

# **The Effect of Different Sugar-Sweetened Beverage Intake by Immature Female Rats on Bone Mineralization and Strength**

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## ABSTRACT

### **The Effect of Different Sugar-Sweetened Beverage Intake by Immature Female Rats on Bone Mineralization and Strength**

**Embedzayi Tsanzi**

Consumption of sugar-sweetened beverages has increased among adolescents. This may have health implication because sugar consumption has been suggested to lead to decreased calcium (Ca) intake and increased urinary Ca output and these effects on Ca balance may have a negative impact on bone. Also, the type of sugar added to beverages has also changed from sucrose to high fructose corn syrup resulting in higher fructose consumption. Although sugar-sweetened beverage intake by adolescent has increased and the type of sugar consumed has changed; the effects of sugar-sweetened beverage consumption during the bone growth stage on skeletal acquisition and adult bone health are unknown. The objectives of this study were to determine the effect of feeding growing rats different sugar-sweetened beverages and its impact on bone and the possible mechanisms of action.

Immature (28 days old) female Sprague-Dawley rats (n=8-9/group) were individually housed in metabolic cages and randomly assigned to be given a non-sugar beverage consisting of deionized distilled water (ddH<sub>2</sub>O, Control), or ddH<sub>2</sub>O containing 13% w/v common commercial caloric sweeteners of either: 1) glucose, 2) sucrose, 3) fructose or 4) high fructose corn syrup (HFCS-55). Assigned beverages were provided *ad libitum* throughout the 8 weeks study. Food intake, urine and fecal output were measured weekly. At the end of the study, the tibiae and femurs were collected. Bone mineral density (BMD) and Bone mineral content (BMC) were determined by dual x-ray absorptiometry (DEXA) and bone strength was measured using three-point bend testing. Bone turnover markers serum osteocalcin and urinary deoxypyridinoline (DPD) were determined using enzyme immunoassays. Urinary/fecal Ca and phosphorus (P) levels were determined using inductively coupled plasma mass spectrometry.

There were no differences in BMC, BMD and bone strength between rats fed sugar-sweetened beverages and those fed non-sugar sweetened beverages. There were significant differences among rats fed the different sugar-sweetened beverages. Tibial and femoral BMD of rats fed glucose were reduced ( $p < 0.001$ ) by 3-8% compared to rats fed the other sweetened beverages. Despite reduced BMD in glucose-fed rats, bone strength was not significantly reduced at the end of the study. There were no differences in serum osteocalcin and DPD levels among the treatment groups. However, calcium intake and retention was lower ( $p < 0.001$ ) in glucose-fed rats compared to the other treatment groups which may explain their lower bone mass. Femoral and tibia total BMC and total BMD were

significantly higher in fructose-fed animals compared to glucose-fed animals. . The higher BMD and BMC in fructose-fed animals may be due to higher Ca intake and retention compared to glucose-fed animals.

Focus has been on the potential negative impact of higher fructose consumption. Based on our study results glucose rather than fructose appears to have the most deleterious effect on mineral balance and bone mass.

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## LIST OF ABBREVIATIONS

Ca	Calcium
P	Phosphorus
dd H <sub>2</sub> O	Deionized distilled water
HFCS-55	High fructose corn syrup-55
BMD	Bone mineral density
BMC	Bone mineral content
DEXA	Dual energy x-ray
absorptiometry	
DPD	Deoxypyridinoline

## **1.0 INTRODUCTION**

Osteoporosis is a disease characterized by a deficiency of bone tissue relative to the volume of the bone (Matkovic et al., 1994). This reduction in bone density increases the susceptibility of individuals to fractures. Bone fractures can result in prolonged or permanent disability or even death. Bone fractures resulting from osteoporosis costs the US health care system 18 billion dollars annually (Surgeon Generals Report, 2004). In the US, 4 in 10 women and 1 in 10 men over age 50 will suffer from osteoporosis (Surgeon Generals Report, 2004). Research directed towards the prevention of bone loss merits high priority given the staggering health and economic cost of osteoporosis.

A key factor in osteoporosis is peak bone mass (PBM). PBM is defined as the amount of bony tissue present at the end of skeletal maturation (National Osteoporosis Foundation, 2004). During early childhood, bone formation exceeds bone resorption resulting in increased bone mass but after this stage bone resorption exceeds bone formation resulting in steady bone loss (Saggese et al., 2001). An increase in PBM of 10% has been associated with a 50% reduction in bone fractures (National Osteoporosis Foundation, 2004). Therefore, maximizing bone mass during the bone formation stage protects against future risk of osteoporosis.

Nutrition is an important modifiable factor that can influence PBM. Dietary prevention of bone loss has focused on calcium and factors that affect calcium balance. There have been suggestions that high sugar intake can negatively influence calcium balance (Holl and Allen, 1987; Milne and Nelson,

2000; Ivaturi and Kies, 1992). Determining the effect of sugar on bone is important because in the US there has been a steady increase in the consumption of added sugars over the past thirty years as observed by food supply data and nationwide food consumption survey data (Johnson and Frary, 2001). Along with the increase in the consumption of added sugars, there has been a gradual shift from sucrose as the main form of added sugar to corn-derived sweeteners, mainly in the form of high fructose corn syrup (HFCS) (Elliot et al, 2002). Despite continual rising sugar consumption among children and adolescents (Cavadini et al., 2000) and a shift in the type of sweeteners from sucrose to HFCS, little is known about the impact of high sugar intake during the bone formation stage.

