accompanied by alterations in glucose and insulin levels suggesting an overall decrease in the risk for diabetes induced osteopenia.

CHAPTER THREE

Effects of Dietary Intake on Bone Health

Introduction: Obesity is a chronic clinical condition that has a severe impact on quality of life and causes other health complications such as diabetes. It is now also known that obesity might contribute to the deterioration of the skeleton as well and have implications for the onset and severity of osteoporosis (15, 19). The longstanding dogma that excess adiposity has a protective effect on the skeleton is now being questioned by new evidence which suggests that increased adipogenesis may reduce osteogenesis because both cell types originate from the same stem cell source in the bone marrow(25). While an effective cure for osteoporosis and obesity has not been found, increased deposition of bone during young adulthood is recommended as a precaution to ameliorate severity of bone loss later on. It is, therefore, important to identify the factors which interfere with the healthy deposition and growth of bone tissue. Studies which have been carried out to establish a relationship between bone mineral content/density and fat mass have come up with opposing results (14, 17, 19). For example, it is unclear if the protective effects of obesity, estrogen production and load bearing can combat the catabolic effects that increased leptin production has on bone mass(21). Further, it has been shown that fatty acids, found in high-fat diet, directly interact with peroxisome proliferator-activated receptors in the bone marrow to modulate gene expression and cause bone resorption(24). Clearly there are many factors which modulate the fat-bone relationship.

Diabetes and the associated glycemic imbalance have also been associated with obesity and recently with osteoporosis (44, 49). Sugars, such as fructose and sucrose, are known to alter urinary calcium retention(58), mineral content(31), breaking strength of the tibia and femur (36) and reduce calcium content besides increasing osteoclast activity(37). Increased carbohydrate consumption also leads to impaired glycemic control (82-83), which may be another mechanism for high-fat-fructose diets to alter the bone formation-resorption balance. Insulin has been shown to affect the function of osteoblasts (84) and abnormal levels of insulin/glucose cause diabetes which is associated with osteopenia and poor bone material properties (44, 49). Hyperglycemia has been shown to have an association with reduced bone mass and reduced BMD (44, 47) perhaps, because glucose

interferes with effective calcium uptake. Glucose intolerance can also cause muscle wastage which can interfere in bone maintenance(80). Diet induced changes in insulin and glucose levels must therefore be included in any study that examines the relationship between diet, fat and bone.

The goal of this study was to determine if changes in density or micro-architecture of bone are accompanied by changes in fat tissue accumulation and in insulin/glucose levels. We also wanted to examine whether diabetes related weight-loss causes unloading of bone. Most studies in this field have drawn correlations only from BMD or BMC measurements using DXA. How high-fat and high-fructose diet consumption affects details of trabecular and cortical bone morphology remains unclear. Further, hardly any studies have tried to correlate body-weight, bone and fat accumulation parameters with insulin and glucose levels. Comparison of long term effects versus short term effects of diets have not been experimentally documented. These questions formed the basis for the first part of this project namely

1) To use micro-computed tomography to compare the effects of a high-fat and a high fructose-diet on bone structure, mass and density.
2) To quantify differences in abdominal fat volumes of different diet groups and examine association of abdominal fat content with parameters of bone structure and mass.
3) To measure insulin and glucose levels along with fat volume to determine whether changes in bone/fat are due to induction of a diabetic state or enzymatic imbalance.
4) To examine differences in results at two different time-points (6 weeks and 15 weeks).

Methods

Animals and Diets: 30 BALB/c mice for the 6 week study and 30 BALB/c mice for the 15 week study were ordered from Jackson Laboratories and randomly assigned into either a high-fat diet (HF), high-fructose diet (CH) or a control diet (SC) group (n=10/group in each study). The high-fat diet was in form of pellets obtained from Purina Test Diet Inc., which is part of the "van-heek" series of lab animal research diets (Purina van-heek 58V8 diet) and provides 45% k cal from fat. For the 6 week group of animals on the high-fructose regimen, diet was in form of pellets obtained from Harlan Teklad and was a 60% fructose diet that provided 66.8% kcal from carbohydrates. However, the food intake of the animals was significantly lower than control over the entire period of the study, leading to weight loss. Hence, for the 15 week study, the high-fructose regimen consisted of 10% w/v powdered fructose being administered by mixing with drinking water. Animals were hence fed standard chow diet and 10% high-fructose water. The control group at both time-points consisted of animals fed a standard lab-chow diet. Animals were housed in individual cages with a 12 hr light/dark cycle and given free access to food and water at all times. All animals were 7 weeks old at the beginning of the study and were treated for 6 weeks or for 15 weeks.

Body weight and food intake: Body weight and food intake in both experiments were recorded on a weekly basis. In the fructose group, in the 8^{th} week of the 15 week experiment one mouse had to be euthanized because of excessive weight loss. In an attempt to monitor the amount of water being consumed every week in the fructose group the standard water-bottles were replaced with smaller graded containers. However, the animals were not able to acclimatize themselves to using these, which led to water deprivation for 3-4 days. Animals in the fructose group saw a sudden decrease in body mass. However, after resorting back to old water containers all animals, except one, recovered their body weight in the span of about a week, after which they showed normal cage activity, appearance and food intake.

In-vivo Micro-Computed Tomography to Quantify Abdominal Adiposity: The abdominal regions (lumbar L1 to L5) of all animals were scanned in-vivo using micro-computed tomography to quantify central adiposity. For the 15 week experiment, n=10

for all groups, other than the fructose group where n=9. Animals were scanned using Viva-CT 75 scanner, Scanco Medical at a resolution of 82 µm. Animals in the 6 week treatment group were scanned at 12 weeks of age and sacrificed at 13 week of age, after 6 weeks of treatment. Animals in the 15 week treatment group were scanned at 20 weeks of age, and sacrificed at 22 week of age. Before scanning, isoflurane inhalation (1%) was used to induce general anesthesia. Animals were scanned by securing them to an animal bed with both hind-limbs stretched out and secured. The resolution and energy settings (82 µm, 45 KV, 133µA, 300 ms integration time) were chosen taking into account factors such as scan time, exposure of animal to X-ray radiation and required tissue detail.

The gray scale scans were then reconstructed into 3 dimensional images of the visceral and subcutaneous fat, contained in the torso of each animal, using an automated separation script written in the image processing language (IPL) and the software provided by Scanco. The landmarks used to define the region of interest for analysis were the lumbar L1 to L5 vertebral disks. The script was used to apply image thresholding (to delineate fat from lean and bone tissue), Gaussian filtering (for noise removal) and edge detection techniques on the selected region of interest, to quantify the total fat volume and separate the visceral adiposity from the subcutaneous adiposity. The functioning of the script is described in detail elsewhere (85)

Tissue Collection and Preparation: All animals were sacrificed at the 6 week or 15 week time-points. Blood was collected via cardiac puncture with the animal under anesthesia after overnight fasting. Plasma was isolated via centrifugation at 14,000 rpm, 4 degrees for 15 mins. Mice were euthanized via cervical dislocation. Right femurs and tibiae were harvested and preserved in 70% ethanol for micro-computed tomography scanning. The weights of the epididymal fat pad, brown fat pad were recorded.

Ex-vivo Micro-Computed Tomography to Establish Bone Phenotype: The morphology of the proximal tibia and distal femur was examined using micro-computed tomography scanning. The proximal tibia and distal femur are regions where effects of nutrition have been previously found to be manifested to a significant degree.

The distal metaphysis of the femur was scanned ex-vivo using micro-computed tomography (Micro-CT 40, Scanco Medical) at a resolution of 12 µm. This resolution is sufficiently accurate to provide detailed data on cortical and trabecular BMD, mass and micro-architecture, while not being excessively high so as to introduce unnecessary noise.

Femurs were carefully cleaned of adherent tissue and held in place in the sample holder using foam. An ethanol medium was maintained throughout the scan. For the distal femur, a tissue volume of 1500 µm was chosen for analysis. For identification of the start and end of the region of interest, the landmark used was the point where the growth plate starts to disappear. This point was chosen qualitatively, by visual slice by slice examination of the gray scale scans. After identifying the slice where a break appears in the growth plate, a region of 600 µm was subtracted from this point so as to ensure that the ROI is entirely away from the growth plate and in the metaphysis. A region of 1500 µm was then chosen and submitted for analysis using an automated separation algorithm that delineates cortical and trabecular bone and reproduces 3 dimensional images of the gray scale scans. A detailed explanation of this algorithm can be found in the publication by Lublinsky et al (86).

For the proximal tibia a region of 1080 µm was chosen for analysis. Bones were prepared for analysis as described above. Similar to the protocol followed for the femur for identification of the start and end of the region of interest, the landmark used was the point where the growth plate starts to disappear. 300 µm distal to this point, a region of 1080 µm was chosen for analysis. Bones that were damaged/broken during extraction or cleaning were excluded from analysis.

Determination of Plasma Glucose Concentrations: Plasma samples were sent to the Metabolic Mouse Phenotyping Centre at the University of Cincinnati for assaying the plasma concentrations of glucose. The assay was carried out using a glucose assay kit from Diagnostic Chemicals Limited. Standards were obtained from NERL diagnostics.

Briefly, the reagent was prepared by adding 100 ml of de-ionized water and mixing gently as per instructions on kit. 200 µl of reagent was mixed with 2 µl of either

deionised water or standard or the sample plasma and the plate was left to incubate at room temperature for 10 minutes. Absorbance of the standards and each sample was determined at a wavelength of 505nm. The glucose concentration in the samples was determined from the standard curve. The standard dilutions consisted of 300, 200, 100, 50, 25 and 0 (mg/dl).

Determination of Plasma Insulin Concentrations: Plasma samples were sent to the Metabolic Mouse Phenotyping Centre at the University of Cincinnati for the insulin assay. The assay was carried out using a mouse endocrine lincomplex kit from Linco (Millipore) (87).

The endocrine standard was reconstituted with 250 µl of deionised water to give a 10,000 pM concentration of standard solution. 180 µl of this solution was mixed with 220 µl of Assay buffer to give a 4500 pM standard concentration. This standard solution was then further diluted using assay buffer to give 1500, 500, 166.7, 55.6, 18.5 and 6.2 pM standard concentrations. Only assay buffer was used for the 0 pM standard (background). The endocrine controls provided with the kit were reconstituted with 250 µl water. 30 ml of wash buffer was mixed with 270 ml of deionized water. The insulin antibody-immobilized beads provided were prepared for assay by mixing 0.15 ml of beads with 2.85 ml of deionized water. Finally the plasma matrix was prepared by adding 1 ml of water to the vial provided. For the assay, the filter plates were blocked using assay buffer, mixed, vacuumed and dried. 10 µl of assay buffer was added to the 0 pM standard (background) and to each sample well. 10 µl of standard or control was added to the appropriate well. 10 µl of matrix dilutent and 25 µl of bead solution were added to standard, control and sample wells. 10 µl of plasma samples were added to appropriate wells. The plate was incubated on a shaker overnight, after which the fluid was removed and plate was washed with wash buffer. 50 µl of detection antibody was added to each well and incubated for 60 minutes at RT. 50 µl of Streptavidin-Phycoerythrin was added to each well, incubated for 30 minutes, following which all contents were vacuumed out. Finally, the plate was washed using buffer and sheath fluid was added to each well. The plate was read using a Luminex Bioanalyzer instrument. Values of insulin levels were calculated using the standard curve.

Determination of Plasma Triglyceride Concentrations: For the 6 week experiment, samples were sent to the Mouse metabolic phenotyping centre for triglyceride analysis. The assay was carried out using a kit from Randox (88).

Briefly, the reagent was prepared by reconstituting the vial with buffer. 2 µl of deionized water, standard or sample was added to appropriate wells. 200 µl of reagent was added to each well and gently mixed and then incubated for 5 minutes at RT. Absorbance values were determined by reading the plate at 500 nm. The values for samples were calculated using the standard curve. 200,100, 50, 25, 12.5, 6.25, 0 (mg/dl) concentrations were used as standards. For the 15 week studies, the assay was run using a plasma triglyceride kit by Sigma-Aldrich (25). Briefly, the glycerol standard was used to obtain a standard curve concentration of 0, 0.09, 0.166, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.5 µg/µl. The volume was totaled to 15 µl in each well. The free glycerol reagent and triglyceride reagent was reconstituted with ddH20 following kit instructions. 160 µl of free glycerol reagent and 40 µl of triglyceride reagent were added to each well followed by standards, 15 µl of ddh2o for blanks and 15 µl of samples in appropriate wells. The samples were found to be too concentrated, so the assay was repeated after diluting the samples using deuterated water in 3:1 ratio. Plate was placed on a shaker for 30 minutes and absorbance values were determined at 540 nm. Concentrations of triglyceride were calculated using equations obtained after plotting known concentrations and optical density from standard curve.

Statistical analysis: The three diet groups standard chow (SC), fructose carbohydrate (CH) and high-fat (HF) were compared using a one-way ANOVA followed by Fischer's least significant difference post-hoc tests.

Results

Body Weight: In the 6 week experiment, all groups had similar body weights for the first three weeks. Starting at week 4 and continued through week 5 and 6, the body weight of fructose group (CH) was significantly lower than control (SC) and high fat (HF). The SC and HF groups, at end of week 4 were 2.6 grams (10%) and 2.4 grams (9%) heavier than the CH group ($p<0.005$), at end of week 5, 4.0 grams (15%) and 3.7 (14%) grams heavier than the CH group ($p< 0.001$) and at end of week 6, 5 grams (18%) and 4 grams (16 %) heavier ($p< 0.001$). However, the SC and HF groups were not significantly different from each other in body weight throughout the study.

In the 15 week experiment, up until end of week 13 there were no significant differences between the body weights among the three groups. Only at the end of week 14 was the HF group heavier (2.8 grams, 9%) than control although this difference was only marginally significant. (F statistic 0.05, p=0.01). In both experiments the weights of HF did not differ from controls (SC).

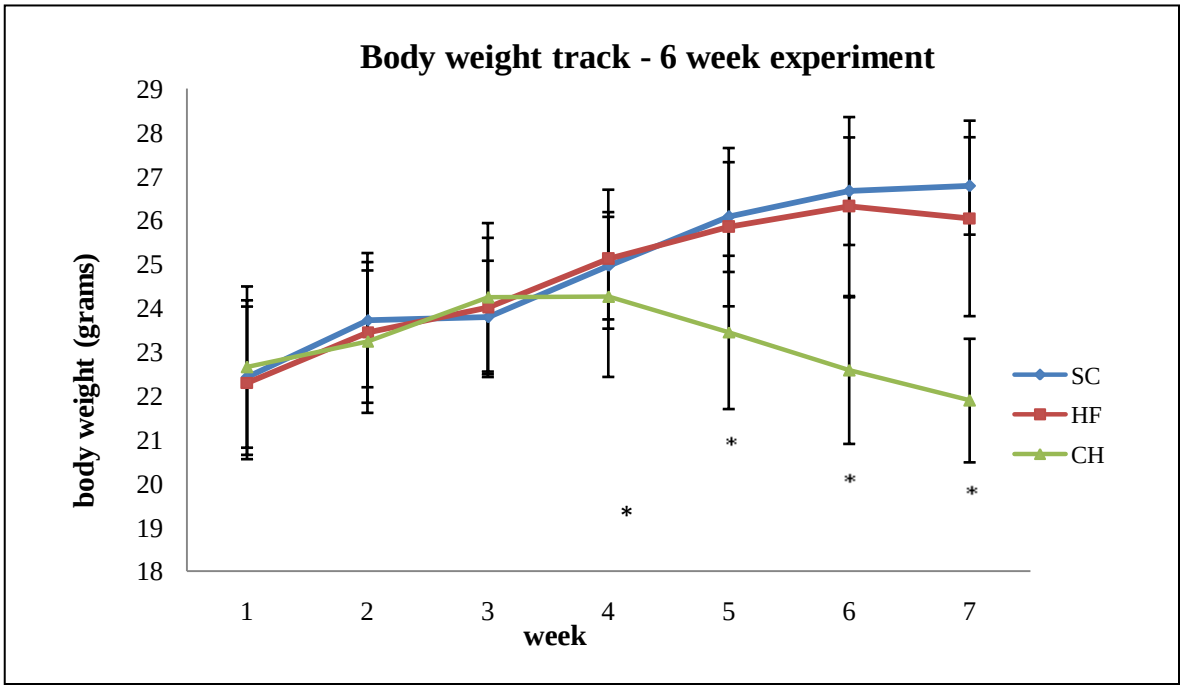


Figure 1(a): In the short term study starting at week 4 the fructose group saw significant weight loss resulting from decreased food intake and causing loading-loss induced bone deterioration. (*-CH significantly different from the other two groups)

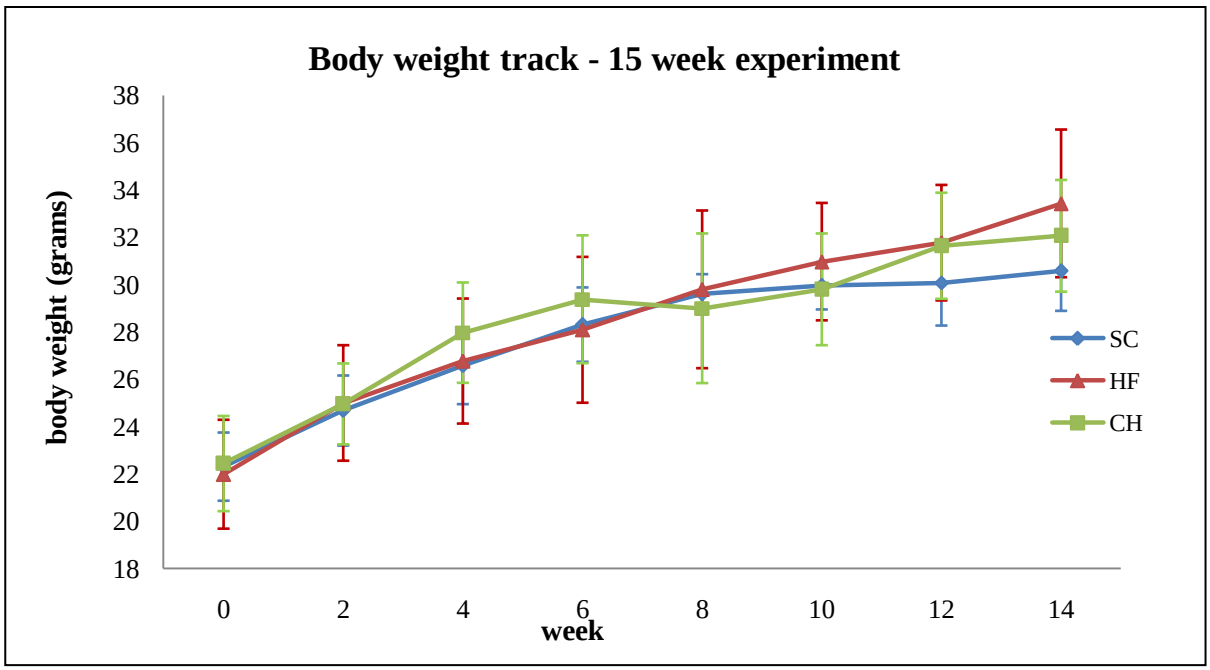


Figure 1(b): In the long term study (right) there were no differences in weights of all three groups allowing for a direct comparison of bone properties.

Food Intake: During the first week of the 6 week experiment the food intake of all groups was significantly different from each other with both CH and then HF being lower than control animals ($p<0.05$). However, once the animals had acclimatized to the food pellets, for the next three weeks the HF group had significantly lower food intake than the other two groups. By week five, the food intake for the HF group was 16 grams compared to 28 grams and 34 grams for the SC and CH groups ($p<0.005$)- almost a 50% difference. The low food intake of the HF group did not seem to have a detrimental effect on the body weight (not significantly different at any time) or general health of the animals. However, animals in the CH group experienced significant weight loss despite having the same or much higher food intake compared to control animals (At week 5 weight was >15% lower and food intake was >41% higher compared to SC; $p<0.05$). The food intake of the mice within the fructose group was very variable with high standard deviations with some individual mice having a dangerously low food intake and others having excessively high food intake (which brought the average food intake values close to the other groups). The mice also saw a significant reduction in body weight.

Therefore in the 15 week experiment, the high fructose diet pellets were replaced by control diet and fructose was administered through water instead. Similar to the 6 week experiment, the HF group had significantly lower food intake than the other two groups throughout the 15 week experiment ($p<0.02$). Again, this did not affect body weight, general health, appearance, activity of the animals. After the fructose diet pellets were replaced with regular diet, food intake of CH animals was almost same as control and administration of the fructose sugar via water did not seem to have any adverse effect on appetite/body weight.

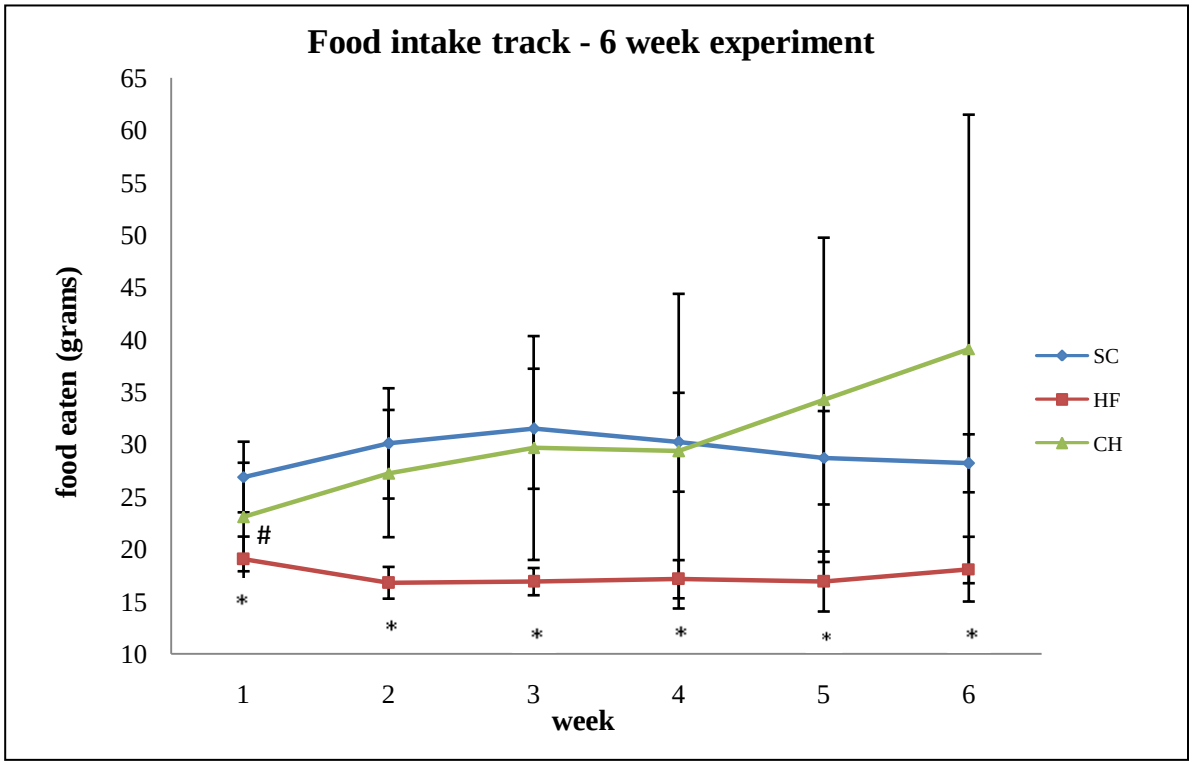


Figure 2(a): The CH group had erratic food intake values with high-standard deviations which resulted in loss of body mass in the short term study. The high-fat diet group had a significantly lower food intake than the other two groups (almost 50% lower at end of 6 weeks) possibly due to leptin induced appetite control. (* - HF significantly different from control; # - CH significantly different from control)

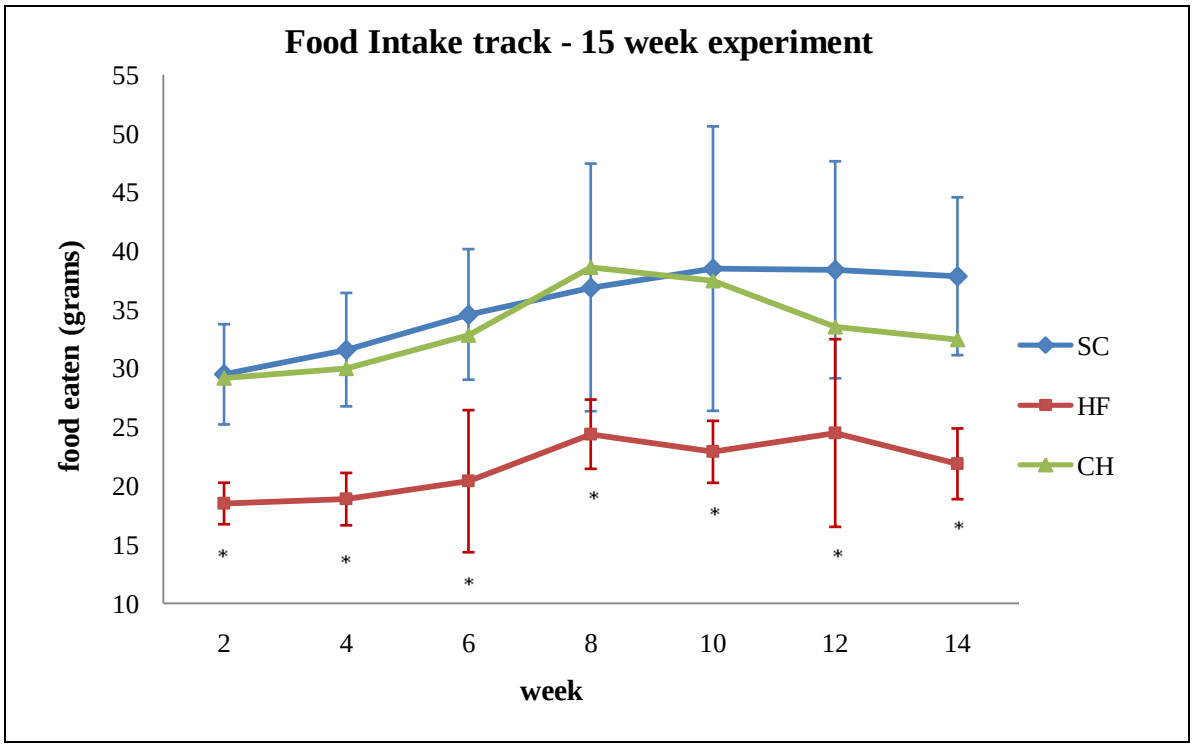


Figure 2(b): In both studies the high-fat diet group had a significantly lower food intake than the other two groups (almost 50% lower at end of 6 weeks) possibly due to leptin induced appetite control. In the long term study the food intake of the CH group was same as control (* - HF significantly different from control).