Guthrie and Morton (2000), using data from the US Department of Agriculture (USDA) 1994-1996 Continuing Survey of Food Intakes by Individuals, reported that soft drinks are a major contributor of added sugars (33%). There is evidence that soft drink consumption negatively impacts bone by decreasing bone mineral density (BMD) and increasing the risk of bone fractures (Wyshak, 1994; Wyshak and Frisch, 2000). Although regular soft drinks are high in sugar content, attention has been given to other ingredients in soft drinks, such as: phosphoric acid and caffeine. Other studies have cited the mechanism for bone loss with increased soft drink consumption as the replacement of calcium rich food such as milk with soft drinks by adolescents (Whiting et al., 2001; Harnack et al., 1999). Soft drinks are high in sugars (13%), often in the form of HFCS; thus, studies on soft drink intake may provide some evidence of the effects of sugar consumption on bone. However, studies that directly determine the

effect of sugar consumption on bones and potential mechanisms whereby sugar may affect bone are needed. Providing a better understanding of the role of sugar consumption on bone will assist in the determination of dietary recommendations to improve bone health and reduce the future risk of osteoporosis.

## **2.0 STUDY OBJECTIVES**

### **Hypothesis**

Different sugar sweetened beverages will negatively affect mineral balance, bone mass, bone mineral content and bone mineral density leading to decreased bone strength when consumed by rats at the same concentration (13%) as that available in soft drinks.

In order to test the hypothesis the study objectives were:

**Objective 1.** To determine whether feeding sugar-sweetened beverages containing different sugars to immature female rats affects bone mineralization and bone strength at maturity.

**Objective 2.** To determine the whether feeding sugar-sweetened beverages containing different sugars to immature female rats affects mineral balance that can compromise bone mineralization and bone strength at maturity.

### **3.0 LITERATURE REVIEW**

#### **3.1 Bone Biology**

The skeleton has several important functions that include: providing support for the body, protection of internal organs, providing sites of attachment for muscles and a reservoir for calcium and phosphate ions. Bone is structured into several segments. The diaphysis is the bone's shaft or body, which is the long cylindrical main portion of the bone (figure 1). The epiphyses are the proximal and distal ends of the bone. The metaphyses are the regions in a mature bone where the diaphysis joins the epiphyses. In a growing bone, the metaphyses are regions that include the epiphyseal plate, the point at which cartilage is replaced by bone. Cartilage covers the bone where the bone forms a joint with another and the rest of the bone is covered by the periosteum, which is a tough sheath of dense irregular connective tissue. The medullary cavity, also called the marrow pit is the space within the diaphysis that contains the fatty yellow bone marrow and the marrow pit is surrounded by endosteum.

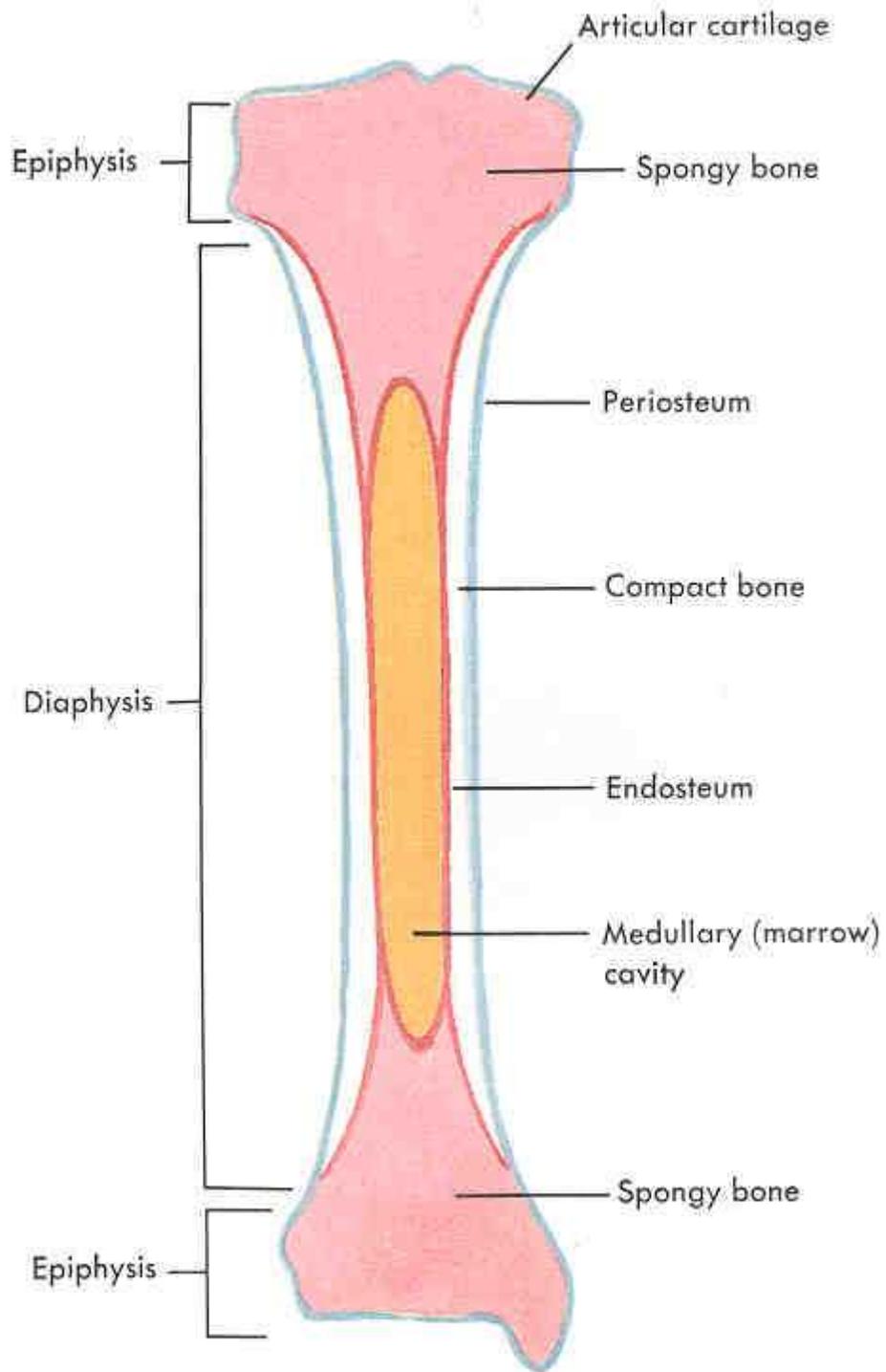


Figure 1. Structure of a long bone adapted from Anthony, C. P. and Thibodeau, G. A., 1984.

The endosteum is a membrane that also contains bone forming cells..

There are two basic types of bone, namely cortical and cancellous bone. Cortical, also referred to as compact bone is well suited to the supporting, protective and mechanical functions of the bone. Cortical bone makes up the shaft of the long bones and it constitutes ~80% of the skeletal mass. Cancellous or trabecular bone has a honeycombed structure and it provides a large surface area that serves as a reservoir for minerals. Cancellous bone makes up the inner parts of the bone and the ends of the long bones. Cancellous bone also provides the sites for bone forming cells.

Bone tissue has three components; an organic matrix, bone minerals and bone cells. The organic matrix is made up mainly of type-1 collagen (90%), with the remaining 10% consisting of other proteins such as osteocalcin. The skeleton contains 99% of the body's total calcium. The calcium is stored in the bones as hydroxyapatite  $[Ca_{10}(PO_4)_6(OH)_2]$ .

There are three types of bone cells, osteoblasts, osteocytes and osteoclasts. Osteoblasts are the bone-forming cells. Osteoblasts act at the bone surface secreting unmineralized collagen, modulating the crystallization of hydroxyapatite and influencing the activity of osteoclasts. Along with collagen type-1, osteoblasts express a number of products that include osteocalcin and alkaline phosphatase that may be used to quantify the activity of the osteoblasts (Compston, 2001). Osteocytes are basically osteoblasts that have become embedded within the mineralized regions of the bone. These cells are involved in the sensing and translation of information about the internal bone environment,

including response to mechanical stimuli and initiating an appropriate modeling or remodeling response via a number of chemical messengers (Compston, 2001).

Osteoclasts are large multinucleated bone resorbing cells (Compston, 2001). Osteoclasts attach to bone surfaces and a ruffle border forms beneath the osteoclast forming a subcellular space. The osteoclasts generates and releases hydrogen ions, lactate, and proteolytic enzymes into the formed subcellular space, which results in breakdown of the protein matrix and the subsequent release of calcium and other bone minerals (Watts, 1999).

### **3.2 Bone Turnover**

Bone tissue is continually being broken down and reformed, and this process is referred to as bone turnover. Bone turnover begins with the recruitment of osteoclasts from bone marrow precursors (Watts, 1999). The osteoclasts attach to the surface of the bone that is to be resorbed and releases proteolytic enzymes, hydrochloric acid and lactate that degrades the bone matrix forming a resorption pit or lacuna (Watts, 1999). After osteoclasts have formed a lacuna, osteoblasts differentiate from connective-tissue precursors. Osteoblasts produce collagen and coat the bone with an adhesive substance to bind the collagen to the bone. Osteoblasts are also responsible for the mineralization of the bone resulting in the complete formation of new bone tissue.

The process of bone resorption is coupled to bone formation; however, this does not mean that there is a balance between the two processes. In the first two decades of life in humans and while the growth plates are still open in

animals, bone formation exceeds bone resorption resulting in bone growth (Colston, 2001). After the age of 35-40 in humans, bone resorption exceeds bone formation resulting in a net loss of bone (Watts, 1999). In rats, bone continues to grow until age 6 months after which bone growth slows (Kalu, 1991).

### **3.2.1 Bone Turnover Markers**

Bone health can be evaluated by a variety of techniques that include histomorphometry, densitometry, measurement of calcium fluxes and bone turnover markers (Watts, 1999). Histomorphometry is not favorable due to its invasive nature. Densitometry is precise and non-invasive but it is slow to reveal changes. Bone turnover markers are non-invasive and they respond to intervention/change more rapidly than densitometry. Thus, bone turnover markers can provide a means of complementing the other techniques or provide direct information (Watts, 1999).