Abdominal Fat Volumes: In the six week experiment the high fat and the high-fructose diets caused major changes in the fat content. The HF group had 39 % greater fat volume than SC. The CH group had a mean fat volume which was the lowest of all three groups- 67 % lower than the control group and 80 % lower than the HF ($p<0.002$ for all three comparisons).

The total fat volume in the abdomen was separated into visceral and subcutaneous parts. These values showed similar trends to the total fat volume, with the HF group always being the highest, the SC group being an intermediate and the CH group always having the lowest fat volume.

Scans from the 15 week animals showed that the HF group had an increase of 53% over the SC group and 42% over the CH group ($p<0.003$ for both). There were no significant differences between the fat volumes of the SC and the CH groups.

Group	SC	CH	HF
Total fat (mm^3)	687.2±223.8	229.1±119.3	1130.5±348.4
Visceral (mm^3)	418.7±161.4	75.3±67.9	686.5±251.6
Subcutaneous (mm^3)	268.6±81	153.8±61	443.9±113.9

Table 1(a): Means and standard deviations 6 week

Comparison	SC vs CH	SC vs HF	CH vs HF
Total fat volume	<0.001	0.001	<0.001
Visceral fat	<0.001	0.003	<0.001
Subcutaneous fat	0.008	<0.001	<0.001

Table 1(b): p-values for individual comparisons 6 week

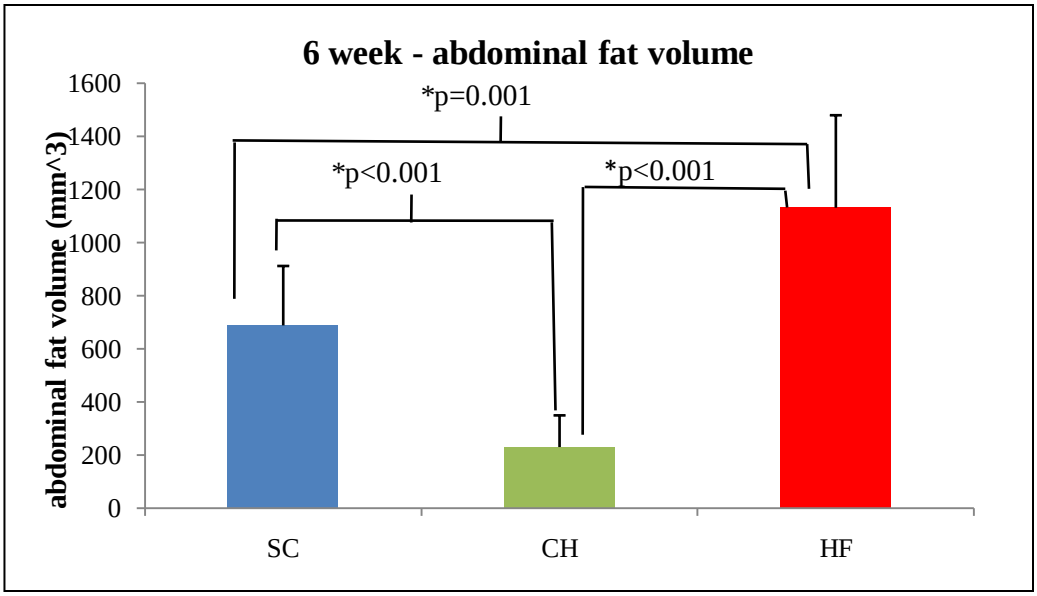
Group	SC	CH	HF
Total fat (mm^3)	647.3±116.6	797±123.7	1368.9±110
Visceral (mm^3)	373±217.1	453.7±256.7	818.8±237.9
Subcutaneous (mm^3)	274.3±88.6	343.3±143	550.1±183

Table 2(a): Means and standard deviations 15 week

Comparison	SC & CH	SC & HF	CH & HF
Total fat volume	0.38	<0.001	0.002
Visceral fat	0.49	<0.001	0.003
Subcutaneous fat	0.33	<0.001	0.006

Table 2(b): p-values for individual comparisons 15 week

3a)



3b)

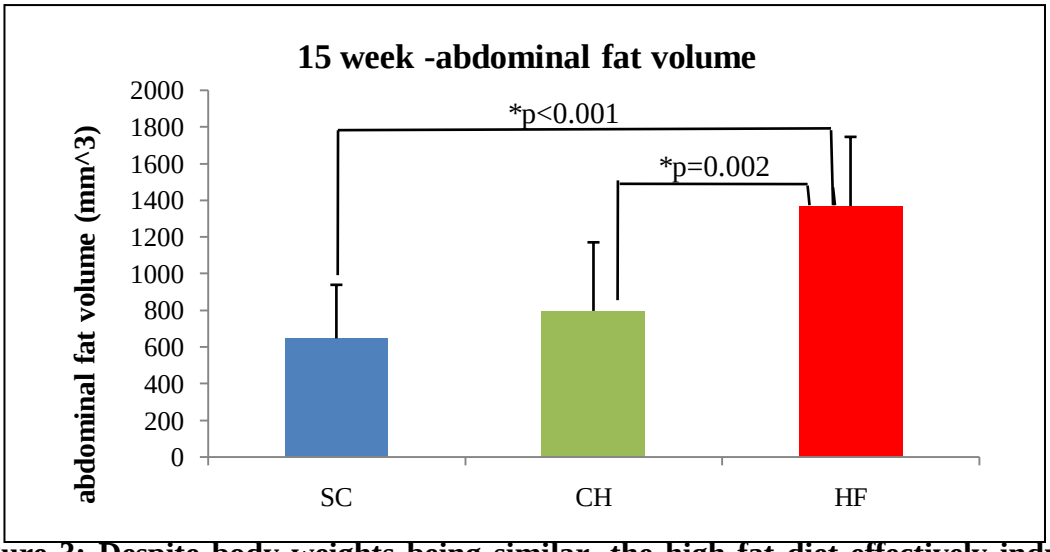
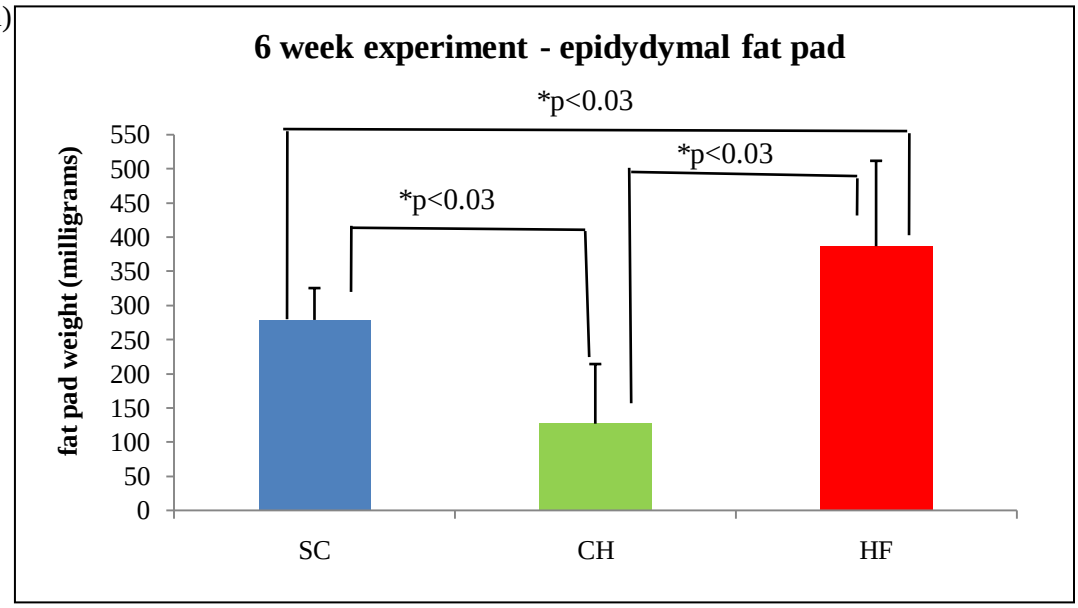


Figure 3: Despite body weights being similar, the high-fat diet effectively induced increased fat deposition in the HF group in both the 6 week (figure 3a) (39.2% higher) and 15 week (figure 3(b) (52.7% higher) experiments compared to control. The CH group on the other hand saw a sharp decrease in fat deposition in the 6 week experiment (67% lower than SC) possibly due to erratic food intake of the CH group.

Fat Pad Weights: In the 6 week experiment the CH group had the lowest epididymal fat pad weight among all three groups of 126.8±87.3 milligrams which was 55% lower than the SC value of 278.7±46. The HF group had the highest epididymal fat pad weight of 386.5±125 which was 28% higher than SC ($p<0.03$ for all three comparisons). The CH group had the lowest brown fat pad weight as well- 68.1±34.8 milligrams, 22% lower than the SC value of 87.3±13.3. The high-fat diet group had the highest value of 113.4±27. However, only the CH and HF groups were significantly different from each other ($p<0.02$).

In the 15 week experiment, the CH group again had the lowest epididymal fat pad weights of 156.6±56.6, 57% lower than the SC value of 367.9±144.1 and the high-fat diet group had the highest value of 839.6±258.2 which was 56% higher than control ($p< 0.03$ for all three comparisons). The brown fat pad weight of the CH group was significantly higher than the others. The means were SC=157.3±34.9, CH=403.4±210.4 and HF=245.8±99. The SC and HF groups did not significantly differ from each other.

4a)



4b)

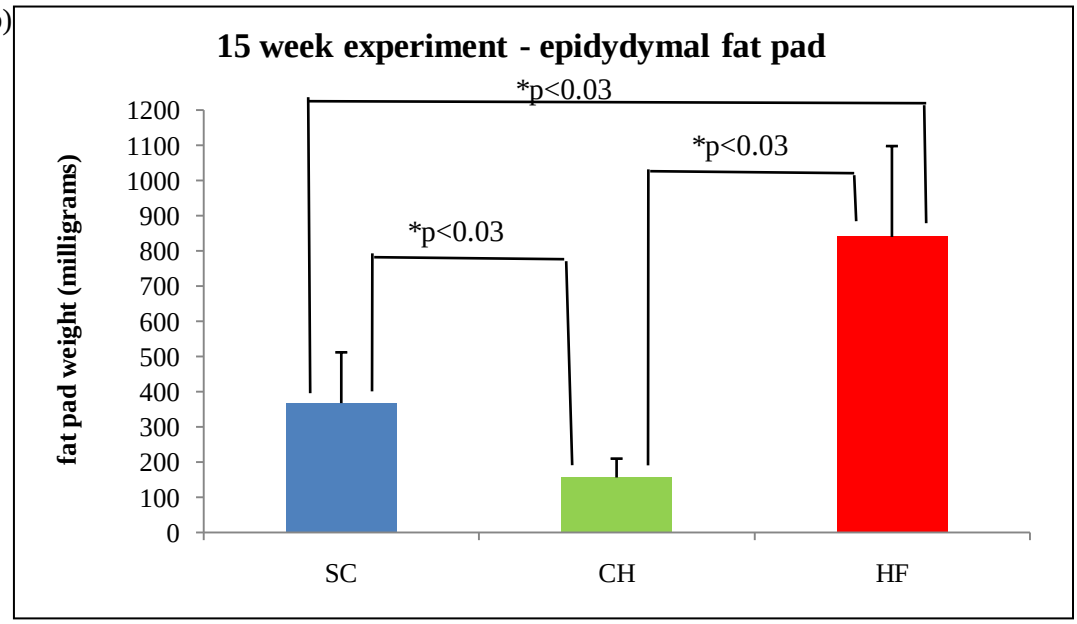


Figure 4: Epididymal fat pad weights showed similar trends to abdominal fat volumes. The high-fat diet consumption resulted in significantly heavier epididymal fat pads whereas the fructose group again had low fat deposition in both the 6 week (4a) and 15 week (4b) experiments.

Plasma Insulin, Glucose and Triglyceride Concentrations: In the 6 week experiment, CH group had 16.6% higher concentration of glucose compared to SC ($p<0.05$) and the triglyceride levels of the CH group were elevated to a large degree (82.4%) compared to SC ($p<0.05$). Compared to SC the HF group saw a 55 % increase in levels of plasma insulin ($p<0.05$). A comparison of the experimental groups showed that the CH group had 26.7% higher levels of glucose ($p<0.05$), 8.6% lower levels of insulin ($p<0.05$) and

85.2% increase in levels of triglycerides ($p<0.05$). Means, standard deviations and p-values are summarized below.

In the 15 week experiment there were no significant changes in plasma insulin, glucose or triglyceride levels in any of the groups compared to SC. The CH group had a 23.8% higher triglyceride concentration in plasma compared to HF, this difference was suggestively significant ($p=0.06$).

Parameter	Insulin (ng/ml)	Glucose (mg/dL)	Triglycerides (ug/ul)
Standard Chow	0.59±0.3	117.5±16.8	0.51±0.2
Fructose	0.17±0.05	140.9±22.3	2.9±0.4
High Fat	1.3±0.8	103.3±18.6	0.43±0.1

Compare	Insulin	Glucose	TG
SC & CH	0.09	0.03	<0.001
SC & HF	0.008	0.189	0.604
CH & HF	<0.001	0.002	<0.001

Tables 3 (a), (b): Effects of diet on plasma insulin, glucose and triglyceride concentrations (a) and p-values (b) for diet group comparisons- 6 week study

Parameter	Insulin (ng/ml)	Glucose (mg/dl)	Triglycerides (ug/ul)
Standard Chow	0.94±0.6	158.9±46.9	3.7±1.1
Fructose	1.8±1.3	180.7±27.6	4.2±1.4
High Fat	1.3±1	183.6±36.6	3.2±0.7

Compare	Insulin	Glucose	TG
SC & CH	0.15	0.41	0.32
SC & HF	0.69	0.34	0.33
CH & HF	0.56	0.98	0.06

Tables 4(a), (b): Effects of diet on plasma insulin, glucose and triglyceride concentrations and p-values for diet group comparisons- 15 week study

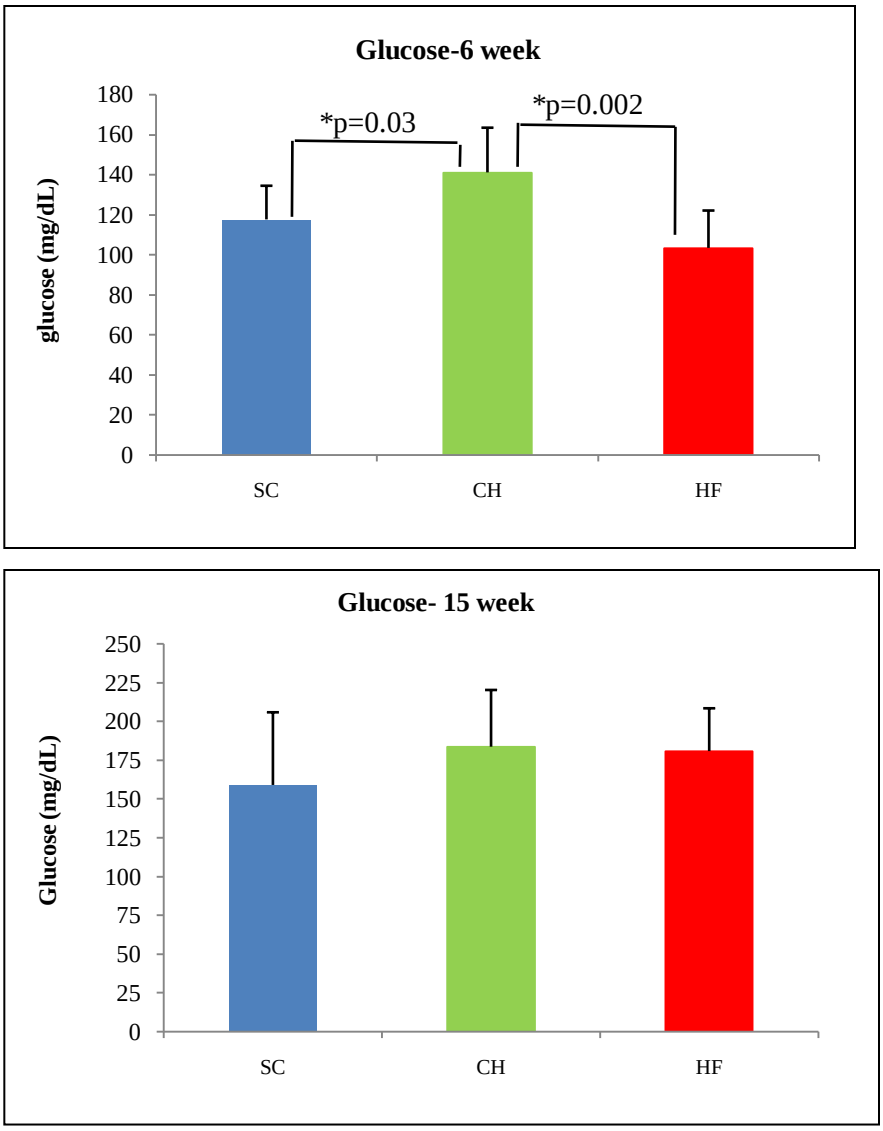
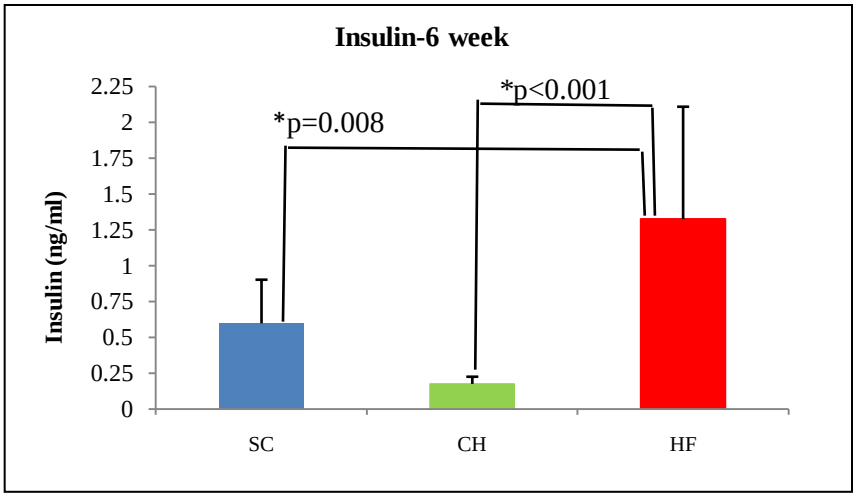


Figure 5: In the 6 week study (top) the fructose diet resulted in elevated glucose levels in plasma where as the high fat diet did not have any effect compared to control. In the 15 week study (bottom), there were no significant differences between groups although the glucose levels for the fructose groups were still highest

6a)



6b)

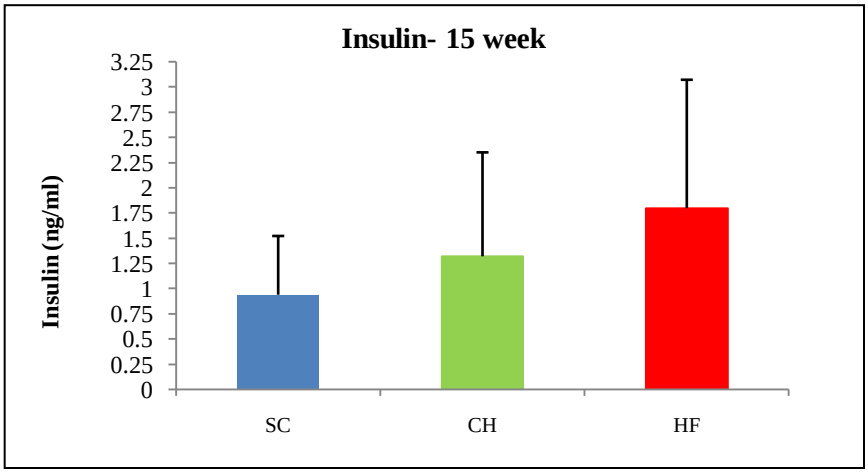
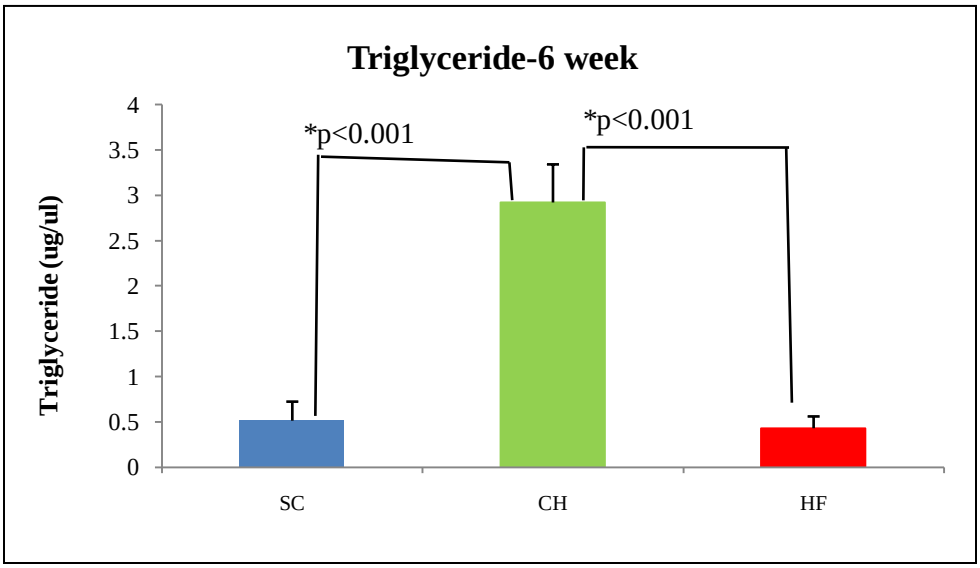


Figure 6: In the 6 week study (top), insulin levels of all three groups in plasma were significantly different from each other with fructose diet causing severe insulinopenia and high-fat diet increasing the levels of insulin (correspondingly affecting glucose levels). In the 15 week experiment (bottom) there were no significant differences though the high-fat diet still had highest insulin values.

7a)



7b)

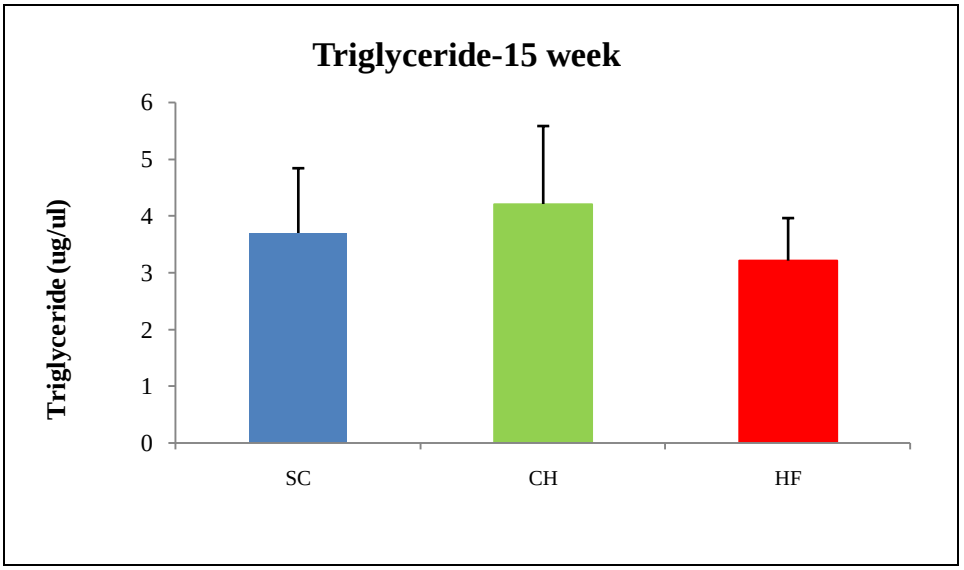


Figure 7: In the 6 week experiment (top 7a) the fructose diet caused significant elevation of plasma triglycerides whereas the high-fat diet did not have any effect despite increased fatty depots in the HF group. In the 15 week experiment (bottom 7b) there were no significant differences.