Bone turnover markers can be classified into 3 groups: a) enzymes or proteins that are secreted by the cells involved in the turnover process, b) breakdown products that are produced during the resorption process and c) byproducts produced during the time of bone formation (Watts, 1999). Bone turnover markers can be measured in both urine and blood.

### **3.2.2 Bone Resorption Markers**

Bone resorption markers include acid phosphatase (an enzyme), calcium and several collagen breakdown products. Acid phosphatase and urinary calcium

are not specific indicators of resorption since acid phosphatase is also released by erythrocytes during clotting and urinary calcium is affected by the diet and renal function (Delmas, 1993). Of the collagen breakdown products, deoxypyridinoline (DPD) is specific for bone, and thus it provides the most accurate way of quantifying bone resorption (Watts, 1999). DPD can be measured both in urine and blood using enzyme immunoassays.

### **3.2.3 Bone Formation Markers**

Bone formation markers include alkaline phosphatase (an enzyme), and byproducts of bone synthesis that include osteocalcin and procollagen extension peptides. Alkaline phosphatase is present in osteoblast but is also found in the liver and intestines. The concentration of procollagen extension peptides is not specific for bone since it also increases with increased turnover on non-skeletal collagen such as the skin and muscle (Watts, 1999). Osteocalcin, the major non-collagen protein of bone matrix is widely used as a marker of bone formation as it is relatively specific for bone (Delmas, 1993). Osteocalcin can be measured by enzyme immunoassay in plasma or serum.

### **3.3 Biomechanics of the Bone**

Bone susceptibility to fractures can be defined by biomechanical properties. Bone fractures occur as a result of different reasons and so there are several different biomechanical definitions of bone fragility. Biomechanical indices of bone fragility includes: strength, brittleness, work to failure and

stiffness which is also used to assess mechanical integrity of the bone (Turner, 2002). These indices are derived from a biomechanical test in which force is applied to a bone until it breaks. A force-displacement curve (Figure 2) is generated from the biomechanical test. Bone strength (ultimate force) is defined as the height of the curve. Work to failure is defined as the area under the curve whilst brittleness can be estimated from the reciprocal of the curve (Turner, 2002). Ultimate bending stress and Young's modulus are also some of the biomechanical properties used to determine bone properties. Ultimate bending stress is a normalized calculated force that takes into account the size of the bone and Young's modulus is normalized stiffness that takes into account bone size (Yuan and Kitts, 1992).

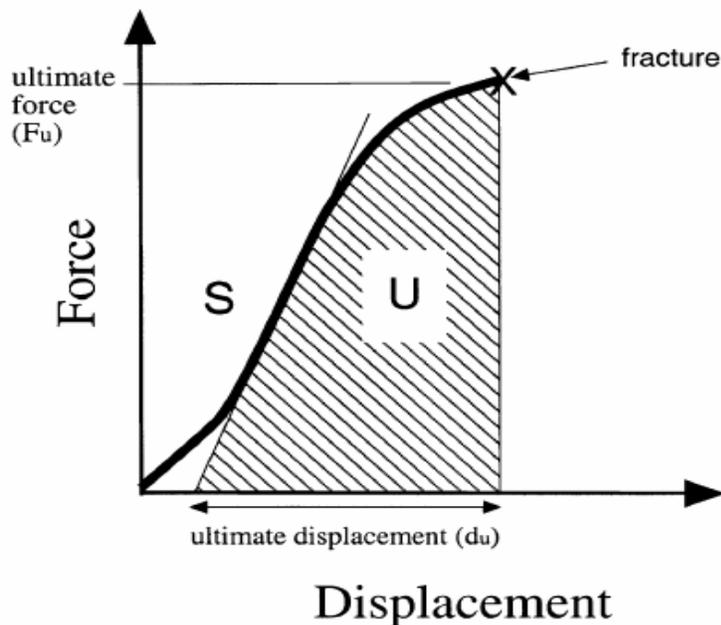


Figure 2. A force-displacement curve resulting from a biomechanical test of a bone specimen. The height of curve represents ultimate force ( $F_u$ ), area under

the curve (U) represents work to failure energy, the slope of the curve is the stiffness (S) and the width of the curve is the ultimate displacement (du). (Turner, 2002)

### **3.4 Peak Bone Mass**

Peak bone mass (PBM) is defined as the highest level of bone mass achieved as a result of normal growth (Matkovic et al., 1994). Bone mass is an important determinant of bone strength (Heaney, 2000). Strength is a combination of mass and the geometric arrangement of the components in space. Strength of a structure varies approximately as the square of its structural density (Heaney, 2000). Thus, it follows that bones of lower density will break with less force applied. Low BMD is known to increase fracture risk in adults (Eastell et al., 1989; Cummings et al., 1993; Marshall et al., 1996) and in adolescence (Goulding et al., 1998).

For individuals with normal body weight, PBM is achieved a few years after the fusion of the long epiphyses (Heaney, 2000). During early childhood and adolescence bone formation exceeds bone resorption resulting in increased bone mass but after about the age of 30, bone resorption exceeds bone formation resulting in steady bone loss (Saggese et al., 2001). Therefore, achieving a high PBM can be protective against late-life fragility fractures.

PBM is probably a result of the interaction of endogenic (genetic and endocrine) and exogenic (nutrition, and physical activity) factors (Matkovic, 1991). Exogenic factors are the most amenable to therapeutic manipulation and

nutrition has been shown to play a significant role in maximizing PBM (Heaney et al., 2000).