Diet induced changes in load bearing bones- 6 week experiment

Diet induced changes in proximal tibia:-

Cortical Region of Tibia: The fructose diet caused significant alterations in bone volume (BV), cortical thickness (Ct. Th.) and bone mineral density (BMD). The bone volume of the CH group was lowest, 14 % less than SC and 13 % less than HF ($p<0.005$ for both comparisons). Similarly the cortical thickness of the CH group was lowest, 9 %

lower than control and 5 % lower than HF ($p<0.05$ for both comparisons). The bone mineral density was lowest for the high fat diet group although this difference was small and was significant only when compared to CH group (HF<CH by 2.2 %, $p<0.02$). Comparison of the two experimental groups showed that the bone volume of the HF group was 11.2% higher than the CH group ($p<0.01$). There were very small differences in BMD- the CH group was 2.1% higher than the HF group ($p<0.03$).

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
Standard Chow	0.89±0.06	0.77±0.02	0.11±0.0	1062.8±16.3
Fructose	0.77±0.03	0.75±0.03	0.1±0.0	1077.8±14.7
High-fat	0.87±0.07	0.75±0.03	0.12±0.0	1054.2±11.5

Table 5(a): Differences in cortical tibia parameters of different diet groups-6 week experiment.

Parameter Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)CH	<0.001	0.03	<0.001	0.05
SC(vs.)HF	0.419	0.085	0.041	0.317
CH(vs.)HF	0.005	0.829	0.103	0.012

Table 5(b): p-values for the F-statistic of diet effects and p-values of individual group comparisons.

The CH group had significantly lower body weights compared to the other two groups and body weight was found to significantly correlate with cortical bone volume, cortical thickness and tissue mineral density (significant Pearson's correlations). Since weight as a loading factor can influence bone density and structure, it was taken as a covariate while comparing groups. After adding body mass as a covariate, only cortical thickness was found to significantly differ between groups. The cortical thickness was 10% lower in the CH group compared to SC and 5% lower in the HF group compared to SC ($p<0.05$ for both comparisons). The two experimental diet groups were not significantly different from each other.

Parameter	Bone volume (mm^3)	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
Standard Chow	0.89±0.06	0.11±0.0	1062.8±16.3
Standard Chow adjusted	0.85±0.02	0.11±0.0	1070.3±6.2
Fructose	0.77±0.03	0.1±0.0	1077.8±14.7
Fructose adjusted	0.85±0.03	0.1±0.0	1064.7±8.6
High-fat	0.87±0.07	0.11±0.0	1054.2±11.5
High-fat adjusted	0.83±0.02	0.11±0.0	1061.7 ±7.4

Table 6(a): Means, standard deviations before adjusting for body weight and the estimated means after adjusting for body weight for parameters that were found to vary with weight (significant Pearson correlation).

Parameter Comparisons	Bone volume	Cortical thickness	Tissue mineral density
SC(vs.)CH	<0.001	<0.001	0.05
SC(vs.)HF	0.419	0.041	0.317
CH(vs.)HF	0.005	0.103	0.012
Adjusted			
SC(vs.)CH	0.98	0.01	0.67
SC(vs.)HF	0.31	0.04	0.28
CH(vs.)HF	0.57	0.149	0.82

Table 6(b): p-values for diet group comparisons before and after adjusting for body weight.

Trabecular Region of Tibia: In the trabecular region, the fructose diet caused significant damage to the bone volume and structure. Compared to SC, the bone volume (-43.2% p=0.002), bone volume fraction (-46.2%,p<0.002), connectivity density (-53.6%,p<0.001), trabecular number (-21.2%,p<0.001), trabecular thickness (-6.8%,p=0.006) were decreased in the CH group while the TRI-SMI and trabecular separation increased by 17.6% (p<0.001) and 22.4% (p<0.001) compared to SC. Higher TRI-SMI values are indicative of an eroded rod like structure of trabeculae as compared to a more structurally sound plate like structure.

Comparison of the HF and CH groups revealed that the fructose diet was more detrimental to trabecular bone than the high-fat diet. The connectivity density of the CH group was 57.7% lower than the HF group ($p<0.005$). The HF group did not show any noticeable changes in any of the parameters compared to SC.

Parameters	Standard chow	Fructose	High-Fat
Bone volume (mm^3)	0.25±0.05	0.15±0.04	0.29±0.1
Bone volume fraction	0.13±0.2	0.07±0.02	0.13±0.04
Connectivity Density (1/mm^3)	132.6±27.3	61.5±26.5	145.6±73.1
TRI-SMI	2.5±0.2	3.1±0.2	2.5±0.4
Trabecular Number (1/mm)	5.1±0.3	4.0±0.3	5.1±0.7
Trabecular thickness (mm)	0.04±0.0	0.04±0.0	0.05±0.0
Trabecular separation (mm)	0.19±0.0	0.25±0.02	0.2±0.03
Tissue mineral density(mg HA/ccm)	847.6±17.1	873.1±16.2	850.6±31.7

Table 7(a): Differences in trabecular tibia of different diet groups-6 week experiment.

Parameters	SC&CH	SC&HF	CH&HF
Bone volume	0.002	0.384	0.001
Bone volume fraction	<0.001	0.635	<0.001
Connectivity Density	0.002	0.57	0.002
TRI-SMI	<0.001	0.73	<0.001
Trabecular Number	<0.001	0.939	<0.001
Trabecular thickness	0.006	0.43	0.003
Trabecular separation	<0.001	0.90	<0.001
Tissue mineral density	0.02	0.79	0.07

Table 7(b): p-values for diet group comparisons.

Similar to the cortical region, the differences in trabecular tibia between diet groups were primarily due to the differences in body weights among the groups. After body weights were taken as covariate the groups were not significantly different in any respect. The estimated means of bone volume fraction, connectivity density, trabecular number and trabecular thickness were still lowest in the CH group.

Parameter	SC	SC adjusted	CH	CH adjusted	High-Fat	HF adjusted
Bone volume (mm^3)	0.25±0	0.2±0.02	0.15±0.04	0.24±0.03	0.3±0.1	0.24±0.03
Bone volume fraction	0.13±0	0.11±0.0	0.07±0.02	0.02±0.01	0.1±0.04	0.12±0.0
Connectivity Density (1/mm^3)	132.6±27	103.3±15.4	61.5±26.5	112.6±21.6	145.6±73.1	116.7±18.6
TRI-SMI	2.53±0.2	2.7±0.1	3.1±0.2	2.8±0.13	2.5±0.35	2.6±0.1
Trabecular Number (1/mm)	5.1±0.3	4.8±0.	4±0.3	4.6±0.21	5.1±0.73	4.8±0.2
Trabecular thickness (mm)	0.04±0	0.04±0.0	0.04±0.0	0.04±0.0	0.05±0.0	0.05±0.0
Trabecular separation (mm)	0.19±0	0.21±0.01	0.25±0.02	0.23±0.01	0.2±0.03	0.2±0.01
Tissue mineral density (mg HA/ccm)	847.6±17	854.9±9	873.1±16	860.4±12.6	850.6±31.6	857.8±10.9

Table 8(a): Means, standard deviations before adjusting for body weight and the estimated means after adjusting for body weight for parameters that were found to vary with weight (significant Pearson correlation).

Parameter	SC&CH	SC&CH Adjusted	SC&HF	SC&HF adjusted	CH&HF	CH&HF Adjusted
Bone volume	0.002	0.46	0.384	0.25	0.001	0.95
Bone volume fraction	<0.001	0.62	0.635	0.57	<0.001	0.42
Connectivity Density	0.002	0.77	0.57	0.5	0.002	0.9
TRI-SMI	<0.001	0.62	0.73	0.68	<0.001	0.48
Trabecular Number	<0.001	0.56	0.939	0.9	<0.001	0.53
Trabecular thickness	0.006	0.29	0.43	0.44	0.003	0.15
Trabecular separation	<0.001	0.29	0.90	0.9	<0.001	0.35
Tissue mineral density	0.02	0.77	0.79	0.8	0.07	0.89

Table 8(b): p-values for diet group comparisons before and after adjusting for body weight.

Diet induced changes in distal femur-6 week experiment:-

Cortical Region of Femur: Statistical analysis showed that the fructose diet caused a reduction in bone volume of the distal cortical femur which was 8% lower compared to SC (p=0.004), bone volume fraction (-1.4% compared to SC, p=0.031) and cortical thickness (0.7% less than SC, p=0.028). There were no differences in any other parameters between groups. The high-fat diet did not cause any alterations in bone volume, cortical thickness or tissue density compared to SC. The means were almost equal to each other. Means, standard deviations and p-values for comparisons are summarized in the table below. Comparison of the two experimental groups revealed no differences in volume or thickness or density of the cortical bone.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
Standard Chow	1.2±0.05	0.85±0.0	0.14±0.0	1117.7±12.7
Fructose	1.1±0.04	0.84±0.0	0.135±0.0	1112.1±21.9
High-fat	1.2±0.08	0.84±0.0	0.136±0.0	1108±8.4

Table 9(a): Differences in cortical femur of different diet groups-6 week experiment.

Parameter	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)CH	0.004	0.031	0.028	0.47
SC(vs.)HF	0.92	0.17	0.07	0.25
CH(vs.)HF	0.01	0.49	0.79	0.64

Table 9(b): p-values for the F-statistic of diet effects and p-values of individual group comparisons.

The bone volume was found to vary with weight as seen by significant Pearson correlations. Thus body weight was taken as a covariate and groups were again compared to each other after which there were no significant differences. The means, standard deviations before adjustment for differences in weight and means and standard errors after adjustment for differences in body weight along with corresponding p-values are summarized in the tables below.

Parameter	Bone volume (mm^3)
Standard Chow	1.2±0.05
Standard Chow adjusted	1.1±0.02
Fructose	1.1±0.04
Fructose adjusted	1.1±0.02
High-fat	1.2±0.08
High-fat adjusted	1.1±0.02

Table 10(a): Means, standard deviations before and after adjusting for body weight.

Parameter Comparisons	Bone volume
SC(vs.)CH	0.004
SC(vs.)HF	0.92
CH(vs.)HF	0.01
Adjusted	
SC(vs.)CH	0.49
SC(vs.)HF	0.88
CH(vs.)HF	0.43

Table 10(b): p-values before and after adjusting for body weight.

Trabecular Region of Distal Femur: Similar to the changes observed in the tibia, the fructose diet caused alterations in the trabecular region of the femur as well. The bone volume fraction (33.33%, p=0.004), connectivity density (42 %, p=0.01) and trabecular number (17 %, p=0.001) of CH were all significantly reduced compared to SC. This was accompanied by a significant increase in the trabecular separation and the TRISMI (15%, p=0.004) indicating severe erosion of the trabecular struts. There were no significant differences in the trabecular thickness or bone mineral density.

The high-fat diet consumption did not have any influence on trabecular femur. There were no changes in the bone volume fraction, connectivity density and trabecular structure or bone density with the means almost being identical in some cases.

Comparison of the two diet groups showed that the fructose diet was much more harmful to trabecular bone volume and structure than the high-fat diet. The bone volume (-38%, p=0.003), bone volume fraction (-35%, p=0.003) connectivity (-53%, p=0.001), trabecular number (16%, p=0.003) and separation (+19%, p=0.003) of the CH group were significantly changed when compared to HF. The means and standard deviations along with p-values are summarized in the table below.

Parameter	Standard chow	Fructose	High-Fat
Bone volume (mm^3)	0.26±0.05	0.18±0.06	0.29±0.06
Bone volume fraction	0.12±0.02	0.08±0.02	0.12±0.03
Connectivity Density (1/mm^3)	133.2±40.8	76.0±40.9	162.1±44.4
TRI-SMI	2.3±0.2	2.8±0.25	2.2±0.2
Trabecular Number (1/mm)	4.7±0.34	3.9±0.52	4.6±0.4
Trabecular thickness(mm)	0.04±0.0	0.04±0.0	0.04±0.0
Trabecular separation(mm)	0.21±0.02	0.26±0.04	0.21±0.02
Tissue mineral density (mg HA/ccm)	848.3±19.2	854.8±26.5	866.3±19

Table 11(a): Means and standard deviations-trabecular femur 6 week experiment.

Parameter	SC&CH	SC&HF	CH&HF
Bone volume	0.16	0.29	0.003
Bone volume fraction	0.004	0.67	0.003
Connectivity Density	0.01	0.2	0.001
TRI-SMI	0.004	0.45	0.002
Trabecular Number	0.001	0.89	0.003
Trabecular thickness	0.17	0.66	0.41
Trabecular separation	0.001	0.98	0.003
Tissue mineral density	0.57	0.14	0.34

Table 11(b): p-values for diet group comparisons

The bone volume, bone volume fraction, connectivity density, trabecular number and trabecular separation of the femur were found to have correlations with body weight. When weight was taken as a covariate for analysis, it was found that the groups were no longer significantly different in terms of bone volume or density or trabecular characteristics. Means, estimated means and p-values from the ANOVA and ANCOVA analyses are compared in the tables below.

Parameter	SC	SC adjusted	CH	CH adjusted	HF	HF adjusted
Bone volume (mm^3)	0.3±0.0	0.23±0.02	0.18±0.06	0.24±0.03	0.29±0.06	0.27±0.02
Bone volume fraction	0.12±0.0	0.11±0.0	0.08±0.02	0.1±0.01	0.12±0.03	0.12±0.0
Connectivity Density (1/mm^3)	133.2±41	114.2±15	76±40.9	107.5±18.7	162±44.4	148.6±16.3
TRI-SMI	2.3±0.25	2.44±0.09	2.7±0.25	2.6±0.12	2.2±0.3	2.3±0.1
Trabecular Number (1/mm)	4.7±0.34	4.46±0.15	3.9±0.52	4.4±0.18	4.7±0.4	4.5±0.2
Trabecular separation (mm)	0.21±0.02	0.23±0.0	0.26±0.04	0.24±0.01	0.21±0.02	0.2±0.0

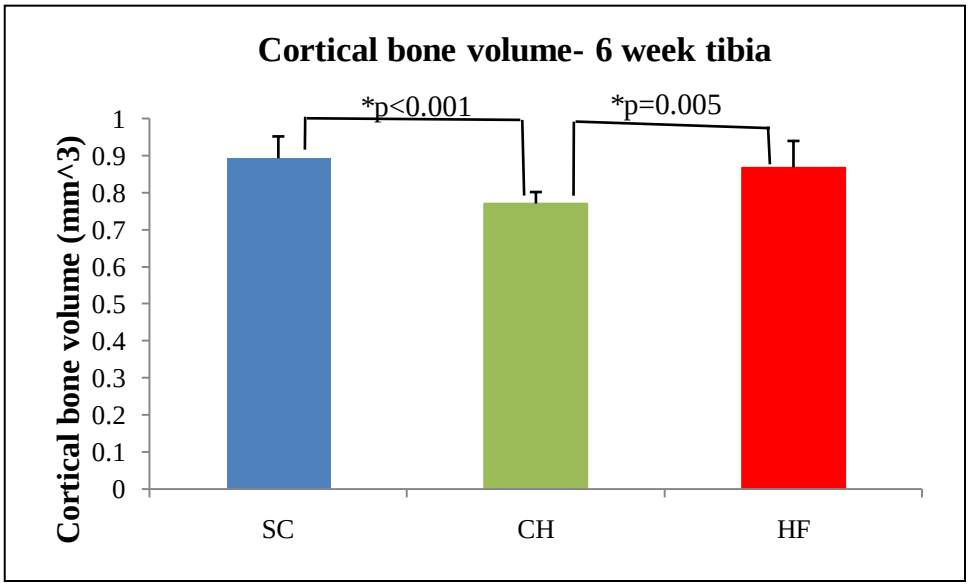
Table 12(a): Means, standard deviations before adjusting for body weight and the estimated means after adjusting for body weight for parameters that were found to vary with weight (significant Pearson correlation).

Parameter	SC&CH	SC&CH adjusted	SC&HF	SC&HF adjusted	CH&HF	CH&HF adjusted
Bone volume	0.16	0.68	0.29	0.1	0.003	0.42
Bone volume fraction	0.004	0.55	0.67	0.46	0.003	0.26
Connectivity Density	0.01	0.81	0.2	0.1	0.001	0.15
TRI-SMI	0.004	0.43	0.45	0.31	0.002	0.13
Trabecular Number	0.001	0.42	0.89	0.86	0.003	0.35
Trabecular separation	0.001	0.49	0.98	0.76	0.003	0.36

Table 12(b): p-values for individual group comparisons before and after adjusting for body weight.

In the six week study the effects of fructose diet were predominant in causing detriment to bone mass and morphology. Figures below illustrate the effects of diet on cortical and trabecular bone volume of load bearing bones.

a)



b)

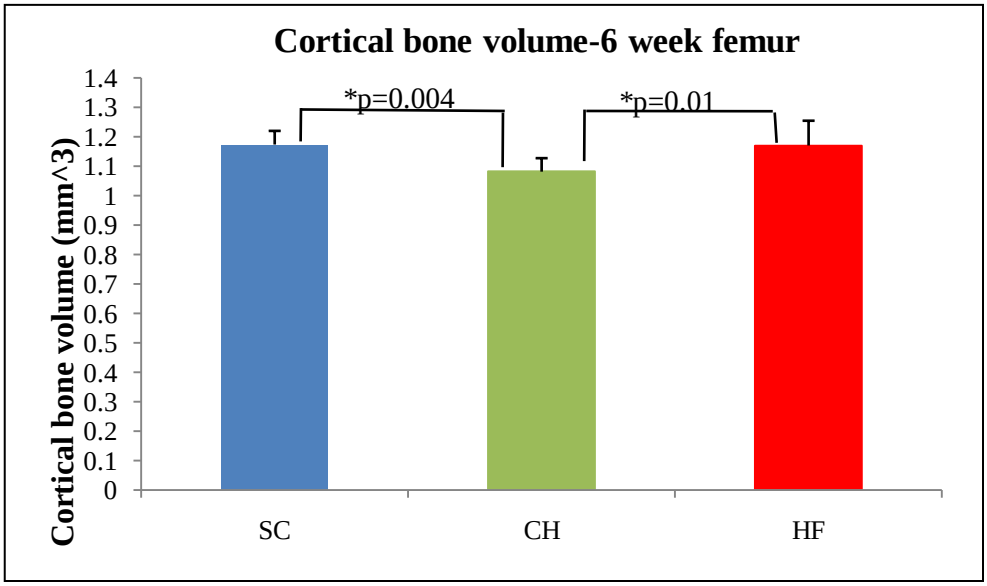
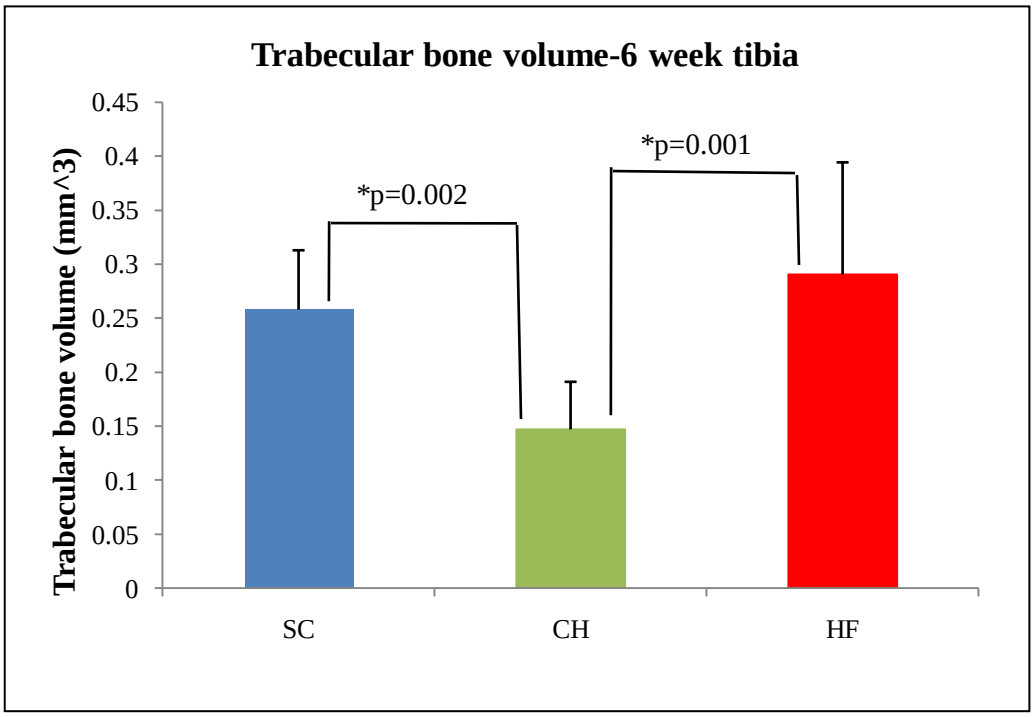


Figure 8: Compared to the other two groups, fructose diet consumption significantly decreased the cortical bone volume of both tibia (Fig 8a) and femur (Fig 8b). (*- difference was significant at the 0.05 level)

9a)



9b)

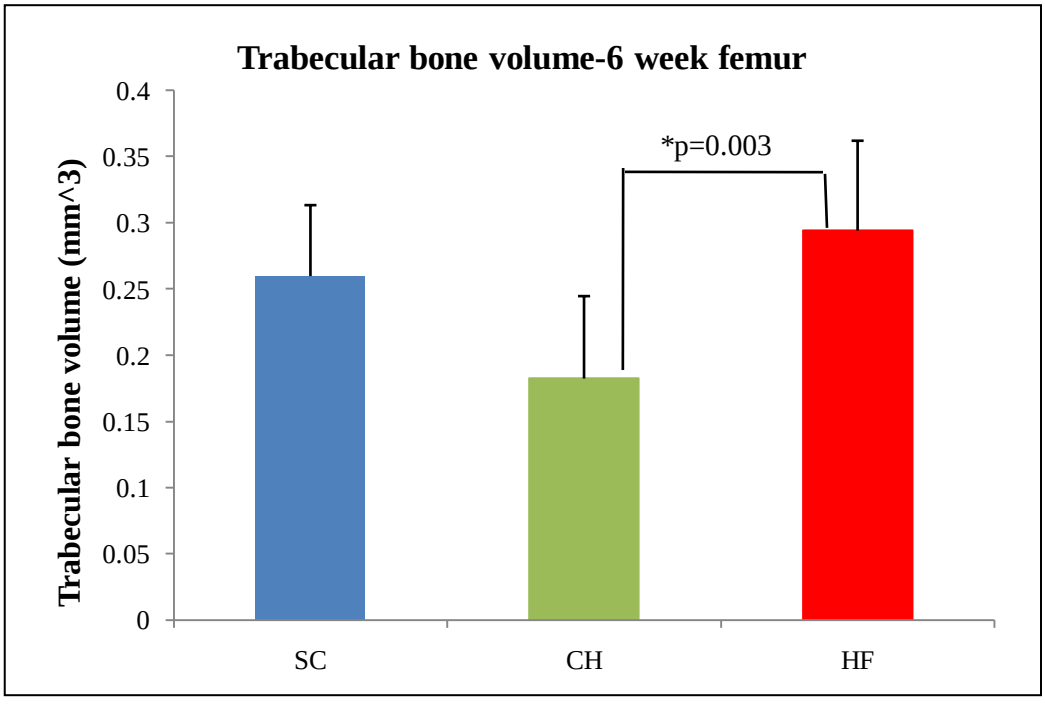


Figure 9: Fructose diet consumption significantly decreased trabecular bone volume of both tibia (Fig 9a) and femur (Fig 9b). (*- difference was significant at the 0.05 level)

Effects of diet on load bearing bones – 15 week experiment

Effects of diet on proximal tibia – 15 week experiment:

Cortical Region of Tibia*:* In the long term study, the high fat diet was more detrimental to bone than the fructose diet. In the cortical region of the proximal tibia the bone volume (7%, p=0.002), bone volume fraction (6%, p<0.001), cortical thickness (10%, p<0.001) and tissue mineral density (3%, p<0.001) of the HF group were significantly reduced in comparison to SC.

The CH group had a reduced cortical thickness (-3%, p=0.052) and bone mineral density (-1.7%, p=0.001) compared to SC. In all other parameters there were no differences. A comparison of the two experimental groups showed that the bone volume (-6%, p=0.01), bone volume fraction (-5%, p=0.004), cortical thickness (-8%, p<0.001) and tissue mineral density (-1.3%, p=0.006) of the HF group were all significantly lower compared to the CH. The means, standard deviations and p-values for comparisons are summarized below.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
Standard Chow	0.95±0.0	0.77±0.0	0.12±0.0	1104.3±9.5
Fructose	0.94±0.05	0.76±0.	0.11±0.0	1084.8±9.9
High-fat	0.9±0.0	0.73±0.0	0.1±0.0	1069.6±12

Table 13(a): Means and standard deviations for cortical tibia- 15 week experiment.

Parameter Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)CH	0.6	0.46	0.052	0.001
SC(vs.)HF	0.002	<0.001	<0.001	<0.001
CH(vs.)HF	0.011	0.004	<0.001	0.006

Table 13(b): p-values of diet group comparisons.

Trabecular Region of Tibia: The SMI for the high-fat group was elevated (14 %, p=0.007) compared to the fructose group. Other than this, there were no differences in bone volume/density or trabecular characteristics between the three groups.

Parameters	Standard chow	Fructose	High-Fat
Bone volume (mm^3)	0.26±0.05	0.29±0.05	0.27±0.04
Bone volume fraction	0.13±0.03	0.14±0.02	0.12±0.02
Connectivity Density (1/mm^3)	132.3±43.2	128.4±38.6	107.6±31.4
TRI-SMI	2.3±0.3	2.1±0.23	2.5±0.2
Trabecular Number (1/mm)	5.1±0.5	5±0.4	4.9±0.5
Trabecular thickness (mm)	0.04±0.0	0.04±0.0	0.04±0.0
Trabecular separation (mm)	0.2±0.0	0.2±0.02	0.2±0.02
Tissue mineral density (mg HA/ccm)	859.7±9.9	864.8±18	874.4±15.9

Table 14(a): Means and standard deviations for trabecular tibia- 15 week experiment.

Parameters	SC&CH	SC&HF	CH&HF
Bone volume	0.19	0.56	0.44
Bone volume fraction	0.52	0.35	0.13
Connectivity Density	0.83	0.15	0.26
TRI-SMI	0.22	0.08	0.007
Trabecular Number	0.51	0.43	0.92
Trabecular thickness	0.02	0.09	0.48
Trabecular separation	0.54	0.49	0.96
Tissue mineral density	0.47	0.035	0.18

Table 14(b): p-values of diet group comparisons.

Effects of diet on distal femur- 15 week experiment:

Cortical Region of Femur: The bone volume (-5 %, p=0.04), bone volume fraction (-5%, p<0.001), cortical thickness (-9%, p<0.001) and tissue mineral density (-2%, p<0.001) were all reduced significantly compared to SC.

When CH group was compared to SC there were no differences in bone volume or cortical thickness or density with the means being very close to each other in many cases. Comparison of the two experimental groups showed that the high-fat diet was much more detrimental to the bone compared to the CH having reduced bone volume fraction (-3%, p=0.007), cortical thickness (-6%, p=0.006) and tissue mineral density (-1.7%, p<0.001). Means and standard deviations along with p-values are listed in the table below.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
Standard Chow	1.3±0.05	0.85±0.01	0.14±0.0	1152.3±8.1
Fructose	1.3±0.09	0.84±0.02	0.14±0.0	1146.1±12.4
High-fat	1.2±0.07	0.81±0.02	0.13±0.0	1126.1±10.9

Table 15(a): Means and standard deviations for cortical femur- 15 week experiment.

Parameter Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)CH	0.53	0.14	0.22	0.21
SC(vs.)HF	0.04	<0.001	<0.001	<0.001
CH(vs.)HF	0.15	0.007	0.006	<0.001

Table 15(b): p-values of diet group comparisons.

Trabecular Region of Femur: In the trabecular region the bone volume fraction of the HF group was significantly reduced compared to SC (18%, p=0.032). There were no other statistically significant differences. There were no differences between the CH and SC groups in any of the parameters. Compared to the CH the HF group had significantly reduced bone volume fraction (-18%, p=0.041).

Parameters	Standard chow	Fructose	High-Fat
Bone volume (mm^3)	0.3±0.06	0.3±0.06	0.26±0.04
Bone volume fraction	0.12±0.03	0.12±0.02	0.1±0.01
Connectivity Density (1/mm^3)	120.3±34.6	108±29	94.4±30
TRI-SMI	2.2±0.3	2.2±0.25	2.5±0.2
Trabecular Number (1/mm)	4.6±0.4	4.4±0.3	4.3±0.4
Trabecular thickness (mm)	0.4±0.0	0.4±0.0	0.43±0.0
Trabecular separation (mm)	0.21±0.02	0.2±0.02	0.23±0.02
Tissue mineral density (mg HA/ccm)	880.6±12.8	877.1±15.3	882±12.4

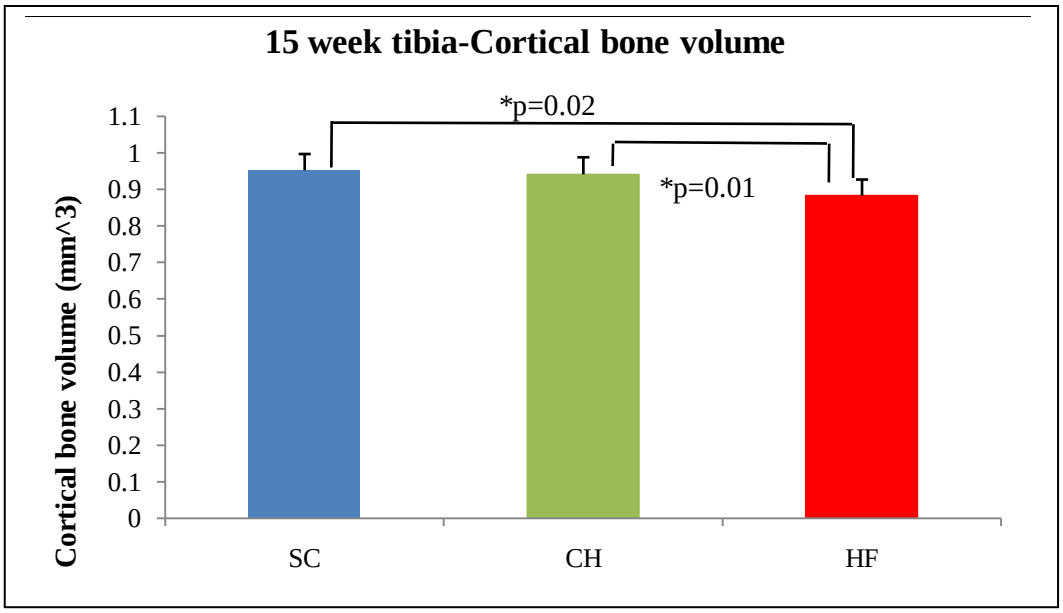
Table 16(a): means and standard deviations trabecular femur-15 week experiment.

Parameters	SC&CH	SC&HF	CH&HF
Bone volume	0.77	0.51	0.36
Bone volume fraction	0.95	0.032	0.041
Connectivity Density	0.4	0.07	0.35
TRI-SMI	0.92	0.005	0.008
Trabecular Number	0.31	0.09	0.51
Trabecular thickness	0.2	0.51	0.064
Trabecular separation	0.35	0.09	0.46
Tissue mineral density	0.58	0.81	0.44

Table 16(b): p-values of diet group comparisons.

Thus in the 15 week experiment the effects of high-fat diet consumption were predominant in altering bone mass and morphology in the load bearing bones. The figures below illustrate the effect of the experimental diets on cortical and trabecular bone volume of the femur and tibia.

10a)



10b)

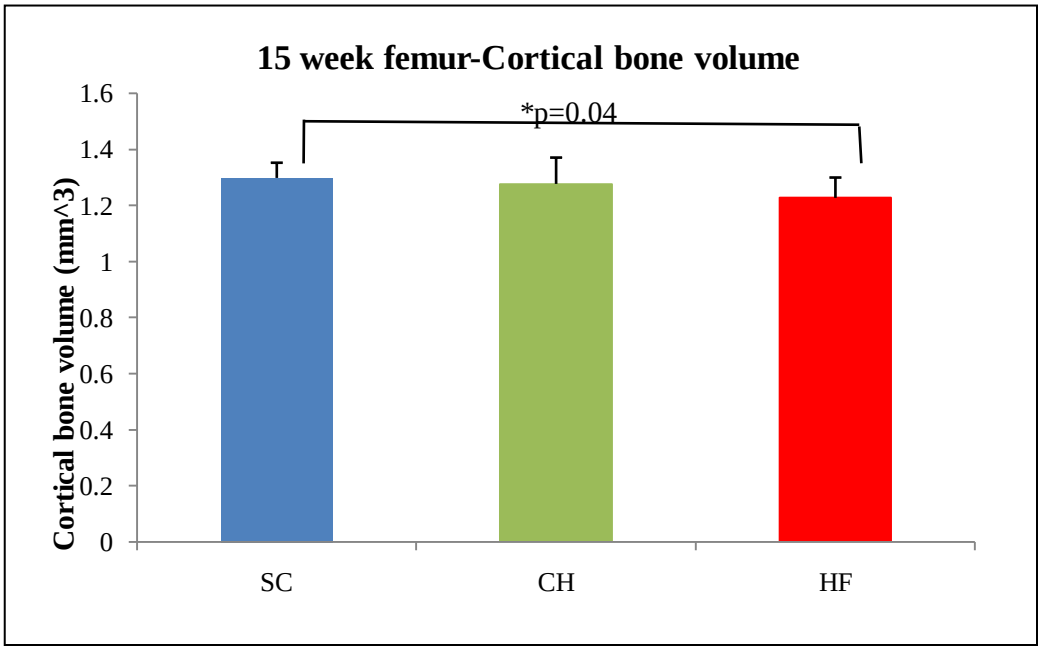
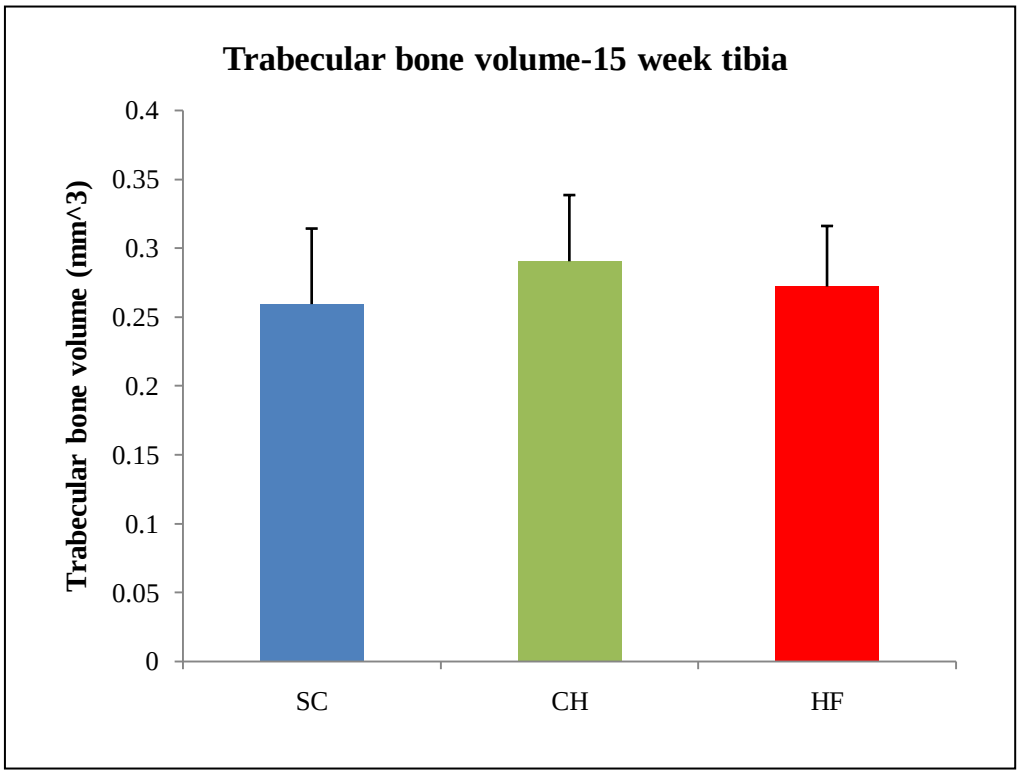


Figure 10: In the 15 week study high fat diet consumption significantly affected cortical bone volume of tibia (Fig 10a) and femur (Fig 10b). (*- difference significant at the 0.05 level)

11a)



11b)

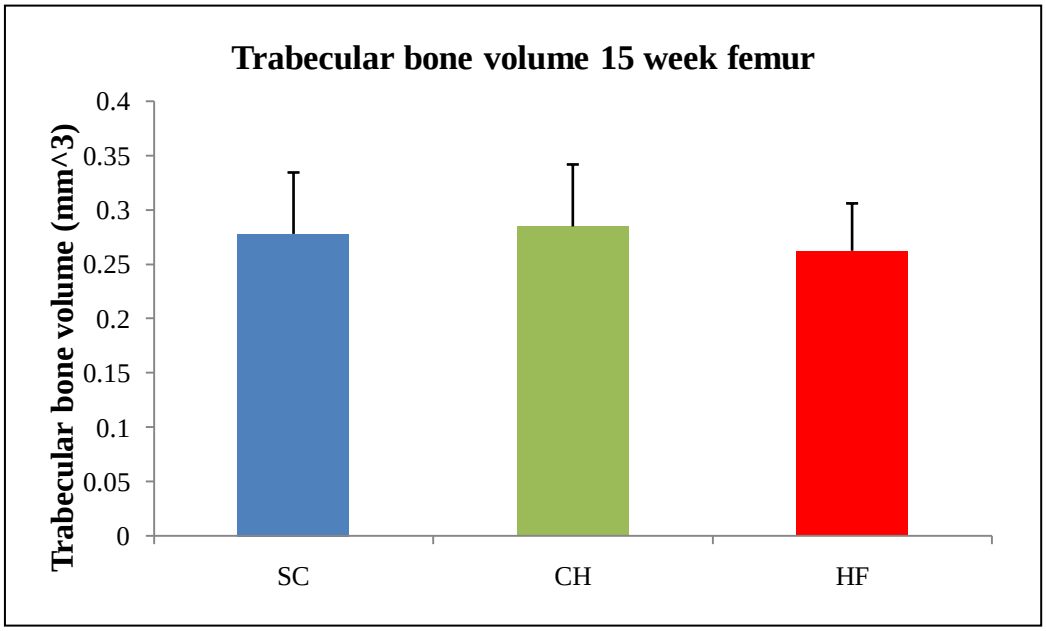
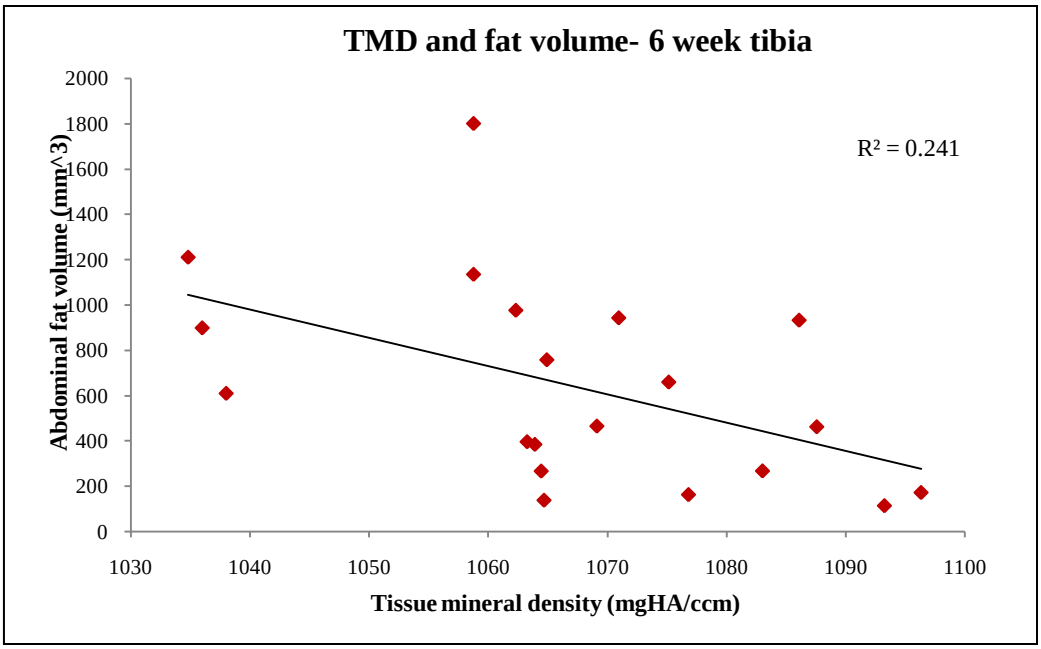


Figure 11: There were no significant differences in trabecular bone volume among diet groups in tibia (Fig 11a) and femur (Fig 11b). Bone volume of HF was still lowest.

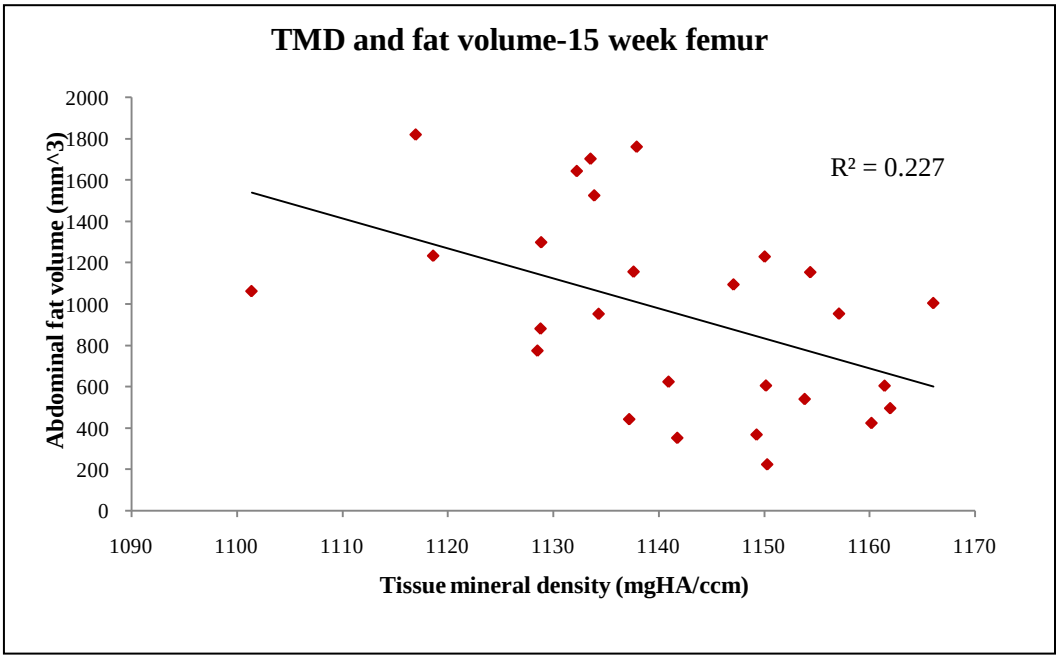
Correlations- Abdominal Fat Volume and Bone Mineral Density: Pearson's correlations revealed that in the 6 week experiment and across all three groups fat volume significantly varied with bone mineral density in the cortical region of the tibia (r= -0.49,

p=0.02). In the 15 week experiment fat volume correlated significantly with tissue mineral density of the cortical tibia (r= -0.514, p=0.007) and cortical femur (r=-0.477, p=0.012).

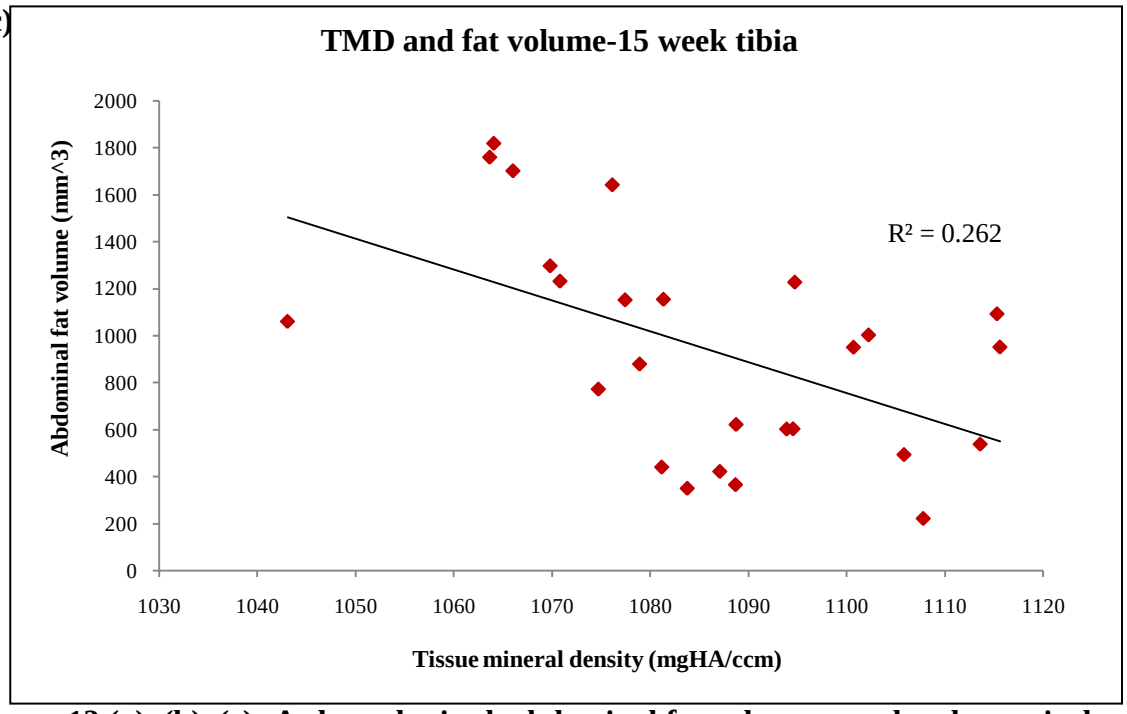
12a)



12b)



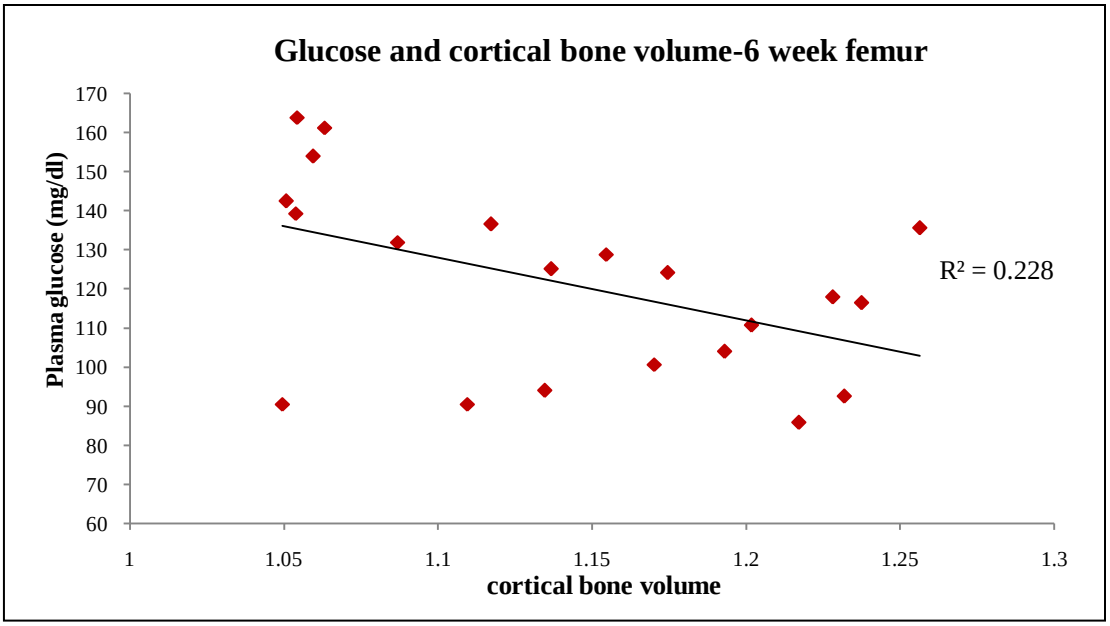
12c)



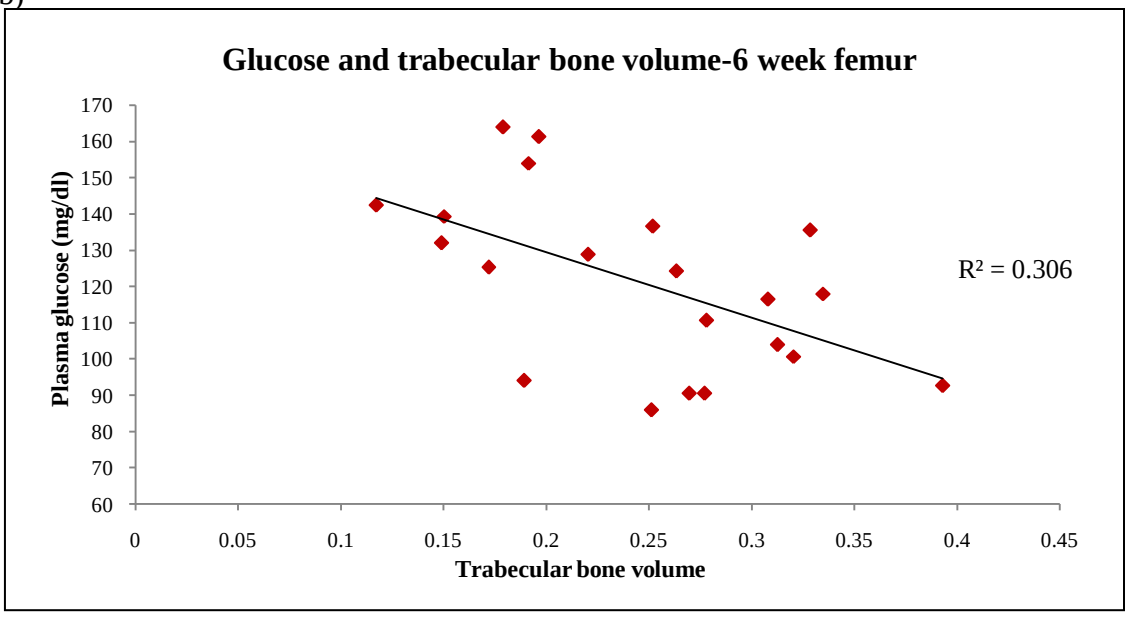
Figures 12 (a), (b), (c): As hypothesized, abdominal fat volume correlated negatively with tissue mineral density in the load-bearing bones of both the 6 week (a) and 15 week study across all three groups (b, c) suggesting an inverse adipogenesis-osteogenesis association.