Calcium intake is an important determinant of PBM (Matkovic et al., 1990). Forbes and colleagues (1979) observed that the amount of bone accumulated during growth in young rats is dependent to some extent on the amount of calcium in the diet. Calcium deficiency during skeletal growth has been observed to decrease the PBM (Chan et al., 1984; Goulding et al., 1998). Several studies have observed a positive correlation between the bone mineral density of adult women and the amount of calcium consumed during their childhood and adolescence (Halioua and Anderson, 1989; Sandler et al., 1985; Soroko et al., 1994). Heaney et al. (2000) noted that it is possible that decreased calcium early in life could account for as much as a 5-10% reduction in PBM.

Several nutrients and food constituents interfere with calcium balance by affecting either calcium absorption or obligatory calcium excretion. Phosphorus is a mineral that is important for bone health, however, high phosphate intake and abnormally low dietary calcium to phosphate ratio has been implicated in the development of bone loss in laboratory animals (Heaney et al., 2000). In humans, the consumption of soft drinks which are high in phosphoric acid have been linked to bone fractures and low bone density in children and adolescence (Calvo et al., 1990; Wyshak and Frisch, 1994). However, it is not clear if the phosphoric acid content of soft drinks is responsible for the observed increased risk in bone fractures due to the presence of other ingredients in the soft drinks that could possibly result in bone loss as well.

Several other nutrients/ food constituents have been observed to increase urinary calcium excretion in humans, these include; high caffeine intake (Bergman et al., 1990), high protein intake (Heaney, 1995; Heaney, 1993) and high sugar intake (Holl and Allen, 1990; Lennon et al., 1970; Lemann et al., 1969). The role of high sugar intake in bone acquisition in children/adolescence requires attention given that data shows a continued increase in sugar consumption in the US and adolescents consuming more added sugars as a percentage of total energy compared to any other group (Frary et al., 2004).

### **3.5 Sugar Consumption**

According to US Food Supply Data the consumption of added sugars increased by 23% between 1970 and 1996 (Kantor, 1998), and more recently consumption of added sugars increased by 22 percent between 1980 and 2000 (Putman, 2002). Thus, there has been a continual increase in the consumption of added sugars. Using data from the US Department of Agriculture (USDA) 1994-1996 Continuing Survey of Food Intakes by Individuals, Guthrie and Morton (2000) observed that for both males and females, consumption of added sugars was highest in adolescence. On average, consumption of added sugars contribute 20% of total energy for both male and female adolescents compared to 12-14% in adults older than 35 (Guthrie and Morton, 2000).

Added sugars are defined as sugars that are eaten separately at the table or used as ingredients in processed/prepared foods such as: cakes, cookies, candy and soft drinks (Johnson and Frary, 2001). Some of the commonly used added

sugars include: sucrose (table sugar), corn syrup, high fructose corn syrup, and fructose. Added sugars do not include sugars naturally found in foods such as lactose in milk.

### **3.6 Classification of Sugars**

Sugars are classified into mono-, di-, tri- and higher saccharides depending on the number of sugar units present in that sugar. Monosaccharides are the simplest sugars that contain a single sugar unit. Examples of monosaccharides are: glucose, galactose and fructose. Disaccharides consist of 2 sugar units joined by a glycosidic bond. Examples of disaccharides are: maltose, lactose and sucrose. Sucrose is made up of a ratio of 50:50 glucose and fructose, lactose is made up of galactose and glucose and maltose consists of 2 molecules of glucose.

Many commonly used sweeteners such as corn syrups contain trisaccharides and higher saccharides. High fructose corn syrup (HFCS) is the most commonly used sweetener in the food manufacturing industry today (Hein et al., 2005). HFCS is similar to sucrose in that both sweeteners are composed of glucose and fructose. However, the glucose and fructose molecules in sucrose are joined by a glycosidic bond that is hydrolyzed by the enzyme sucrase in the small intestines. Contralaterally, HFCS is composed entirely of glucose and fructose in their monosaccharide forms (they are not bonded). Several scientists have suggested that the human body utilizes HFCS and sucrose in the same way (Hein et al., 2005; Schorin, 2005). However, the presence of higher order saccharides in HFCS could change the body's utilization of HFCS.

There are 3 available forms of HFCS namely; HFCS-42, HFCS-55 and HFCS-90, where the numbers represent the percentage of fructose in the sweetener. HFCS-55, the form that is used the most in the beverage industry in the US has 55% fructose, 42-44% glucose and 1-3% trisaccharides and other higher order saccharides (Schorin, 2005).

### **3.7 Soft Drink Consumption and Bone Health**

The largest source of added sugars in America's diet is regular soft drinks which account for a third of the total added sugars consumed (Murphy and Johnson, 2003). Data from food consumption surveys carried out between 1965 and 1997 showed that regular soft drink consumption increased by 187% for adolescent boys and 123% for adolescent females (Cavadini et al., 2000). This increase in soft drink consumption raises concern given that several researchers have observed decreased BMD and increased risk of fractures in adolescents with high intakes of soft drinks (McGartland et al., 2003; Wyshak G, 1994; Wyshak and Frisch, 2000; Whiting et al., 2001) and decreased bone strength in animal studies (Garcia-Contreras et al., 2000).

Garcia-Contreras and colleagues (2000) observed that ovariectomized Sprague-Dawley rats (aged 9 months) consuming soft drinks (~140ml/day) had significantly lower femoral BMD compared to animals consuming water. These results suggest that high soft drink consumption has the potential to reduce BMD in older animals. To our knowledge, no studies have been conducted using younger animals. It is important to investigate the effect of consuming high levels

of soft drinks on bone health using animals in the growing stage due to the increase in soft drink consumption among adolescents.

In a study with adolescents, 76 girls and 51 boys, Wyshak (1994) found a strong association between cola beverage consumption and bone fractures in girls (adjusted odds ratio; OR=3.59; 95% CI,  $p<0.02$ ). Wyshak also observed that high calcium intake was protective against the fractures (OR= 0.284, 95% CI,  $p<0.05$ ). The results of this study were confirmed by results from a similar study by Wyshak and Frisch (2000) showing a strong association between carbonated beverage consumption and BMD in adolescent girls (OR=3.14, 95% CI,  $p<0.04$ ). MacGartland et al., (2003) also observed a significant inverse relationship between total soft drink consumption and bone mineral density in girls aged 10-15 years.

Although there is substantial evidence that soft drink consumption is associated with poor bone health, the pathophysiology of how soft drink consumption affects bone has not been clarified. Several researchers have observed that calcium intake is low in individuals with high soft drink consumption as evidenced by a decrease in consumption of milk and milk products (Wyshak, 1994; Whiting et al., 2001; Harnack et al., 1999). Thus, suggesting that the effect of soft drink consumption on bone health can be explained by the displacement of calcium-rich foods by soft drinks. In contrast, McGartland et al. (2003) showed an inverse association between soft drink consumption and BMD in adolescent girls persisted even after adjusting for milk consumption and calcium intake among other factors. This suggests that soft

drink consumption has an effect independent of calcium or milk intake. In a study by Garcia-Contreras and colleagues (2000) rats offered soft drinks had a ~50% reduction in solid food intake compared to rats drinking tap water. To adjust for differences in solid food intake, Garcia-Contreras and colleagues (2000) pair-fed rats. Pair feeding of rats resulted in no significant differences in BMD between rats fed tap water and rats fed soft drinks. This also supported the view that soft drinks have bone effects that are independent of the replacement of calcium and milk products.

Soft drinks contain several ingredients that include sugars, caffeine and phosphoric acid that could possibly affect bone health (Fitzpatrick and Heaney, 2003). In trying to understand the mechanisms responsible for the negative impact of soft drinks on bone, more attention has been given to the caffeine and phosphoric acid. Caffeine is a diuretic and thus, it can result in higher losses of calcium in the urine. However it has not been proven if the losses are enough to cause significant changes in bone. Attention has been given to phosphoric acid due to its impact on the Ca:P ratio which may result in poor bone formation. Although soft drinks contain a significant amount of sugars (13%), little attention has been given to the role these sugars have in bone health. There have been several studies that have shown negative effects of high sugar diets on bone strength in animals (Tjaderhane and Larmas, 1998) and negative macromineral balances in humans (Holl and Allen, 1990; Ivaturi and Kies, 1992).

### 3.8 Glucose and Bone Health

Terada et al. (1998) investigated the growth inhibitory effect of a high glucose concentration independent of insulin *in vitro*. Human osteoblast-like cells (human osteosarcoma line that has osteoblastic phenotype including production of bone formation markers i.e alkaline phosphatase and osteocalcin) were cultured in medium containing various concentrations of glucose. Concentrations tested included; control, 5.5 mmol/L (normal glucose concentration), high doses of 33.0 and 49.5 mmol/L of glucose or iso-osmolar medium consisting of 27.5 or 44 mmol/L mannitol plus 5.5 mmol/L glucose. Mannitol is a sugar alcohol which is used in this study as an isomolar control for the high doses of glucose. Continual exposure of the human osteosarcoma cells for 4 days to the high concentrations of glucose (33.0 and 49.5mmol/L) inhibited cell growth and decreased DNA content of the osteoblast cells in a dose-dependent manner. A dose-dependent inhibition of growth and DNA synthesis was not observed in osteoblast cells cultured in isomolar concentrations of mannitol. The results suggested that high glucose (33.0 and 49.5 mmol/L) concentrations inhibited osteoblast proliferation and this in turn, may impair bone formation resulting in bone loss.

Although cell culture studies have an important role in determining mechanism of action, results obtained from *in vitro* studies may be different from what actually takes place *in vivo* due to the complexity of living organisms. Furthermore, the high glucose concentrations used in this study are unlikely to be found in normal human subjects due to the tight regulation of glucose in the human body, maintaining blood glucose levels around 5.5 mmol/L (~110 mg/dL).

Studies that involve animals and/or human subjects will provide more information. To our knowledge, there are no studies that have investigated the direct effect of high glucose intake on bone biology and the subsequent effect on bone mineral density in normal subjects.

The elevated blood glucose levels in diabetics may indirectly serve as an indicator of the effect of high glucose effects on bone. Several studies have observed decreased bone mineral density (BMD) in individuals with both insulin-dependent diabetes mellitus (Hamlin et al. 1975; McNair et al. 1979; Shore et al., 1981) and non-insulin dependent diabetes mellitus (Imura et al. 1987). There are several hypotheses regarding the pathogenesis of a condition commonly referred to as diabetic osteopenia. The hypotheses include bone loss due to: 1) increased urinary calcium excretion triggered by hyperglycemia, 2) direct reduction of osteoblast activity by elevated insulin levels and 3) direct reduction of osteoblast activity by elevated glucose as discussed by Terada et al. (1998).