Correlations - Plasma Glucose and Bone Volume: In the 6 week study, across all three groups glucose levels showed significant negative correlations with cortical bone volume (r=-0.478, p=0.028), trabecular bone volume (r=-0.554, p=0.009) and connectivity density (r=-0.654, p=0.001) in the femur. Glucose levels also correlated with trabecular bone volume (r=-0.456, p=0.043) and trabecular thickness (r=-0.502, p=0.024) in the tibia. Insulin showed a significant positive correlation with trabecular thickness in the tibia (r=0.474, p=0.035).

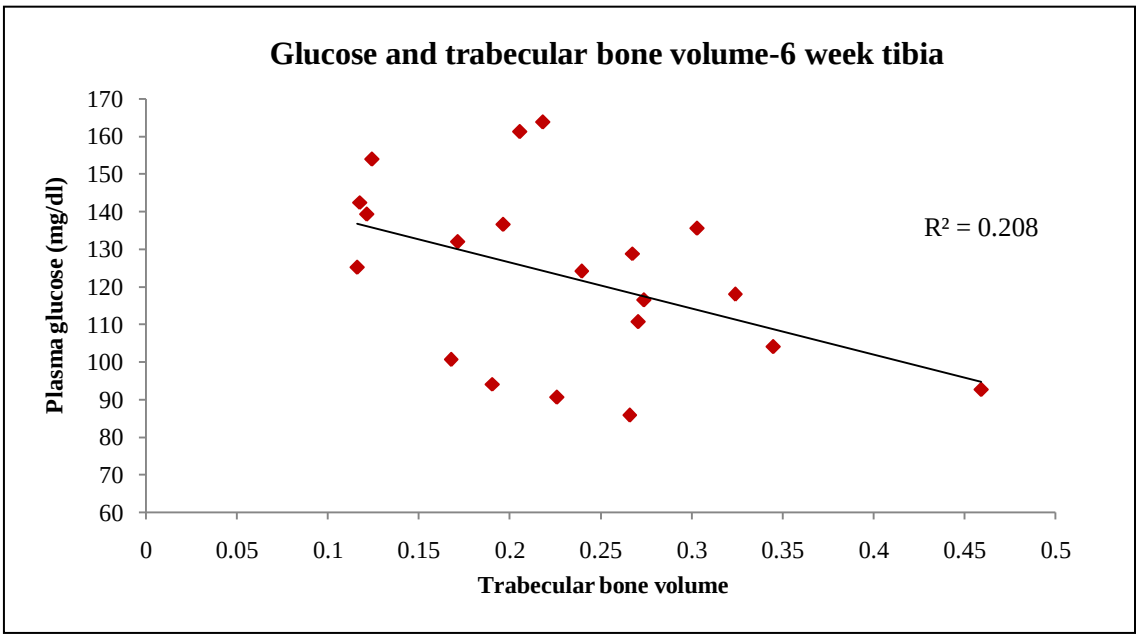
13 a)


Glucose and cortical bone volume-6 week femur
Plasma glucose (mg/dl)
170
160
150
140
130
120
110
100
90
80
70
60
1
1.05
1.1
1.15
1.2
1.25
1.3
cortical bone volume
R² = 0.228

13 b)


Glucose and trabecular bone volume-6 week femur
Plasma glucose (mg/dl)
170
160
150
140
130
120
110
100
90
80
70
60
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
Trabecular bone volume
R² = 0.306

13 c)



Figures 13 (a), (b), (c): In the 6 week experiment, glucose levels showed significant negative correlations with cortical and trabecular bone volume in the femur (a, b) and with trabecular bone volume in the tibia (c) across all three groups suggesting that the mechanisms via which high-fat and fructose diets affect bone mass are not unrelated to changes in glucose levels.

Discussion

Effects of Diet on Load Bearing Bones – Summary of Findings: The overall objective of the diet study was to use micro-computed tomography to quantify, by comparison to a control diet, the effects of a high-fat (HF) and a high-carbohydrate (CH) diet on bone density, mass, cortical thickness and trabecular structure. The hypothesis was that both the diets would prove to be detrimental to bone development in growing mice. After 6 weeks of fructose diet when compared to control, the cortical thickness of tibia was reduced, cortical bone volume of both tibia and femur was less, trabecular bone volume fraction, connectivity, trabecular number were less and trabecular separation was greater in both the tibia and femur of the fructose group. On the other hand, the high-fat diet caused a very small decrease in tissue mineral density (TMD) of the tibia but no other effects were seen. Conversely in the 15 week experiment, the bone volume fraction and cortical thickness of the high fat group was significantly less compared to SC in both tibia and femur of the high-fat diet group. The HF group also had lower trabecular bone volume of the femur, higher trabecular TRISMI of tibia and lower TMD of cortical femur. The fructose diet caused a slight reduction in the cortical thickness of the tibia and no other effects were seen.

Limitations of the Study: While fructose was administered as a diet in the short term study, it was replaced by a water based regimen in the 15 week experiment and this could have caused differences in the results at two time-points. It is not possible to ascertain how different the two treatments were.

In the 15 week study water bottles in the fructose diet group were replaced with graduated plastic bottles to measure weekly water consumption. However, the construction of these bottles was not easy to use and the animals were unable to access water for a few days. Many animals in the fructose group were severely dehydrated. All mice, except one, recovered their body mass and health within a week of switching back to old water-containers, but it is possible that dehydration and loss of body mass may have caused metabolic fluctuations, affected food intake, and depleted fat deposits. There is no method of assessing exactly how this affected results.

High-Fat Diet Consumption Reduced Food Consumption but Increased Fat Deposition: The high-fat diet animals saw a decrease in appetite, which led to prevention of diet induced obesity – the animals had normal body weights and normal triglyceride levels. In both the studies presented here, the animals on the high-fat diet lowered their food intakes and adjusted energy intake such that triglycerides in plasma were not significantly elevated. Leptin based regulatory pathways in the appetite control mechanisms may have been responsible for this. In certain strains of mice, it is found that food intake is responsive to centrally administered leptin up to as long as 11-12 weeks of high-fat diet feeding, after which both peripheral and central leptin resistance develops. (89) The hypophagia observed both in the 6 week and the 15 week study has been previously documented in other strains as being a "middle" stage in development of diet induced obesity (DIO) – a stage of oncoming peripheral resistance to leptin which results in fat deposition, while central leptin sensitivity still attempts to prevent obesity by causing reduction in food intake. (90-91). Hypophagia does not normally begin ~2 weeks after high-fat diet consumption and it is unclear why mice in both studies presented here had a reduced food intake from the very beginning. Energy intake control might function differently in different genetic strains. The BALB/c strain is known to be less susceptible to DIO (92) and is also relatively resistant to metabolic changes caused by obesity (93). Thus while the animals had significantly higher fat deposition and thus presumably also had increased adipogenesis, they were not particularly obese or overweight.

Insulin Levels Modulated the Short-Term Effects of a High-Fat Diet on Bone: The only change caused by the high-fat diet in the six week study was a large and significant increase in insulin levels. In the six week experiment, the high-fat diet group had significantly elevated levels of insulin (54% higher) which led to glycemic regulation, as seen by glucose levels, not significantly different from controls. Other studies have reported similar findings where high-fat diet consumption is accompanied by a continuous increase in insulin levels (41). The increased insulin levels might have had an anabolic effect on bone tissue. Insulin levels have been positively associated with bone mineral density via the action of IGF-1 receptors and their supposed influence on osteoblast function (45, 47). More importantly, lack of insulin causes hyperglycemia which is associated with reduced bone mass, mechanical strength, bone area, osteocalcin

levels and trabecular bone density (44-50). Differences in bone mass, density and structure due to high-fat diet can also be attributed to a shift in the differentiation pathway that mesenchymal cells may opt for leading to increased adipogenesis, which inevitably leads to decreased osteogenesis in the bone marrow, finally manifesting itself as an increase in fat deposition and decrease in bone formation (25). Accordingly, at both time-points there was a large and significant increase in the abdominal fat volumes and fat pad weights of animals in the high-fat group compared to control. Despite increased fat formation, in the six week study, the effects of insulin were sufficient to counteract the detrimental effects of adipogenesis with the net result that load bearing bones were not different from those of controls.

High-Fat Diet Consumption Over a Longer Period Caused Bone Deterioration: In the fifteen week study the high-fat diet did not cause any changes in insulin, glucose or triglyceride values. The food intake was significantly lower compared to the control group suggesting effective leptin action even over a relatively longer period of 15 weeks which led to lack of weight gain. However, there was a very large and significant deposition of fat as seen by abdominal fat scans and fat pad weights (epididymal fat pad weight was ~56% higher). This was accompanied by lower values of bone volume, density, cortical thickness and a weaker trabecular structure. It is very likely that the increase in bodily fat content and deterioration of bone parameters were related to each other. This finding is similar to published reports where increased fat deposits have a negative influence on bone and have been found to be associated with reduced BMD and bone mass (18-19). Studies have also shown that fatty acids found in high-fat diets interact with PPARγ receptors to modulate gene expression in the bone marrow cavity leading to a shift in the osteogenesis versus adipogenesis balance (24). The PPARγ gene itself is also known to interact with BMD-8 to modulate bone formation based on dietary intake (26). High-fat diets also are known to increase osteoclastogenesis in the bone marrow (29) and negatively affect bone material properties(27-28). Therefore in our study, over a longer period (15 weeks), a continuous, sustained and large increase in fat deposition and adipogenesis accompanied by consumption of a diet high in fatty acid content caused significant degeneration of bone mass and structure.

Contribution of Leptin to the Detrimental Effects of High-Fat Diet on Bone: Leptin is an important link in the regulation of bone formation vs. resorption although its ultimate effect on bone is debatable. Leptin receptors have been found on osteoblasts and leptin is secreted by adipocytes- thus a bone marrow high in adipocytes and leptin levels can be expected to increase anabolic bone forming activity by stimulating osteoblast function. Leptin is also said to promote osteogenic differentiation while suppressing adipogenic differentiaton of bone marrow mesenchymal stem cells (94-96). At the same time, centrally administered leptin increases trabecular bone resorption via its action on osteoblast beta2-adregenic receptors(97). The role of leptin in the present research cannot be determined conclusively as of now without additional data. Nevertheless, as is indicated by the significant decrease in food intake, it is probable that high-fat diet animals in the present study had increased leptin levels which contributed to the bone loss/ affected bone development in the 15 week study.

High Fructose Diet Negatively Affected Bone Development by Affecting Body Mass: It is possible that a high-fructose diet does not alter bone directly but rather manifests its effects by altering appetite, body weight and consequently bone. In the 6 week study, food intake of the animals in the fructose group decreased after the first three weeks, with a corresponding drop in body weight. There are multiple reasons why food intake of the animals in the fructose group became temporally variable in the 6 week study, after being stable initially. It is possible that the animals took a dislike to the diet pellets provided, which were prone to degradation at room temperature. It is also possible that increased carbohydrate intake affected appetite. It has been shown in many studies that fructose intake leads to an increased and early feeling of satiety and is associated with reduced calorie consumption (98-100). But this change was observed ~3 weeks after the experiment started, whereas it would be expected earlier. A third explanation is that the animals in the 6 week fructose group saw a marked decrease in insulin levels and leptin production is in part regulated by diet induced changes in insulin levels (83). It is possible that the lower insulin levels interfered with normal leptin activity leading to changes in food intake.

Reduced Weight Bearing Lead to Significant Bone Damage: In any case, an ANCOVA analysis revealed that when body weight was accounted for in the 6 week experiment, differences in most micro-CT bone parameters lost their statistical significance. By what amount the weight bearing on any particular bone needs to be altered to be directly responsible for causing a significant change in bone mass is debatable. In the 3rd week of the 6 week experiment, the mice in the CH group started losing 3.3%, 6.4%, 4.8% of body weight every week which was a net weight loss of 13% over 3 weeks. Even small percentages of weight loss in the developmental stage can have negative effects on bone development. Other studies that have tried to establish a relationship between weight change and tissue mineral density (TMD) have found mixed results. Nevertheless, there is evidence that even a small degree of weight loss can cause a significant decrease in TMD in both older women and young adolescents. But other studies have found that change in body weight has little or no significant effect on TMD (101-107). From our data it is clear that loss of body weight can significantly affect bone loss.

Detrimental Effects of Fructose Diet on Bone were Independent of Adipogenesis: Similar to a high-fat diet, consumption of a high-fructose diet can also lead to weight gain and obesity along with increased fat deposition which as discussed earlier is associated with decreased osteogenesis (108). In the 6 week study, food intake of the animals was too erratic for sustained deposition of fat to take place. In the 15 week experiment, for one week in the study, the animals had restricted access to water and lost body mass and caused dehydration which may have affected fat stores. Thus, in neither study did the fructose consumption result in increase in abdominal fat volume or increase in fat pad weights. In our research, excess adipogenesis, as a factor, can thus be ruled out as being responsible for any fructose induced changes in the load bearing bones. This shows that the effects of carbohydrate/sugar consumption on bone development are not entirely dependent on DIO.

Fructose Diet Influenced Bone via Glycemic Disruptions: Even after accounting for body weight, the cortical thickness of the tibia in the fructose group was still significantly lower than control. While not statistically significant, values of parameters such as bone

volume, density and trabecular thickness were still lowest in the CH group suggesting that body weight, though a significant factor, was not the only factor which caused bone detriment. Irrespective of food intake and body weight, a high-fructose diet can cause glycemic imbalance and affect insulin levels (42). Although there is no consensus in literature on the effects of fructose consumption on fasting insulin levels, which depends on many factors such as exact diet composition, species/strain of animal used etc., fructose is known to cause reduced insulin secretion compared to other carbohydrates such as glucose (42, 82, 109). But in rodents, fructose consumption can cause hyperinsulinemia (83), a finding which is not supported by our data. In the 6 week study presented here, the fructose group saw a significant and large decrease in levels of insulin accompanied by a significant increase in levels of glucose. As discussed above, insulin levels have been positively associated with bone mineral density and lack of insulin causes hyperglycemia, along with reductions in bone mass, mechanical strength and density. In this study, dietary fructose consumption caused a drop in levels of circulating insulin accompanied with and probably contributing to decreases in bone volume, density.

Fructose Consumption Over a Longer Period – Attenuated Bone Damage: In the 15 week experiment, administering fructose via water did not have any effects on food intake, body weight or fat deposition. Fructose intake in the 15 week study also failed to cause a notable change on any bone parameters other than cortical thickness and TMD of the tibia which significantly decreased. Although not statistically significant, the insulin levels in the fructose group were still ~47% higher than control. Higher insulin levels have been associated with higher BMD values as discussed above (45, 47). Insulinopenia due to fructose diet consumption observed in the 6 week study was not repeated in the 15 week experiment and this may have attenuated the detrimental effects of fructose consumption on load bearing bones seen in the shorter study. Nevertheless, fructose consumption via water affected cortical bone thickness and bone density further providing evidence of the damaging effects of increased fructose intake on bone health.

Plasma Glucose Levels Had Negative Effects on Bone Structure and Mass: The theory that changes in metabolic status was responsible for changes in bone volume and

morphology in the 6 week study, was further supported by regression analysis. In the 6 week study, across all three groups, glucose levels showed significant negative correlations with cortical femoral bone volume and with trabecular bone volume, bone volume fraction and parameters describing trabecular structure in both femur and tibia. In the trabecular region of the tibia, insulin showed a significant positive correlation with trabecular thickness. Other studies have found similar associations between blood glucose levels and indicators of bone health such as osteocalcin levels, bone density and bone mineral content (48-49). We have further extended these findings using micro-CT analysis to show that metabolic changes have associations with bone volume and trabecular morphology as well. While correlations do not necessarily imply cause-effect, they indicate that glycemic balance is at-least a link in the mechanism via which fructose diet treatment affects bone health.

Inverse Relationship between Fat Volume and Bone Density: In many diet induced obesity studies which seek to establish a bone-fat relationship, there often arises the problem that along with increase in fat, the animals in the high-fat diet group see an increase in body weight, leading to increased weight bearing for that group and it is often difficult to separate the effects of the high-fat diet from effects of increase in weight (23). In both the experiments in this project, this problem was not encountered because animals in the high-fat diet group adjusted their food intake to significantly lower values than the other two groups, with the result that while they had definite increased fat deposition, their body weights were not different from the other groups, allowing for a direct comparison of the load-bearing bones of the three groups. In the 6 week experiment there was a clear and significant negative correlation between fat volume and bone mineral density in the tibia. Similarly in the 15 week experiment there was a significant negative correlation between fat volumes and the bone mineral density of tibia and femur. These kinds of relationships have been established in clinical studies involving human subjects (15, 18-19) and also in studies proving the inverse relationship between adipocyte and osteoblast differentiation (25). However many of these studies are limited by a high degree of genetic/lifestyle variation in the population tested. Our data further confirms the bone-fat inverse relationship in a controlled animal study.

Summary: In both the six week and fifteen week study, animals on a high-fat diet had significantly higher fat deposition but were not overweight compared to controls. This allowed us to assess the effects of high-fat diet feeding on bone tissue without the confounding factor of load bearing. Accordingly it was found that in the six week study the high-fat diet did not have any catabolic effects on bone deposition. It is possible that the effects of increased insulin in this group were sufficient to counteract the detrimental effects of adipogenesis. However, over a longer period (15 weeks), large increases in fat deposition were accompanied by significant detriment of bone mass and structure. The longer duration of high-fat feeding, increased deposition of fat or possible increases in leptin levels may be responsible for this. Thus in the research presented here high-fat diet feeding significantly modulated bone mass and morphology via mechanisms involving metabolic changes, increased fat formation and possibly leptin action. These changes in load bearing bones were also dependent on duration of high-fat diet feeding. Our results show that although obesity might have a protective effect on the skeleton as has been claimed, when load bearing is taken out of the equation, a diet high in fat content is in-fact detrimental to bone tissue.

In the 6 week study the fructose group saw significant decreases in body mass and possibly weight bearing. It is very likely that the loss of weight bearing was largely responsible for the degeneration of bone mass, morphology and density seen in this group. However, even after taking body mass into account fructose diet consumption had a direct effect on cortical morphology. Bodily fat content in the fructose animals was the same or even lower than controls ruling out adipogenesis as a factor influencing bone tissue. It is possible that drop in levels of insulin and increase in circulating glucose caused by dietary fructose over 6 weeks was responsible for the reduction in cortical thickness. In the 15 week study 10% fructose water consumption reduced cortical thickness and bone density. However, there were no significant changes in metabolic status or fat formation or body mass and weight bearing. This indicates that sweetened beverage consumption can affect bone density via pathways other than adipogenesis and glycemic imbalance only. It is possible that sweetened water consumption may have disrupted the mineral balance in these animals or increased urinary extraction of

phosphorous leading to changes in bone density. Thus a fructose diet can affect bone development by affecting metabolic balance and body mass.

CHAPTER FOUR: WHOLE BODY VIBRATION TREATMENT

Introduction

Exercise and increased physical activity are effective and recommended treatments prescribed to both osteopenic and obese individuals. Exercise increases mechanical loading on bones and depletes fat stores for energy. Recently it has been shown that there may be a third mechanism by which exercise regulates both obesity and bone loss at the same time. Mechanical signals generated by regular physical activity can be transduced by stromal cells into increased bone formation, which automatically leads to reduced fat formation because of the inverse relationship between adipogenesis versus osteogenesis(61). However, despite its efficacy many populations of obese/osteopenic individuals are incapable of regular physical activity or exercise due to age, paralysis or other impairments. For example, in age related osteoporosis bones are often too fragile to be able to tolerate strains generated by vigorous exercise regimens. Recently, low level whole body vibration (WBV) has been proposed as a non-pharmacological and safe treatment. WBV has been shown to bias lineage selection towards increased osteoblast differentiation and decreased adipocytic differentiation and has therefore been developed and tested as a treatment for both osteoporosis and obesity (25, 110). Osteogenic effects of WBV have been documented, both at the cellular level, and at tissue level(25). WBV can cause a population shift in the bone marrow stem cell niche by decreasing the number of hematopoetic stem cells and increasing the number of mesenchymal stem cells, leading to increased bone volume fraction, improved bone morphology and structure and reduced adiposity in rodents(25). This anabolic skeletal effect has been demonstrated to be fairly consistent, successfully promoting osteogenesis in sheep(111) and mice(62).

However, the magnitude of the skeletons response to WBV has been seen to vary across genetic strains(77), hormonal status(78), bone site tested(111), treatment duration, strain and frequency parameters applied(79, 112) and age group(79). To be able to fully utilize the potential of WBV treatment, it is important to study the factors which modulate the sensitivity of the skeleton to these mechanical signals and cause such varied responses. While age and genetic make-up have been proposed to be two major influences which alter this response, the goal of this research is to explore dietary composition as a factor.

WBV been shown to assert its effects even in cases of diet induced obesity and high fat diet consumption in one mouse strain(25). As mentioned in earlier chapters, a high sugar/high-fat diet is a proven cause of low BMD and impairs proper bone formation, via many direct and indirect pathways, and thus may interfere with the response of bone cells to WBV. Considering that the prevalence of such unhealthy food intake is increasing daily, there is a need to examine if WBV, which successfully elicits osteogenic responses in healthy animals, can reverse bone loss caused by high-fat/fructose diets. High-fat and high-sugar diets also cause hyperglycemia and hyperinsulinemia which has been linked to decreased BMD and bone mass (41, 44, 82). Our study aimed at examining whether WBV can have a stabilizing effect on the glycemic imbalance caused by these diets. Finally it is important to see whether consumption of these diets will interfere with the expected anabolic response of the skeleton to WBV. Thus the goals of this study were to:

1) Assess the efficacy of WBV in compensating the negative effects of a high-fat and high-fructose diet consumption on bone density and micro-architecture
2) Document changes in adipose tissue accumulation on application of WBV in the high-fat diet, fructose diet groups.
3) Assess whether modulation of the bone-fat balance using WBV involves changes in levels of insulin and glucose.
4) Compare skeletal, metabolic, anti-adipogenic effects of WBV at two durations of treatment.

Methods

Animals and Diets: 60 BALB/c mice for the 6 week study and 60 BALB/c mice for the 15 week study were ordered from Jackson Laboratories and randomly assigned into either a high-fat diet, high-fructose diet or a control diet group (n=20/group in each study). Each group was further divided into 2 subgroups (n=10), one that was subjected to whole body vibration treatment and one that was not. The high-fat diet was in form of pellets obtained from Purina Test Diet Inc., which is part of the "van-heek" series of lab animal research diets (Purina van-heek 58V8 diet) and provides 45% k cal from fat. For the 6 week group of animals on the high-fructose regimen, diet was in form of pellets obtained from Harlan Teklad and was a 60% fructose diet that provided 66.8% kcal from carbohydrates. However, the food intake of the animals seemed significantly lower than control over the entire period of the study, leading to weight loss. Hence, for the 15 week study, the high-fructose regimen consisted of 10% w/v powdered fructose being administered by mixing with drinking water. Animals were hence fed standard chow diet and 10% high fructose water. The control group at both time-points consisted of animals fed a standard lab-chow diet. Animals were housed in individual cages with a 12 hr light/dark cycle and given free access to food and water at all times. All animals were 7 weeks old at the beginning of the study and were treated for 6 weeks or for 15 weeks.

Body weight and Food Intake: Body weight and food intake in both experiments were recorded on a weekly basis. In the fructose group of the 15 week experiment, in the 8^{th} week of vibration one mouse had to be euthanized because of excessive weight loss and dehydration which resulted from insufficiency of drinking water. In an attempt to monitor the amount of water being consumed every week in the fructose group, the standard water-bottles were replaced with smaller graded containers. However, the animals were not able to acclimatize themselves to using these, which led to water deprivation for 3-4 days. Animals in the fructose group saw a sudden decrease in body mass. However, after resorting back to old water containers all animals, except one, recovered their body weight in the span of about a week, after which they showed normal cage activity, appearance and food intake.

Application of Whole Body Vibration: Animals in the vibrated groups were subjected to daily WBV treatment. WBV were applied via a circular shaped vibrating platform at 90 Hz frequency, 0.2 g acceleration magnitude for 15 min/day, 5 days/week for 6 weeks or 15 weeks. These treatment parameters were chosen based on previous studies that have successfully demonstrated their use as an osteogenic stimulus in rodents(25). Mice were placed in a flat bottomed, transparent, covered plastic container which was divided into 10 slots. The container was then placed onto the vibrating plate and the acceleration magnitude inside each slot was measured using an accelerometer and a LabView program before starting the treatment every day. Mice in the untreated groups were placed in a similar container on the ground next to the vibrating plate. Placement of mice in the slots was rotated clockwise on a daily basis to ensure that any variations that may exist across the surface of the plate don't affect any one set of animals over the entire experimental period. The animals did not have any negative side effects after the treatment as observed by normal activity and general healthy appearance.