Calcium levels are tightly regulated in living organisms due to the critical role of calcium in muscle contractions. Normal blood calcium has to be maintained despite inadequate intake of calcium. If the blood calcium is low, the body increases the rate of calcium absorption and also decreases urinary calcium by increasing calcium reabsorption by the kidneys through the action of parathyroid hormone and vitamin D. When serum calcium is too high, the levels of parathyroid hormone falls and there is an increase in urinary losses of calcium and decreased intestinal calcium absorption. The thyroid gland also secretes calcitonin which decreases calcium losses from the bones. A number of studies

support the hypothesis that increased glucose intake triggers an increase in urinary calcium loss, however the increase in urinary calcium is unlikely a result of decreases in parathyroid hormone as there is also overwhelming evidence that glucose enhances absorption of calcium as discussed below.

Lennon and colleagues (1970) observed significant increases in both urinary Ca (~40%) and urinary Mg (~60%) excretion about 2-3 hours after glucose ingestion (100g) in 7 healthy males. Similarly, Lemann et al. (1969) reported that the intake of 100g of glucose in 14 healthy males increased urinary calcium (~30%) and urinary magnesium (~40%). Lemann et al. (1969) observed that glucose ingestion decreased the glomerular filtration rate, but despite this reduction they observed increased urinary Ca and Mg, thus indicating that glucose could be inhibiting the net tubular reabsorption of these minerals. The authors suggested that the reduced net tubular reabsorption of Ca and Mg is likely due to a direct alteration of the renal cells by glucose; however, the mechanism of action was not evaluated in this study. Although both studies made similar observations of increased urinary Ca and Mg excretion with glucose ingestion; the number of subjects used was too low to make valid conclusions.

Glucose has been reported to increase calcium excretion but, on the other hand, glucose has also been shown to enhance calcium absorption. Wood et al. (1986) investigated the effect of glucose (50 g) and glucose polymers on intestinal calcium absorption in human subjects aged between 25 and 51 years. The efficiency of calcium absorption in the subjects increased by 20% for glucose and 27% for glucose polymers ( $p < 0.01$ ) compared to subjects given tap water.

Enhanced calcium absorption with glucose consumption was observed by a number of investigators studying both humans and animals. In 9 healthy human subjects, Norman et al. (1980) observed that 65-100 mM glucose increased calcium absorption by about two fold. Monnier et al. (1978) found that orally administering 40 g of glucose increased the rate of calcium absorption in 10 diabetic males by 21%.

In animal studies, both Zheng et al. (1985) and Younoszai et al. (1985) observed increased intestinal calcium absorption in both diabetic and normal rats. Zheng et al. (1985) observed a 57% increase in rats (~2 months old) fed 4.0 mg glucose/g body weight compared to control animals receiving tap water. Infusion of glucose (15mmol/L) to young rats (3 weeks old) enhanced calcium absorption by ~40% in both normal and diabetic animals (Younoszai et al., 1985). Younoszai et al. (1985) also noted that the observed increase in intestinal calcium absorption is unlikely due to the action of vitamin D since they used diabetic rats that could not convert vitamin D to its active metabolite 1,25-dihydroxy vitamin D, but still observed enhanced calcium absorption with glucose consumption.

There are a few studies that have not observed enhanced calcium absorption with glucose consumption (Urban and Pena, 1977; Francis et al., 1986); however this might be due to the use of lower doses of glucose.. Urban and Pena (1977) did not observe any significant differences in the rates of calcium absorption in male young rats given 10mM glucose and those not fed glucose (control). Francis et al. (1986) also showed that a 10g oral dose of glucose did not increase calcium absorption in post-menopausal women. In study by Zheng et al.

(1985) using rats, enhanced calcium absorption by glucose was in a dose-responsive manner. The doses of glucose ranged from 0.5-4.0 mg/g body weight and the rates of calcium absorption increased in a linear pattern from 28% at 2 mg/g body weight to 57% at 4 mg/g of body weight relative to calcium absorption at 0.5 mg/g body weight.

With evidence supporting both the enhanced absorption of glucose in the intestines and an increased loss of calcium in urine with glucose consumption, it is difficult to determine the effect of glucose on bone mineralization. It is possible that the observed increase in the loss of calcium after glucose ingestion is a result of the increased calcium absorption. Studies that showed an increase in urinary calcium with glucose intake were all clearance studies that were done in a single day (Lemann et al., 1969; Lennon et al., 1970). It would be of interest to perform studies of longer duration to investigate the effects of long-term mineral losses on bone mineral density.

Most of the studies reviewed above studied either the effect of glucose on urinary calcium excretion or the effect of glucose on calcium absorption independently. There is a need to have a study that investigates calcium retention, taking into account both the effects of glucose on intestinal absorption and total excretion (urinary losses and fecal losses). In our study, we investigate the effect of glucose on calcium absorption, calcium retention and we also look at the effect of glucose on bone properties including calcium content and strength.