In-vivo Micro-computed Tomography to Quantify Abdominal Adiposity: The abdominal regions (lumbar L1 to L5) of all animals were scanned in-vivo using micro-computed tomography to quantify central adiposity. For the 15 week experiment, n=10 for all groups, other than the fructose non-vibrated group where n=9. Animals were scanned using Viva-CT 75 scanner, Scanco Medical at a resolution of 82 µm. Animals in the 6 week treatment group were scanned at 12 weeks of age and sacrificed at 13 week of age, after 6 weeks of treatment. Animals in the 15 week treatment group were scanned at 20 weeks of age, and sacrificed at 22 week of age. Animals were scanned by securing them to an animal bed with both hind-limbs stretched out and secured. Isoflurane inhalation (1%) was used to induce general anesthesia. The resolution and energy settings (82 µm, 45 KV, 133µA, 300 ms integration time) were chosen taking into account factors such as scan time, exposure of animal to X-ray radiation and required tissue detail. The gray scale scans were then reconstructed into 3 dimensional images of the visceral and subcutaneous fat, contained in the torso of each animal, using an automated separation script written in the image processing language (IPL) and the software provided by Scanco. The landmarks used to define the region of interest for analysis were the lumbar L1 to L5 vertebral disks. The script was used to apply image thresholding (to delineate fat

from lean and bone tissue), Gaussian filtering (for noise removal) and edge detection techniques on the selected region of interest, to quantify the total fat volume and separate the visceral adiposity from the subcutaneous adiposity. The functioning of the script is described in detail elsewhere (85)

Tissue Collection and Preparation: All animals were sacrificed at the 6 week (n=54 animals) or 15 week (n=59 animals) time-points. Blood was collected via cardiac puncture with the animal under anesthesia after overnight fasting. Plasma was isolated via centrifugation at 14,000 rpm, 4 degrees for 15 mins. Mice were euthanized via cervical dislocation. Right femurs and tibiae were harvested and preserved in 70% ethanol for micro-computed tomography scanning. The weights of the epididymal fat pad, brown fat pad were recorded.

Ex-vivo Micro-computed Tomography to Establish Bone Phenotype: The morphology of the proximal end of the tibia and distal end of femur was examined using micro-computed tomography scanning. The proximal tibia and distal femur are regions where effects of nutritional and vibrational stimuli have been previously found to be manifested to a significant degree.

The distal metaphysis of the femur was scanned ex-vivo using micro-computed tomography (Micro-CT 40, Scanco Medical) at a resolution of 12 µm. This resolution is sufficiently accurate to provide detailed data on cortical and trabecular BMD, mass and micro-architecture, while not being excessively high so as to introduce unnecessary noise. Femurs were carefully cleaned of adherent tissue and held in place in the sample holder using foam. An ethanol medium was maintained throughout the scan. For the distal femur, a tissue volume of 1500 µm was chosen for analysis. For identification of the start and end of the region of interest, the landmark used was the point where the growth plate starts to disappear. This point was chosen qualitatively, by visual slice by slice examination of the gray scale scans. After identifying the slice where a break appears in the growth plate, a region of 600 µm was subtracted from this point so as to ensure that the ROI is entirely away from the growth plate and in the metaphysis. A region of 1500 µm was then chosen and submitted for analysis using an automated separation algorithm that delineates cortical and trabecular bone and reproduces 3

dimensional images of the gray scale scans. A detailed explanation of this algorithm can be found in the publication by Lublinsky et al (86).

For the proximal tibia a region of 1080 µm was chosen for analysis. Bones were prepared for analysis as described above. Similar to the protocol followed for the femur for identification of the start and end of the region of interest, the landmark used was the point where the growth plate starts to disappear. 300 µm distal to this point, a region of 1080 µm was chosen for analysis. Bones that were damaged/broken during extraction or cleaning were excluded from analysis.

Determination of plasma glucose concentrations: Plasma samples were sent to the Metabolic Mouse Phenotyping Centre at the University of Cincinnati for assaying the plasma concentrations of glucose. The assay was carried out using a glucose assay kit from Diagnostic Chemicals Limited. Standards were obtained from NERL diagnostics. Briefly, the reagent was prepared by adding 100 ml of de-ionized water and mixing gently as per instructions on kit. 200 µl of reagent was mixed with 2 µl of either deionised water or standard or the sample plasma and the plate was left to incubate at room temperature for 10 minutes. Absorbance of the standards and each sample was determined at a wavelength of 505nm. The glucose concentration in the samples was determined from the standard curve. The standard dilutions consisted of 300, 200, 100, 50, 25 and 0 (mg/dl).

Determination of Plasma Insulin Concentrations: Plasma samples were sent to the Metabolic Mouse Phenotyping Centre at the University of Cincinnati for the insulin assay. The assay was carried out using a mouse endocrine lincomplex kit from Linco (Millipore) (87). The endocrine standard was reconstituted with 250 µl of deionised water to give a 10,000 pM concentration of standard solution. 180 µl of this solution was mixed with 220 µl of Assay buffer to give a 4500 pM standard concentration. This standard solution was then further diluted using assay buffer to give 1500, 500, 166.7, 55.6, 18.5 and 6.2 pM standard concentrations. Only assay buffer was used for the 0 pM standard (background). The endocrine controls provided with the kit were reconstituted with 250 µl water. 30 ml of wash buffer was mixed with 270 ml of deionized water. The insulin antibody-immobilized beads provided were prepared for assay by mixing 0.15 ml of

beads with 2.85 ml of deionized water. Finally the plasma matrix was prepared by adding 1 ml of water to the vial provided. For the assay, the filter plates were blocked using assay buffer, mixed, vacuumed and dried. 10 µl of assay buffer was added to the 0 pM standard (background) and to each sample well. 10 µl of standard or control was added to the appropriate well. 10 µl of matrix dilutent and 25 µl of bead solution were added to standard, control and sample wells. 10 µl of plasma samples were added to appropriate wells. The plate was incubated on a shaker overnight, after which the fluid was removed and plate was washed with wash buffer. 50 µl of detection antibody was added to each well and incubated for 60 minutes at RT. 50 µl of Streptavidin-Phycoerythrin was added to each well, incubated for 30 minutes, following which all contents were vacuumed out. Finally, the plate was washed using buffer and sheath fluid was added to each well. The plate was read using a Luminex Bioanalyzer instrument. Values of insulin levels were calculated using the standard curve.

Determination of Plasma Triglyceride Concentrations: For the 6 week experiment, samples were sent to the Mouse metabolic phenotyping centre for triglyceride analysis. The assay was carried out using a kit from Randox (88). Briefly, the reagent was prepared by reconstituting the vial with buffer. 2 µl of deionized water, standard or sample was added to appropriate wells. 200 µl of reagent was added to each well and gently mixed and then incubated for 5 minutes at RT. Absorbance values were determined by reading the plate at 500 nm. The values for samples were calculated using the standard curve. 200,100, 50, 25, 12.5, 6.25, 0 (mg/dl) concentrations were used as standards. For the 15 week studies, the assay was run using a plasma triglyceride kit by Sigma-Aldrich (25). Briefly, the glycerol standard was used to obtain a standard curve concentration of 0, 0.09, 0.166, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.5 µg/µl. The volume was totaled to 15 µl in each well. The free glycerol reagent and triglyceride reagent was reconstituted with ddH20 following kit instructions. 160 µl of free glycerol reagent and 40 µl of triglyceride reagent were added to each well followed by standards, 15 µl of ddh2o for blanks and 15 µl of samples in appropriate wells. The samples were found to be too concentrated, so the assay was repeated after diluting the samples using deuterated water in 3:1 ratio. Plate was placed on a shaker for 30 minutes and absorbance values were determined at 540 nm. Concentrations of triglyceride were calculated using

equations obtained after plotting known concentrations and optical density from standard curve.

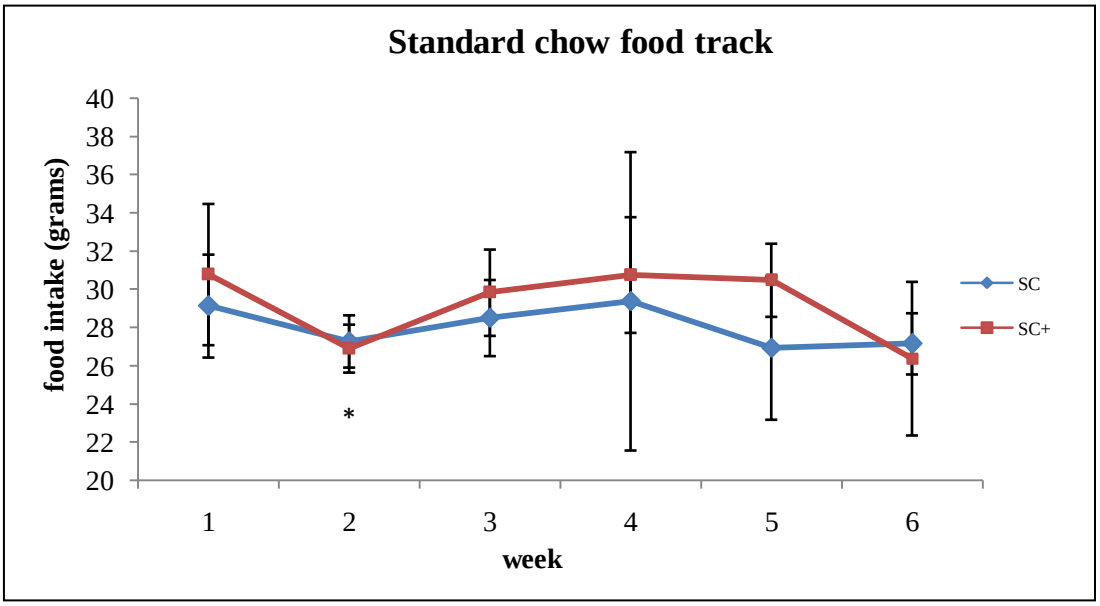
Statistical Analysis: The diet groups were compared to their vibrated counterparts using t-tests to assess effects of vibration. Finally a two-way ANOVA was carried out to check for interaction between vibration and diet treatment.

RESULTS

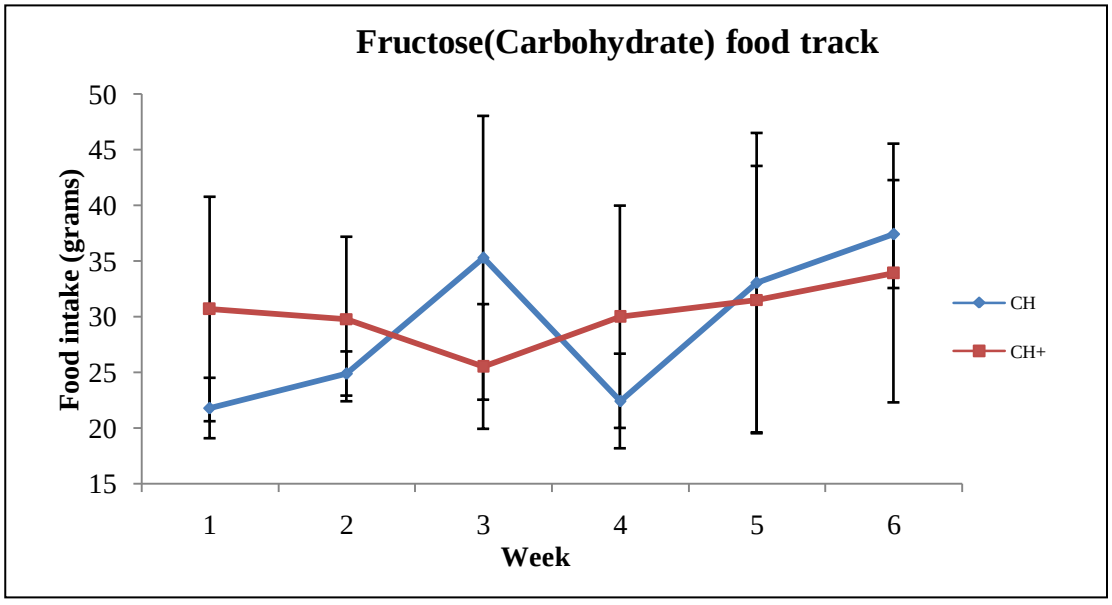
Effects of vibration- 6 week experiment

Food Intake: Comparisons showed that vibrations had no significant effect on food intake in any of the three groups. In the SC group, for week 2 vibrations caused a reduction in food intake ($p<0.05$, SC=30.08 grams and SC+ =26.4 grams). Other than this, the means of the vibrated and control groups were almost identical to each other at most other time-points. 2-way analysis of variance showed that the two factors diet and vibration did not have any influence on each other.

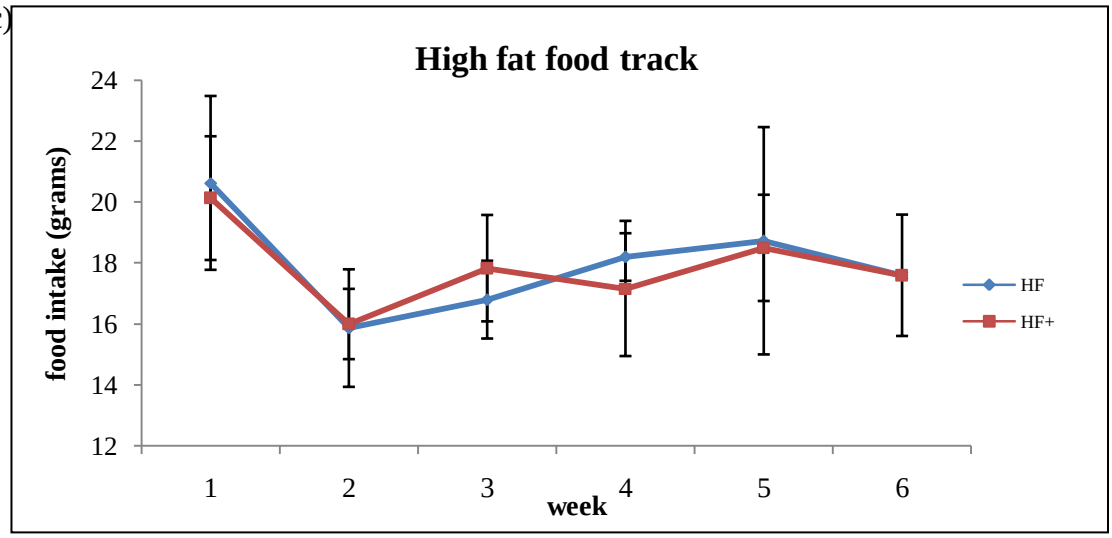
14a)



14b)



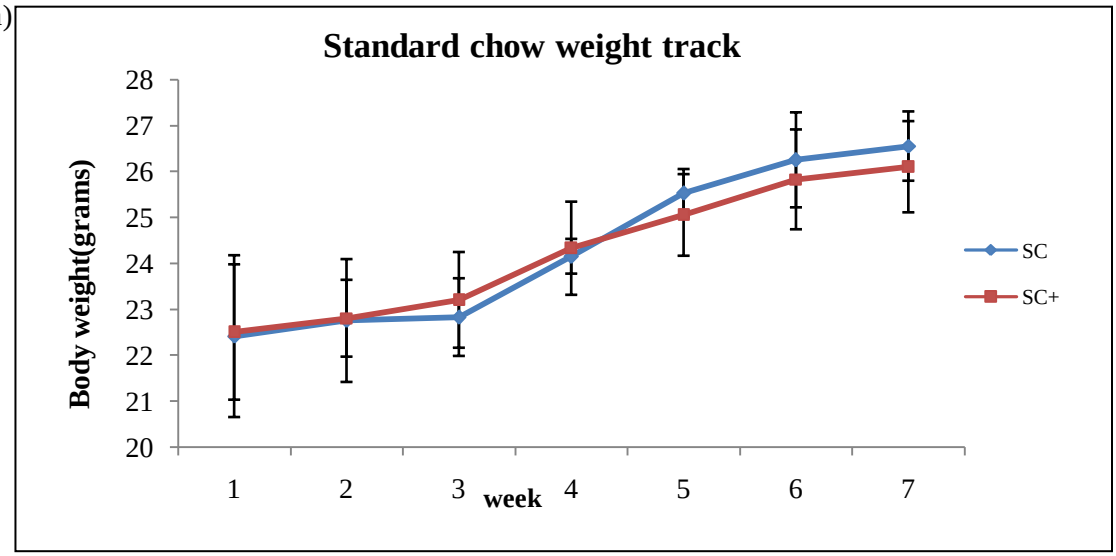
14 c)



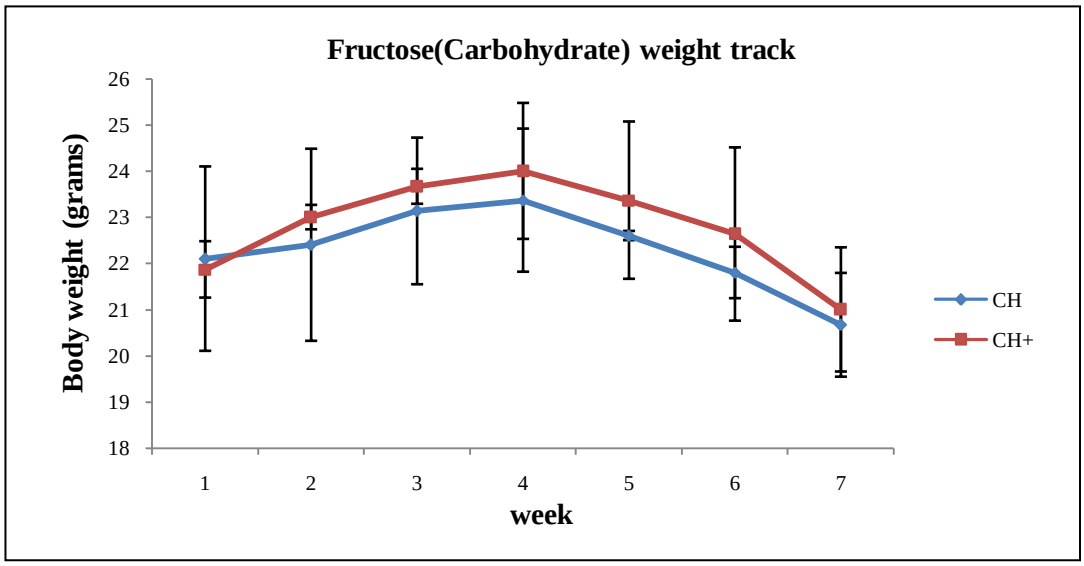
Figures 14 (a), (b), (c): Whole body vibration did not affect the food intake of the SC (figure 14(a)) or CH (figure 14(b)) or HF (figure 14(c)) diet group. In the SC diet group (a), vibrated animals generally tended to eat less but this difference was not statistically significant. (*- SC significantly more than SC+)

Body Weight*:* Comparisons showed that vibrations had no effect on body weight in any of the three groups. The means of the vibrated and control groups were almost identical to each other at most time-points in all the three diet groups and there were no significant differences. 2-way analysis of variance showed that the two factors diet and vibration did not have an influence on each other. The p-values for comparison between control and vibrated at each week are given below. SC, CH, HF represent the diet groups whereas SC+, CH+, HF+ the vibrated diet groups.

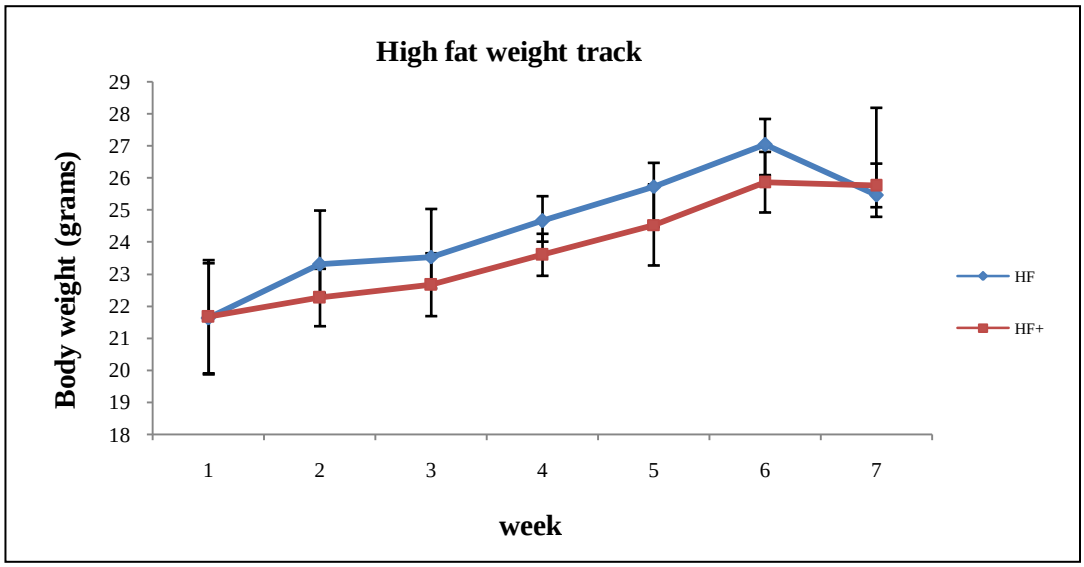
15 a)



15 b)



15 c)



Figures 15 (a), (b), (c): Whole body vibrations did not affect body weight in any of the diet groups. SC (fig 15a) group weights were almost identical between vibrated and control animals. CH (fig 15b) vibrated animals weighed slightly more and HF (fig 15c) animals weighed less but no statistically significant difference existed. Although the weight changes of CH and HF groups after vibration were opposite, no interaction was found between effects of vibration and diet grouping.

Abdominal Fat Volumes: In the SC, CH or HF group vibration did not have any significant effect on total or visceral or subcutaneous fat values. A two-way ANOVA analysis revealed that there was no interaction between the two factors (p value for interaction was 0.7, 0.64, 0.79 for total, visceral and subcutaneous fat. The means and p-values for comparisons are summarized below.

Group	Total fat volume (mm^3)	Visceral fat volume (mm^3)	Subcutaneous fat volume (mm^3)
SC	687.2±234	418.7±161.4	268.6±81
SC+WBV	789.5±263	495.7±198.9	293.8±73
CH	229.1±119.3	75.3±67.9	153.8±61.0
CH+WBV	284.3±117.2	115.6±63.1	168.7±59.1
HF	1130.4±348.4	686.5±251.6	443.9±113.9
HF+WBV	1003.3±397	592±295	411.3±116.7

Table 17(a): Means and standard deviations fat volumes – 6 week study

Comparison	SC vs SC+WBV	CH vs CH+WBV	HF vs HF+WBV
Total fat volume	0.38	0.33	0.49
Visceral fat	0.37	0.21	0.48
Subcutaneous fat	0.48	0.6	0.56

Table 17(b): p-values for vibrated and control comparisons within each diet group

Effects of Vibration on Proximal Tibia-6 week experiment:-

Cortical Region of Tibia: Vibration treatment did not have any effect on the density, volume or cortical thickness of the tibia in the SC, CH and HF groups. Means of most parameters between control and vibrated groups were almost the same and identical in some cases. Means, standard deviations and p-values are summarized below. Two-way analysis of variance showed that there was no interaction between diet and vibration treatments.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
SC	0.89±0.06	0.77±0.02	0.11±0.0	1062.8±16.3
SC+WBV	0.88±0.05	0.79±0.02	0.11±0.0	1072.3±12.3
CH	0.77±0.03	0.75±0.03	0.1±0.0	1077.8±14.7
CH+WBV	0.8±0.08	0.75±0.02	0.1±0.0	1072.3±20.3
HF	0.87±0.07	0.75±0.03	0.11±0.0	1054.2±11.5
HF+WBV	0.86±0.07	0.74±0.05	0.11±0.0	1063.8±12.5

Table 18(a): Means and standard deviations for effects of vibration on cortical tibia-6 week experiment.

Parameter Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)SC+	0.55	0.13	0.65	0.17
CH(vs.)CH+	0.36	0.89	0.50	0.53
HF(vs.)HF+	0.78	0.66	0.80	0.19

Table 18(b): p-values of vibrated and control group comparisons.

Trabecular Region of Tibia: In the trabecular region, vibration treatment was detrimental to bone tissue. For the SC diet group, vibrated animals had lower bone volume (p=0.014, -22%), bone volume fraction (p=0.013, -17%), connectivity density (p=0.027, -22%), decrease in trabecular number (-7%, p=0.01) and an increase in trabecular separation (-8%, p=0.007). In the CH or HF group there were no statistically significant differences. There was no interaction between diet type and vibration treatment. Means, standard deviations and p-values are summarized.

Parameter	SC	SC+WBV	CH	CH+WBV	HF	HF+WBV
Bone volume (mm^3)	0.26±0.05	0.2±0.03	0.15±0.04	0.13±0.05	0.29±0.1	0.2±0.12
Bone volume fraction	0.13±0.02	0.11±0.02	0.08±0.02	0.07±0.02	0.14±0.04	0.13±0.06
Connectivity Density (1/mm^3)	132.6±27	103.7±22.7	61.5±26.5	49.2±33.7	145.6±73.1	120.4±60.1
TRI-SMI	2.5±0.19	2.8±0.2	3.1±0.22	3.1±0.2	2.5±0.4	2.7±0.3
Trabecular Number (1/mm)	5.1±0.3	4.7±0.22	4 ±0.3	3.6±0.8	5.1±0.7	5.3±1.2
Trabecular thickness (mm)	0.04±0.0	0.04±0.0	0.04±0.0	0.04±0.0	0.05±0.0	0.04±0.0
Trabecular separation (mm)	0.19±0.01	0.21±0.01	0.25±0.02	0.3±0.09	0.19±0.03	0.19±0.03
Tissue mineral density (mg HA/ccm)	847.6±17	854.7±13.6	873.1±16.2	863.1±14.4	850.6±31.6	864.24±22.9

Table 19(a): Means and standard deviations effects of vibration on trabecular tibia – 6 week experiment

Parameters	SC&SC+	CH&CH+	HF&HF+
Bone volume	0.017	0.47	0.19
Bone volume fraction	0.013	0.28	0.86
Connectivity Density	0.027	0.41	0.53
TRI-SMI	0.032	0.94	0.38
Trabecular Number	0.01	0.15	0.75
Trabecular thickness	0.11	0.86	0.08
Trabecular separation	0.007	0.12	0.97
Tissue mineral density	0.33	0.2	0.43

Table 19(b): p-values of vibrated and control group comparisons for each diet

Effects of vibration on distal femur 6 week experiment:-

Cortical Region of Femur: In the SC, CH or HF group vibration did not cause any changes in bone volume, density or cortical thickness. Two way ANOVA analyses showed that the two factors diet and vibration did not influence each other. Means, standard deviations and p-values are summarized in tables below.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
SC	1.2±0.05	0.85±0.0	0.14±0.0	1117.8±12.7
SC+WBV	1.2±0.06	0.85±0.0	0.14±0.0	1106.9±19.6
CH	1.1±0.05	0.84±0.0	0.13±0.0	1112.1±21.9
CH+WBV	1.1±0.06	0.85±0.0	0.14±0.0	1116.6±15.4
HF	1.2±0.08	0.84±0.0	0.14±0.0	1108.1±8.3
HF+WBV	1.1±0.07	0.84±0.0	0.14±0.0	1109.9±18.7

Table 20(a): Means and standard deviations for effects of vibration on cortical femur-6 week study

Parameter Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)SC+	0.71	0.41	0.66	0.18
CH(vs.)CH+	0.45	0.12	0.45	0.63
HF(vs.)HF+	0.22	0.89	0.66	0.80

Table 20(b): p-values of vibrated and control group comparisons within each diet.

Trabecular Region of Femur: In the SC and HF group, vibration caused no statistically significant differences. In the CH group vibration caused significant decreases in bone volume fraction (-27%, p=0.046). Two way ANOVA analyses revealed that there was no interaction between diet group and vibration treatment. Means, standard deviations and p-values are summarized below.

Parameters	SC	SC+WBV	CH	CH+WBV	HF	HF+WBV
Bone volume (mm^3)	0.26±0.0	0.23±0.04	0.18±0.06	0.13±0.04	0.29±0.07	0.23±0.07
Bone volume fraction	0.12±0.0	0.11±0.02	0.08±0.02	0.06±0.02	0.13±0.03	0.1±0.02
Connectivity Density (1/mm^3)	133.2±41	122.0±23	76.1±40.9	46.5±27.3	162.1±44	112.9±46
TRI-SMI	2.3±0.26	2.4±0.2	2.8±0.26	3±0.24	2.2±0.28	2.53±0.27
Trabecular Number (1/mm)	4.7±0.35	4.5±0.3	3.9±0.52	3.4±0.53	4.7±0.39	4.36±0.4
Trabecular thickness (mm)	0.04±0.0	0.04±0.0	0.04±0.0	0.04±0.0	0.04±0.0	0.04±0.0
Trabecular separation (mm)	0.21±0.0	0.22±0.02	0.26±0.04	0.3±0.05	0.21±0.02	0.23±0.02
Tissue mineral density (mg HA/ccm)	848.7±19	841.9±21	854.8±26	848.9±20	866.3±19	855.6±20.5

Table 21(a): Means and standard deviations effects of vibration on trabecular femur – 6 week experiment

Parameter	SC&SC+	CH&CH+	HF&HF+
Bone volume	0.25	0.057	0.11
Bone volume fraction	0.29	0.051	0.13
Connectivity Density	0.48	0.11	0.06
TRI-SMI	0.55	0.057	0.06
Trabecular Number	0.19	0.08	0.17
Trabecular thickness	0.24	0.54	0.69
Trabecular separation	0.27	0.09	0.16
Tissue mineral density	0.48	0.61	0.32

Table 21(b): p-values of vibrated and control group comparisons within each diet group

Plasma Insulin, Glucose and Triglyceride Concentrations: In the SC and HF groups, vibration did not have any effect on the levels of plasma insulin, glucose or triglyceride concentrations. In the CH group insulin increased by 27% in the vibrated group compared to non-vibrated (p=0.016). Across groups, there was no interaction between diet and vibration treatment

Parameter	Insulin (ng/mL)	Glucose (mg/dL)	Triglyceride (ug/ul)
SC	0.59±0.3	117.5±16.8	51.4±21.2
SC+WBV	0.57±0.2	115.1±22.6	50.4±19.0
CH	0.17±0.0	141±22.3	292.7±42.3
CH+WBV	0.24±0.0	118±57.5	229.2±99.7
HF	1.3±0.8	103.3±18.6	43.2±13.0
HF+WBV	1.0±0.6	113.5±15.3	43.6±10.3

Compare	Insulin	Glucose	TG
SC & SC+WBV	0.81	0.79	0.92
CH & CH+WBV	0.01	0.34	0.15
HF & HF+WBV	0.46	0.29	0.95

Table 22(a) (b): Means and standard deviations effects of vibration on plasma insulin, glucose and triglyceride concentrations (Left-a); p-values for vibrated and control group comparisons within each diet group (Right-b)

Fat Pad Weights – As seen from weights of epididymal and brown fat pads harvested right after sacrifice, vibration did not have any effect on fat deposition. Means, standard deviations and p-values are given

Group	Epididymal fat (mg)	Brown fat (mg)
SC	278.7±46.6	87.3±13.3
SC+	283.3±86.3	96.8±20.4
CH	126.7±87.3	68.1±34.8
CH+	82.3±33.4	79.5±31.6
HF	386.5±125	113.4±27.0
HF+	367.2±171.8	118.6±23.7

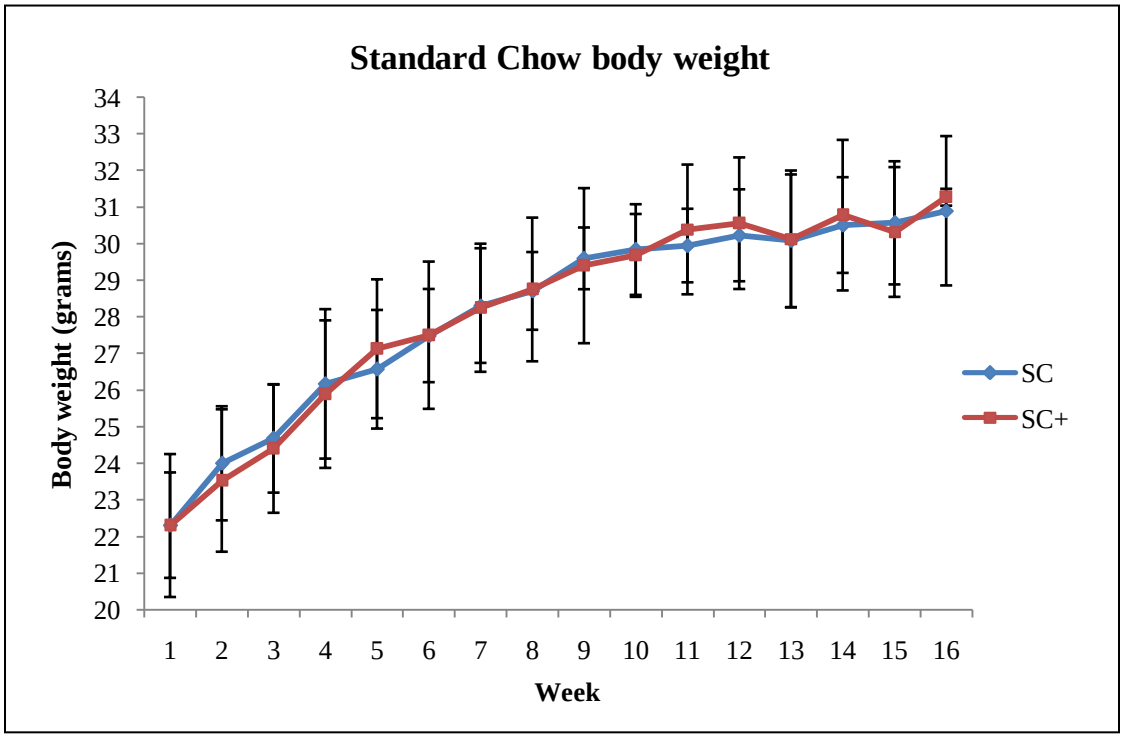
Comparison	Epididymal fat	Brown fat
SC & SC+	0.88	0.30
CH & CH+	0.21	0.52
HF & HF+	0.80	0.74

Table 23(a) (b): means and standard deviation; effects of vibration on fat pad weights 6 week experiment (L); p-values of vibrated and control group comparisons within each diet group (R).

Effects of vibration –15 week experiment

Body Weight: In the SC, CH and HF group vibration did not have any effect on body weight. The mean values of vibrated and control groups were often almost identical.

16a)



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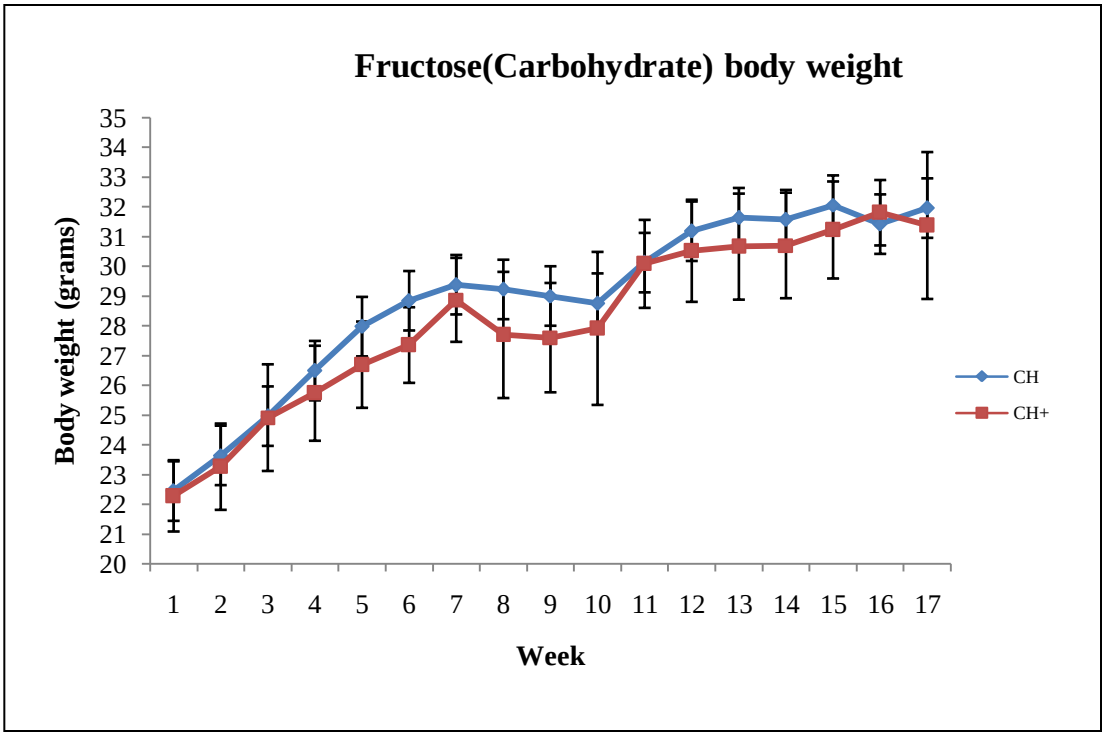


16c)

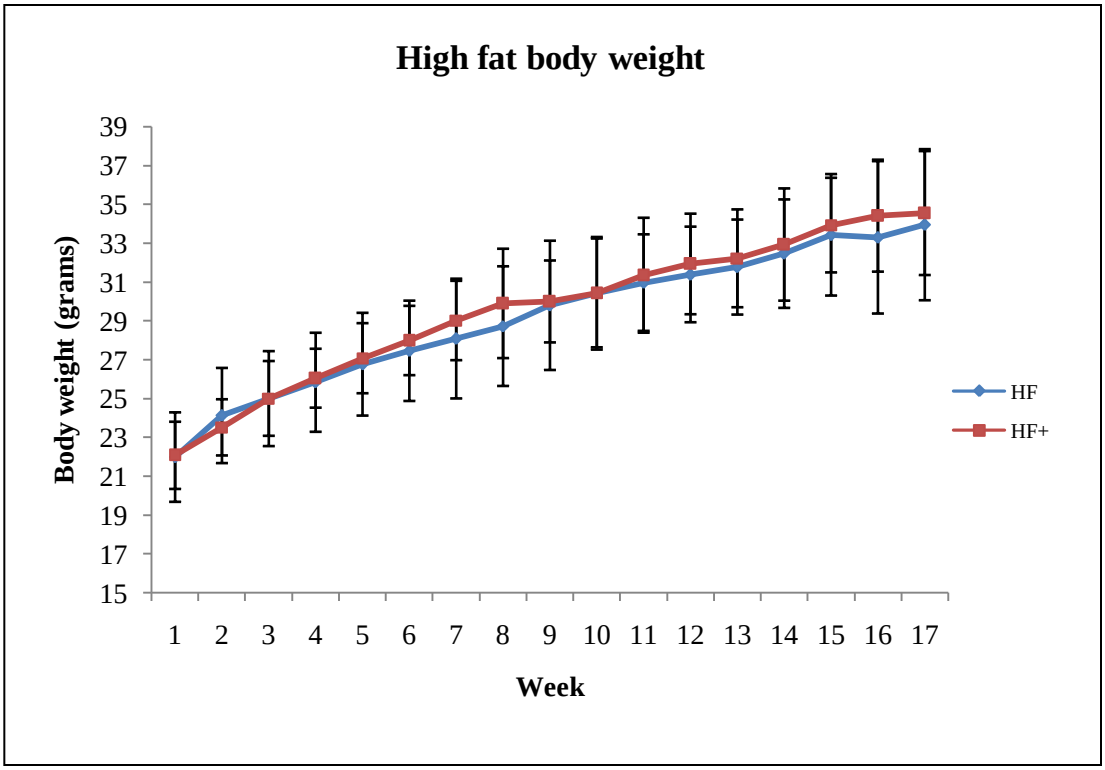
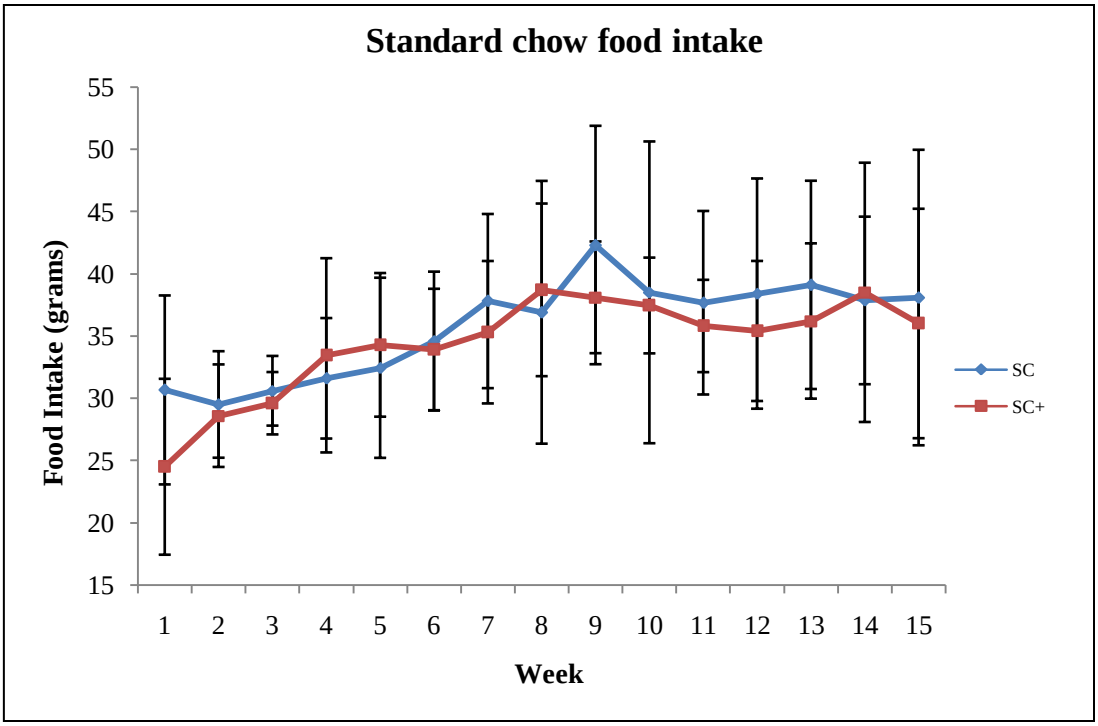


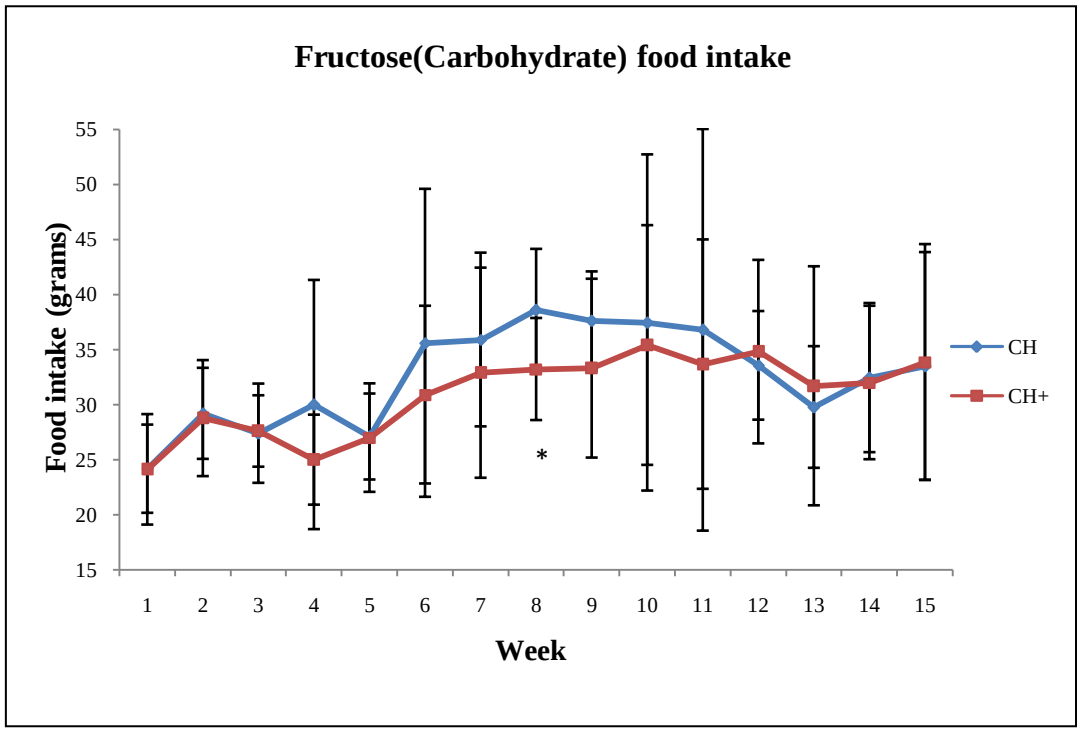
Figure 16 (a),(b),(c): In the 15 week experiment, vibration treatment did not have any discernable effect on body weight in the SC (Fig 16a) or HF (Fig 16b) or CH (Fig 16c) diet group.

Food intake: In the SC group, vibration did not have any significant effect on food intake. In the CH group, in the eighth week the difference was statistically significant with the vibrated animals eating 14% less food than controls ($p<0.05$). In the high-fat diet group in week 9 and week 11 the vibrated group ate ~10% more food than the controls ($p<0.05$). Other than this, there were no statistically significant differences between control and vibrated groups. There was no interaction between vibration and diet treatments.

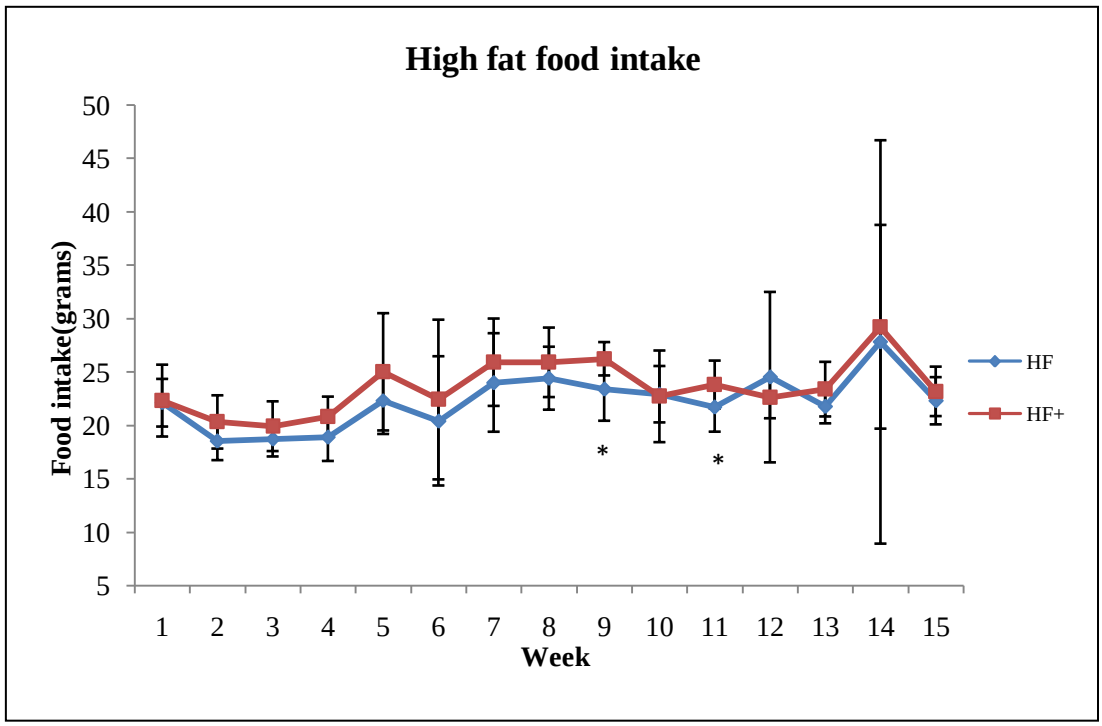
17a)



17b)



17c)



Figures 17 (a), (b), (c): WBV dint have any effect on the food intake of animals in either SC (Fig 17a), CH (Fig 17b) or HF (Fig 17c) groups. (*- vibrated significantly different from control)

Abdominal fat volume: In the SC, CH or HF group vibration did not cause any significant differences in terms of p-value. There was no interaction between vibration treatment and the diet grouping. Means, standard deviations and p-values are summarized below.

Group	Total fat volume (mm^3)	Visceral fat volume (mm^3)	Subcutaneous fat volume (mm^3)
SC	647.3±291.9	373.02±217.1	274.3±88.6
SC+WBV	691.0±263.0	410.3±193.0	280.7±96.0
CH	797.0±373.9	453.7±256.8	343.3±143
CH+WBV	768.5±230.4	449.1±182.9	319.4±82.2
HF	1368.9±376.7	818.8±237.9	550.1±183
HF+WBV	1161.1±515.4	697.4±429.6	463.7±109.3

Table 24(a): means and standard deviations for effects of vibration on abdominal fat volume- 15 week experiment.

Comparison	SC vs SC+WBV	CH vs CH+WBV	HF vs HF+WBV
Total fat volume	0.74	0.85	0.33
Visceral fat	0.7	0.96	0.46
Subcutaneous fat	0.88	0.68	0.22

Table 24(b): p-values for vibrated and control comparisons within each diet group

Plasma insulin, glucose and triglyceride concentrations: In the SC, CH or HF group there were no statistically significant differences. There was also no interaction between diet grouping and vibration treatment. Means, standard deviations and p-values are summarized below.

a)

Parameter	Insulin (ng/ml)	Glucose (mg/dL)	Triglycerides (ug/ul)
SC	0.94±0.6	158.9±46.9	3.7±1.1
SC+WBV	1.0±0.5	187.6±34.6	3.6±0.5
CH	1.3±1	183.6±36.6	4.2±1.4
CH+WBV	1.3±0.7	195.4±41.9	4.7±1
HF	1.8±1.3	180.8±27.6	3.2±0.7
HF+WBV	1.5±0.8	178.9±49.6	3.0±0.9

b)

Compare	Insulin	Glucose	Triglyceride
SC & SC+	0.7	0.13	0.89
CH & CH+	0.91	0.51	0.37
HF & HF+	0.47	0.92	0.68

Table 25(a), (b): means and standard deviations for effects of vibration on plasma insulin, glucose and triglyceride concentrations; p-values for vibrated and control comparisons within each diet group

Effects of vibration on proximal tibia 15 week experiment:-

Cortical region of tibia: In the cortical region of the tibia, vibration did not cause any changes in bone volume or density or cortical thickness in any of the three diet groups that was statistically significant or percent wise significant. There was no interaction between diet grouping and vibration treatment. Means, standard deviations and p-values are summarized below.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
SC	0.95±0.05	0.77±0.02	0.12±0.0	1104.3±9.5
SC+WBV	0.94±0.06	0.76±0.02	0.11±0.0	1094.9±12.1
CH	0.94±0.05	0.76±0.02	0.11±0.0	1084.8±9.9
CH+WBV	0.93±0.08	0.76±0.03	0.11±0.0	1088.3±8
HF	0.88±0.05	0.73±0.02	0.11±0.0	1069.6±12.0
HF+WBV	0.88±0.06	0.72±0.03	0.10±0.0	1070.2±9.3

Table 26(a): Means and standard deviations for effects of vibration on cortical tibia- 15 week experiment

Parameter / Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)SC+	0.51	0.29	0.08	0.07
CH(vs.)CH+	0.84	0.7	0.28	0.44
HF(vs.)HF+	0.96	0.53	0.35	0.9

Table 26(b): p-values of vibrated and control comparisons within each diet group.

Trabecular region of tibia: In the SC or CH group vibration did not cause any statistically significant differences. In the HF group, vibration decreased TRI-SMI by 8% (p=0.03). There was no interaction between diet and vibration treatment. Means, standard deviations and p-values are summarized below.

Parameter	SC	SC+WBV	CH	CH+WBV	HF	HF+WBV
Bone volume (mm^3)	0.29±0.0	0.28±0.06	0.29±0.04	0.27±0.0	0.27±0.0	0.3±0
Bone volume fraction	0.133±0.0	0.14±0.0	0.14±0.02	0.14±0.0	0.12±0.0	0.14±0
Connectivity Density (1/mm^3)	132.3±43.2	127.1±40.2	128.4±38.6	124.2±40	107.6±31	136.2±39
TRI-SMI	2.3±0.3	2.3±0.25	2.1±0.24	2.3±0.3	2.5±0.2	2.3±0.2
Trabecular Number (1/mm)	5.1±0.5	5.1±0.5	5±0.41	5.0±0.5	5±0.49	5.2±0.5
Trabecular thickness (mm)	0.04±0.0	0.04±0.0	0.04±0.0	0.04±0.	0.04±0.0	0.04±0
Trabecular separation (mm)	0.2±0.0	0.2±0.0	0.2±0.01	0.2±0.0	0.2±0.0	0.19±0.
Tissue mineral density (mg HA/ccm)	859.7±9.9	864.2±18.5	864.8±18	876.7±12	874.4±16	869.6±13

Table 27(a): Means and standard deviations for effects of vibration on trabecular tibia- 15 week

Parameters	SC&SC+	CH&CH+	HF&HF+
Bone volume	0.41	0.48	0.07
Bone volume fraction	0.72	0.81	0.09
Connectivity Density	0.78	0.83	0.08
TRI-SMI	0.79	0.33	0.03
Trabecular Number	0.96	0.86	0.27
Trabecular thickness	0.15	0.92	0.61
Trabecular separation	0.94	0.82	0.31
Tissue mineral density	0.5	0.14	0.47

Table 27(b): p-values for vibrated and control comparisons between each diet group- 15 week experiment.

Effects of vibration on distal femur 15 week experiment:

Cortical region of femur: In the SC group, Vibration did not have any effects on bone volume or cortical thickness of the tibia. There was a statistically significant decrease in the bone mineral density but this was very small (0.8%). In the CH and HF groups, there was no change caused by vibration in any of the parameters. There was no interaction between diet and vibration treatments. Means, standard deviations and p-values are listed below.

Parameter	Bone volume (mm^3)	Bone volume fraction	Cortical thickness (mm)	Tissue mineral density (mg HA/ccm)
SC	1.3±0.0	0.85±0.01	0.14±0.0	1152.3±8.2
SC+WBV	1.3±0.1	0.85±0.02	0.14±0.0	1142.3±6.9
CH	1.3±0.1	0.84±0.02	0.14±0.0	1146.2±12.5
CH+WBV	1.3±0.1	0.85±0.03	0.14±0.0	1148.8±13.4
HF	1.2±0.1	0.81±0.03	0.13±0.0	1126.1±10.9
HF+WBV	1.2±0.1	0.80±0.02	0.13±0.0	1121.8±9.6

Table 28(a): Means and standard deviations for effects of vibration on cortical femur- 15 week experiment.

Parameter Comparisons	Bone volume	Bone volume fraction	Cortical thickness	Tissue mineral density
SC(vs.)SC+	0.38	0.91	0.56	0.01
CH(vs.)CH+	0.93	0.50	0.8	0.67
HF(vs.)HF+	0.58	0.46	0.29	0.37

Table 28(b): p-values for vibrated and control comparisons within each diet group- 15 week experiment.

Trabecular region of femur: In the SC and CH or HF groups, vibration treatment did not have any effect on bone volume or density or trabecular structure. Means were almost the same for vibrated and control groups. Means, standard deviations and p-values are listed below.

Parameter	SC	SC+WBV	CH	CH+WBV	HF	HF+WBV
Bone volume (mm^3)	0.3±0.06	0.3±0.09	0.28± 0.06	0.27±0.06	0.26±0.0	0.3±0.05
Bone volume fraction	0.13±0.03	0.13±0.03	0.12±0.02	0.12±0.02	0.1±0.01	0.11±0.02
Connectivity Density (1/mm^3)	120.3±34.6	112.0±42.3	107.9±29	106.7±32.9	94.5±30	107.7±24.6
TRI-SMI	2.2±0.3	2.2±0.36	2.2±0.26	2.3±0.24	2.5±0.2	2.4±0.22
Trabecular Number (1/mm)	4.6±0.4	4.5±0.4	4.4±0.3	4.4±0.3	4.3±0.35	4.4±0.2
Trabecular thickness (mm)	0.04±0.0	0.05±0.0	0.05±0.0	0.04±0.0	0.04±0.0	0.04±0.0
Trabecular separation (mm)	0.22±0.02	0.22±0.02	0.22±0.02	0.23±0.02	0.23±0.02	0.23±0.01
Tissue mineral density (mg HA /ccm)	880.6±12.8	879±10.3	877.1±15.3	879.4±13	882±12.5	886±10.2

Table 29(a): Means and standard deviations for effects of vibration on trabecular femur – 15 week experiment

Parameter	SC&SC+	CH&CH+	HF&HF+
Bone volume	0.63	0.52	0.25
Bone volume fraction	0.90	0.57	0.27
Connectivity Density	0.66	0.93	0.30
TRI-SMI	0.75	0.6	0.14
Trabecular Number	0.69	0.85	0.56
Trabecular thickness	0.59	0.35	0.94
Trabecular separation	0.76	0.85	0.56
Tissue mineral density	0.76	0.74	0.43

Table 29(b): p-values for vibrated and control comparisons within each diet group.

Fat pad weights*:* As seen from weights of epididymal and brown fat pads vibration did not have any effect on fat deposition. Means, standard deviations and p-values are given below.

a)

Group	Epididymal fat (mg)	Brown fat (mg)
SC	365.6±120	143.6±41.7
SC+	328.8±118.8	146.8±31.6
CH	404.1±196.8	160±51.2
CH+	369.2±84.2	156.7±34.01
HF	839.6±258.2	245.8±99.01
HF+	847.8±296.9	225.5±52.5

b)

Group	Epididymal fat	Brown fat
SC&SC+	0.49	0.84
CH&CH+	0.63	0.87
HF&HF+	0.94	0.57

Tables 30(a) (b): Means and standard deviations for effects of vibration on fat pad weights (left-a); p-values for vibrated and control comparisons within each diet group (right-b)

Discussion – Vibration

Hypotheses and Aims of Research: The goal of the second part of this research was to test the effects of whole body vibration (WBV) treatment on load bearing bones in a regular diet group and in conjunction with high-fat and high-fructose diet treatments. The hypothesis was that vibration treatment will result in increased bone volume, density and also cause an improvement in the trabecular micro-architecture. We also hypothesized that WBV would be effective in repairing bone damage caused by the two experimental diets. Another goal of this research was to see if dietary composition is a factor that affects the response of trabecular and cortical bone to anabolic mechanical stimuli. Finally, we wanted to test if whole body vibration can have an anti-adipogenic and osteogenic effect by decreasing fat deposition and triglyceride levels.

Limitations of the Study: While micro-computed tomography is a very powerful tool that is capable of providing detailed information about the quantity and structure of bone tissue, it is certainly not the only one. There are other tools of investigation such as histomorphometry or mechanical testing which must be carried out for a more comprehensive understanding of the characteristics of the femur and tibia. It is possible that other tests may follow different trends than the ones seen in micro-CT data presented here. There have been studies where anabolic effects of WBV were reflected in results of procedures such as histomorphometry but there was no effect on any micro-CT based bone parameter (77). Secondly, although all possible care was taken to ensure that the acceleration measured across the vibration plate was uniform, some variation might have existed due to the construction of the plate. Mice were vibrated, one group at a time, since only ten mice can fit on the plate and the plate was placed on the same position on the floor every day. Mice were also rotated in the container slots to ensure that any variations across the plate will not particularly affect one set of animals over the entire experiment. Despite all these precautions it is unlikely, but possible, that very small unavoidable variations in acceleration might have existed across the plate. Finally, it is possible that in-vivo scan results in the 15 week study were affected to a certain extent by technical difficulties while carrying out the in-vivo abdominal scans which resulted in noise and motion artifact in some of the gray scale images.

WBV Had No Effect on Body Weight/Food Intake: In the second week of vibration, in the 6 week experiment, the food intake of the control vibrated (SC+WBV) group was significantly but slightly lower than the food intake of the control untreated group (SC). However, this was most likely a result of the animals adjusting to the vibration regimen. In all other time-points of both studies, WBV did not influence food intake. In both the 6 week and 15 week experiment, WBV also did not have any influence on body weights. WBV might have an effect on food intake if it modulates leptin levels. In a study by Maddalozo et al., WBV decreased serum leptin levels and body weights and yet had no effect on food intake (113). Most other studies in literature support our findings, where even if vibration loading has any effect on body mass, it is usually transient, very small and often influenced by other factors (25, 77, 114-115). One study which used localized constrained tibial vibration saw a small decrease in body mass of the treated group, but this was more an effect of anesthesia than the vibration treatment and was reversed (116). While there were no statistically significant differences in the fructose diet group, the vibrated animals weighed slightly more than untreated throughout the study, whereas in the high-fat diet group the vibrated animals weighed slightly less than the untreated throughout the study, suggesting that there could be some interaction between the effects of vibration treatment and the type of diet consumed. In conclusion in this research WBV did not influence body mass or energy intake in the BALB/c mouse strain.

WBV Had No Effects on Adiposity/Fat Accumulation in BALB/c Mice: In the 6 week or the 15 week study, WBV did not have any effect on fat deposition in the control or fructose or high-fat diet groups. There were also no differences in circulating triglyceride levels. The effects of vibration therapy on fat content have been tested before with varying results. For example Luu et al. found that in C57BL/6 mice, on a high fat diet WBV at 0.2g, significantly reduced fat volumes and weights of fat pads (25) and Madalozzo et al. found a similar reduction in whole body fat content in case of regular diet consumption at a larger vibration magnitude (110). But at the same time, other studies have found that fat pad mass is unaffected by WBV at 0.3g in C57BL/6 mice (79), showing that even within the same strain of animals, a slight change in the loading parameter can affect response. Similarly genetic variations which determine predisposition to obesity and mechanisms affecting adipogenesis may result in different

response of fat accumulation to mechanical stimulation. As mentioned above WBV has worked in reducing adipogenesis in the C57BL/6 mouse model, while our results show that this does not seem to be the case in BALB/c mice (25). It is known that C57BL/6 mice are easily predisposed to developing severe DIO whereas, BALB/c mice are resistant to obesity and its metabolic effects (92-93). Accordingly, mice in the control group had normal, and fructose group had normal and below-normal body weights and fat deposits. Therefore our data suggests that in this research, sensitivity of fat tissue to WBV was affected by sensitivity of the animal to developing obesity in the first place. In summary, 6 weeks or 15 weeks WBV did not have any effect on adipogenesis of control, fructose or high-fat diet fed BALB/c mice.

Dietary Composition Interfered with Anti-Adipogenic Effects of WBV: WBV decreased the abdominal fat volume in high-fat diet group of 6 week and 15 week study by ~11% and 15%, but this difference was not statistically significant. Mechanical signals reduce adipogenesis by suppressing expression of PPARγ and promoting osteoblastogenesis instead. Despite high-fat diet feeding, this mechanism of action works in C57BL/6 mice (25, 117). But, fatty acids found in high-fat diets are known to directly influence PPAR action to modulate gene expression (24). Others have similarly shown that increased dietary fat intake alters BMD and bone mass by activating PPARγ mechanisms (26). Hence it is likely that in our study, mechanical stimulation based inhibition of PPARγ activation had to compete with effects of increased fatty acid action, due to continuous high-fat diet feeding. Genetic strain might further affect the magnitude of such interactions. Thus although there were decreases in fat volumes in the vibrated high-fat group, they were not statistically significant. In case of high-fat diet feeding, mechanical signals of altered parameters such as perhaps higher strain or longer duration might be required to assert an anti-adipogenic influence. In our study, WBV failed to prevent diet induced fat deposition in BALB/c mice.

Vibration as a Potential Tool for Maintaining Glycemic Balance: Few studies have been performed to see if whole body vibration has any effect on glycemic control. Studies in human subjects and rodent models have found that vibration exercise does reduce glucose to a small degree but does not significantly affect insulin levels (81, 116,

118-119). Results from this study supported the use of WBV to maintain glycemic control. In the 15 week study presented here, diet or vibration did not cause any changes in insulin/glucose. In the 6 week study, there were no effects of diet or of vibration on glucose/insulin levels in high-fat or standard diet groups. But the fructose diet, in the 6 week study, caused metabolic changes similar to those seen in Type-1 diabetes – there was a severe drop in circulating insulin levels and an associated significant increase in levels of glucose compared to control diet. WBV was able to attenuate this effect to some degree – the CH+WBV group saw a 27% increase in levels of insulin compared to CH and a drop in level of plasma glucose which was not statistically significant, but became almost identical to that of the control group (CH+WBV= 117.62mg/dL, SC=117.52mg/dL). It has been suggested that the anabolic effect of mechanical loading on bone is largely attributable to increased muscle contractions and forces (120-121). Also, metabolic systems are said to modulate responses of bone cells to mechanical signals (122). In our study, it is possible that the WBV necessitated an increase in glucose uptake by cells, thus causing an increase in levels of insulin, since insulin is required for glucose uptake. These findings show that WBV treatment could be a potential non-pharmacological method of maintaining glycemic control.

WBV Effects on Load Bearing Bones: WBV treatment had very little anabolic effect on load bearing bones in both the short term and long term studies. Contrary to our hypothesis, the cortical bone of both femur and tibia in all three diet groups and at both time-points was entirely unresponsive to vibration. In the six week study, WBV either had no effect or was detrimental to trabecular bone. In the fifteen week study, vibration had some anabolic effect on the trabecular region of the tibia of HF animals, while the other two diet groups did not undergo any changes in the tibia or femur. There is no single general conclusion about WBV effects on bone that can be applicable over the two time-points or across the different diet groups. Response to the mechanical stimulation varied from being catabolic to no-response at one time-point and anabolic to no-response at another time-point with each diet group responding in a different manner as is described below.

WBV Had a Detrimental but Transient Effect in the SC Group: In the control diet group, the tibia of the vibrated animals had a reduced trabecular bone volume fraction, connectivity density and trabecular number and increased TRI-SMI and trabecular separation. Without considering effects that diet type might have on WBV, it is surprising that vibration in the six week group was detrimental to bone in the control diet group. It might be possible that the genetic make-up of BALB/c mice resulted in a response to vibration that is different from the anabolic responses seen in other strains such as C57BL/6 (25). There are some other studies which have found that vibration treatment has no effect on trabecular or cortical bone of the tibia or on bone formation rates when a vibration regimen at slightly higher acceleration (0.3g) is used (79). In some other studies which have used the same vibration regimen as ours and the same strain of mouse, it was found that although vibration increased mineralizing surface, data parameters describing trabecular bone morphology such as trabecular bone volume fraction or number/thickness remain unchanged (62). However in our study, BALB/c mice were definitely not entirely unresponsive to vibration- the response was simply not as hypothesized. But this catabolic response of trabecular bone to vibrations was not sustained and was not repeated in the 15 week experiment, where vibrations had no effect on cortical or trabecular tibia or femur other than decreasing TMD of the femur by 1%. The same vibration equipment with the same settings was used in both experiments and it is unlikely that the different responses were due to variations in treatment. It appears as though the catabolic effect in the short term was transient and was attenuated over the 15 week period. Nonetheless, the hypothesis that WBV treatment is anabolic to skeleton of regular diet fed BALB/c mice at 90 Hz and 0.2g was not supported in this study.

Poor Nutrition Induced Bone Loss Influenced Bone Response to Loading: In the fructose diet group, fructose consumption over six weeks caused severe bone loss and erosion of trabecular structure in both the tibia and femur. Whole body vibrations did not attenuate this effect in our study. Studies using WBV have shown good results in healthy, young animals (25, 62, 71-72). But it may be possible that WBV treatment regimens must be altered in terms of strain and duration when used in individuals already suffering from severe bone attrition. Some studies have found that WBV treatment over six weeks does not restore or improve bone loss induced by ovariectomy (123). Others have

proposed that bone loss increases the sensitivity of bone tissue to WBV (78), which is safe and anabolic, even at higher magnitudes than used in our study. While the interactions of effects of poor nutrition induced bone loss with effects of vibration treatment have not been tested, we found that in the fructose group bones that were already fragile were unresponsive to the regular application of WBV.

Insulin Played a Major Role in Bone Adaptation to WBV: It is possible that the large metabolic imbalances caused by the fructose diet interfered with the effects of vibration. WBV may cause an anabolic effect on bone development in part because of the increases in muscle fiber contractions and the resulting strains exerted on bone (120). But muscle contractions require energy and glucose uptake which in turn requires insulin. In absence of appropriate insulin action muscle fiber cells might be unable to function normally and be incapable of transducing mechanical loading signals into increased bone formation. Recently, it has been proposed that muscle activity is not always required and vibration signals are directly transmitted to the skeleton (124). Nonetheless, insulin-glucose imbalance is known to cause increase in bone resorption and decrease in bone formation, thus reducing bone deposition (125-127). It is possible that affects of WBV are attenuated in bones that have an increased resorption rate. Therefore in both the 6 week and 15 week studies presented here, fructose consumption negatively affected insulin levels and glycemic control and these metabolic imbalances interfered with bone response to mechanical stimulation.

Dietary Fat Content Interfered with Bone Response to WBV: In the six week group, vibrations did not cause any change in cortical or trabecular bone volume or structure of the femur or tibia. Obesity/high-fat diet consumption has been linked to insulin resistance (128). In the six week experiment, high-fat diet consumption significantly increased insulin levels to maintain slightly above normal glucose levels, suggesting that perhaps insulin mediated transport of glucose in skeletal muscle might have been affected. This may have led to a lack of response in the high-fat diet group to WBV. As has been discussed earlier WBV shifts the adipogenesis-osteogenesis axis towards increased bone formation by inhibiting PPARγ expression (25) (117). High fat content in diets is known to directly affect PPARγ expression as well besides increasing osteoclastogenesis in the

marrow (24, 26, 29), possibly interfering with the anabolic action of WBV. It is possible that, in such a case, WBV must be applied over a longer period or at different magnitudes so as to assert its influence on PPARγ even in presence of fatty acid-PPARγ interaction. In the study by Luu et al. WBV, over 6 weeks, was sufficient to counteract the adipogenic affects of high-fat diet consumption. Our results indicate that this response was not seen in BALB/c mice where 6 weeks of WBV failed to elicit an anabolic response in load bearing bones.

WBV Decreased Bone Resorption Caused by a High-Fat Diet over 15 Weeks: Our hypothesis that high frequency whole body vibrations are anabolic to the skeleton held true only in the 15 week high-fat diet group. WBV decreased TRI-SMI in the tibia suggesting reduced osteoclast activity. There were also increases in bone volume and improvements in connectivity of trabeculae, although these differences were not statistically significant. Luu et al have documented similar results in C57BL/6 mice on high-fat diets over a time-frame of both 6 weeks and 14 weeks (25). Our data indicates that in BALB/c mice, such anabolic responses need a longer period (15 weeks) to significantly cause phenotypic improvements in bone.

Bone Loss Affected Sensitivity of Skeleton to Mechanical Stimuli: It is interesting to note that, similar to observations in the fructose diet groups, vibration resulted in changes in the high-fat diet group only when the high-fat diet by itself was detrimental to bone, suggesting that an increased sensitivity to applied stimuli results when bone cells are already actively being affected by other factors. For example, it has been suggested that bone loss caused by ovariectomy increases the sensitivity of the skeleton to mechanical signals and that levels of hormones such as estrogen modulate the effect of mechanical loading (78). From a comparison of the two diets in this study, it is probable that insulin was a major mediator which affected the end result of application of WBV. In the fructose group which had insulinopenia, WBV worsened bone loss where as in the high-fat diet group which had normal insulin levels, WBV had a reparative effect. This research further emphasizes the need to take into account dietary composition and metabolic state while testing the anabolic effects of WBV treatment on osteogenesis.

Summary: WBV did not influence body mass, energy intake or fat deposition in any of the diet groups. In the HF group, it is possible that mechanical loading based inhibition of adipogenesis and PPARγ action had to compete with effects of increased fatty acid consumption on PPARγ. Genetic strain might further affect the magnitude of such interactions. In any case, the use of WBV as a tool to inhibit adipogenesis is not supported in this research. In all three diet groups of the 15 week study and in the SC and HF group in the 6 week study, WBV did not cause changes in insulin/glucose levels. But in the fructose diet group of the 6 week study WBV was able to improve glycemic control significantly. It is possible that WBV has an effect on circulating insulin and glucose levels because it increases the utilization of glucose by increasing muscle contractions. Further studies are needed in this regard to isolate and develop the mechanisms by which WBV can regulate insulin-glucose levels. The cortical bone of tibia and femur in all three diet groups at both time-points was unresponsive to WBV. In the 6 week study, WBV had no effect in the two experimental diet groups and was detrimental to trabecular bone in the control diet group. In the fifteen week study vibration had some anti-resorptive effect on the trabecular tibia of HF animals suggesting that WBV must be applied over a longer period so as to assert its influence on osteogenesis even in presence of fatty acid-PPARγ interaction. Results also suggest that in the fructose group, bones that were already fragile were incapable of responding to mechanical signals. It is also possible that fructose consumption caused metabolic imbalances which interfered with bone response to mechanical stimulation. This research suggests that dietary composition, metabolic influences and nutritional intake can interact with and affect the response of load bearing bones to mechanical signals. These interactions must be addressed while designing WBV treatment regimens for individual patients.

CHAPTER FIVE

Conclusions

Summary: The goal of the first study presented in this thesis was to characterize the skeletal effects of poor nutritional intake in a controlled animal study. Accordingly, we tested two diets- one high in fructose content and one high in fat content- both of which may have negative implications on bone health and the risk for obesity. The high-fructose diet proved detrimental to bone quantity, density and structural integrity over a short (6 week) period and the deterioration of bone health was found to have a statistically significant association with disruption of glycemic balance. The high-fat diet was detrimental to bone quality, density and structural integrity over a longer (15 week) period and was accompanied by a significant increase in fat deposition indicating the onset of obesity. Importantly, at both time-points bone mineral density was found to have negative correlations with bodily fat content. These findings have isolated two potential lifestyle related factors which influence bone accrual and skeletal health in the developmental stage and have also thrown some light on the mechanisms via which they hamper skeletal development. This data highlights the complexity of the diet-bone relationship and the importance of studying the effects of dietary composition on bone development. While a cure for osteoporosis is elusive, such research can help develop precautionary guidelines to promote maximum bone deposition at an early age.

The second study presented in this thesis was designed to test the efficacy of whole body vibration treatment in promoting/restoring increased volume and stronger structure of load bearing bones in case of regular diet/ imbalanced diet consumption. A vibration regimen of 90 Hz, 0.2 g, 15 minutes/day was applied. Results showed that response to WBV was highly variable at a short (6 weeks) vs. long (15 weeks) interval and also variable across diets. Cortical bone was unresponsive to WBV treatment in all three diet groups and at both time-periods. In the regular diet group WBV was detrimental to trabecular bone over 6 weeks and did not have any effect over 15 weeks. In the fructose group, WBV further exacerbated trabecular bone deterioration caused by the diet in the six week study and did not have any effect over 15 weeks. In the high-fat diet group, WBV did not have any effect over 6 weeks but improved trabecular bone structure over

15 weeks. WBV did not have any effect on fat development or triglyceride production but was able to attenuate the insulin imbalance caused by fructose consumption to a certain extent. This research shows that the outcome of WBV treatment is affected not only by genetic make-up but also has interactions with characteristics such as dietary composition and metabolic state. These details must be taken into account to modulate treatment parameters on a per patient basis.

Future studies: In this research, high-fructose and high-fat diets were chosen because the consumption of such diet types is increasing daily and is a proven lifestyle related cause of many health conditions such as diabetes and obesity. It has been proposed by some that high-fat and high-fructose diets affect bone development because of their associations with obesity and with mineral/calcium imbalance. Further there is evidence that sugar sweetened drinks have a detrimental effect on bone mineral density. Future studies investigating the effects of both high-fat and high-fructose consumption in the same group will provide additional relevant information since the consumption of these two diets is often in conjunction. Similarly the addition of a baseline group would provide helpful data on how bone formation and fat deposition changed over time because of the diets.

As has been discussed, leptin is a hormone that has associations with all the outcome parameters of this study such as food intake, body weight, fat accumulation, bone formation. It will be interesting if future studies examine the plasma concentrations of leptin and try to correlate them with the other findings of this study. This will be a major contribution in further understanding and interpreting the present data. Finally there is a need in the future to look at indicators of bone health other than micro-CT such as material properties using mechanical testing, histomorphometry or calcein labeling.

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