Although glucose is used in the manufacture of sport drinks, its intake is mainly in the form of sucrose (table sugar) and high fructose corn syrup. It is interesting to also look at the effects of these sweeteners on bone.

### **3.9 Sucrose and Bone Health**

Several studies have investigated the direct effects of sucrose on bone morphology and biomechanics. Li et al. (1990) studied the effect of diets containing different combinations of sugar and fat on cortical bone morphology and mechanics in rapidly growing, female Sprague Dawley rats (8 weeks old) for 10 weeks. The authors reported that rats fed the high-fat-sucrose (HFS) diet (saturated fat; 1909 g/kg; sucrose; 472 g/kg) had significantly reduced bone strength indicated by: lower maximum loads, failure energies, stresses for tibia when compared to animals fed a low-fat-complex-carbohydrate (LFCC) diet (saturated fat 17.7g/kg; sucrose 0g; starch 649 g/kg). In the metatarsals, LFCC diet resulted in significantly higher density ( $1.95 \text{ g/cm}^3$ ) than HFS diet ( $1.75 \text{ g/cm}^3$ ). Metatarsals from rats fed the LFCC diet also had a significantly higher elastic modulus (9 GPa) than HFS (4GPa).

In a similar study, Zernicke et al. (1995) investigated the long-term (2 years) effects of feeding HFS diet (saturated fat; 1909 g/kg; sucrose; 472 g/kg) on bone morphology, mineral content and mechanical properties of the femoral neck (FN) and sixth lumbar vertebra (L6) in female Fischer rats (4 weeks old). Zernicke et al. (1995) observed that feeding rats HFS diet long-term significantly

reduced BMC and bone strength, indicated by decreased loads and stiffness, for both FN and L6 compared to rats fed LFCC diet. The results suggested that HFS diet decreased bone strength which increases the risk of bone fractures.

Several mechanisms may have contributed to the observed negative effects of the HFS diet. High sucrose diets may lead to hyperinsulinemia and elevated insulin has been linked to increased urinary calcium excretion in human subjects (Hall and Allen, 1987). Increases in urinary calcium has been suggested to be due to inhibition of renal cell calcium reabsorption by insulin (Lemann et al., 1970). Zernicke et al. (1995) found that during the 2 year study that animals fed HFS diet were hyperinsulinemic (439 pmol/L) compared to LFCC animals (98 pmol/L). Thus, it is possible that the high sucrose levels in the diet may have been responsible for the observed negative results on bone. However, it is also possible that the high fat content may have contributed to the deterioration of bone in the animals fed a HFS diet.

In a study using broilers (age 3 weeks), Atteh and Leeson (1984) observed that diets high in saturated fat (palmitic acid, C16:0) resulted in significantly lower bone weight and bone calcium content compared to animals fed a diet high in unsaturated fats (oleic acid, C18:1). The high fat diet used by both Li et al. (1990) and Zernicke et al. (1995) consisted of lard (190g /kg body mass) which is high in palmitic acid. Atteh and Leeson (1984) suggested that saturated fats are capable of forming intestinal soaps that can inhibit mineral absorption. In support, Tadayyon and Ludwark (1968) observed that soaps formed by saturated fats inhibited absorption of calcium in the intestines. In order to understand the

mechanism responsible for reduced bone mineral and strength associated with the consumption of a high-fat-sucrose diet, there is need to investigate the sugar and fat components in the high-fat-sucrose diet individually.

Tjaderhane and Larmas (1998) investigated the effect of a high sucrose (43 g/100g) diet on bone growth, composition and mechanical strength in young (3 weeks old) growing male and female Wistar rats. The authors observed feeding female rats a high sucrose diet significantly reduced the weights and density of both the tibia and femur compared to rats fed a high starch diet (control) ( $p < 0.05$ ), but no differences were observed for the male rats. Calcium and phosphorus concentrations were significantly lower in female rats for both femur and tibia of the high-sucrose group compared to the high starch group. The authors suggested that the effect of sucrose might be mediated by altered urinary calcium excretion. In support, Ericsson et al. (1990) observed increased urinary calcium with high sucrose intake in adult human subjects. However, Tjaderhane and Larmas, (1990) did not consider the amount of urinary calcium before and after the high sucrose consumption in the rats. Studies that combine determining the effect of sucrose on bone properties and investigation of possible mechanisms of actions on bone by examining calcium balance and bone markers are required. In our study, we investigate the effect of sucrose on bone properties and mechanisms of action by determining calcium balance and the effect of sucrose on bone markers.

Tjaderhane and Larmas (1998) suggested that there might be a metabolic difference in the response to dietary alterations between males and females.

Smith and colleagues (2000) did not observe any significant differences in bone composition and mechanical properties in male rats aged 3, 8, 16 or 56 weeks fed a high sucrose diet, a low fat diet or a high fat diet for 5 weeks. The authors suggest that their results indicate that the duration of the study (5 weeks) might have been too short in order to observe any effects. However, the use of male rather than female rats in this study may also have resulted in different observations. In this study we used female rats. Females have been observed to be at higher risk of bone loss compared to their male counterparts (Hannan et al., 2000). Most of the studies that have observed negative effects of high sucrose on bone have only used females (Li et al., 1990; Zernicke et al., 1995, Tjaderhane and Larmas, 1998).

Although sucrose is still widely used in the US, there has been a major shift from sucrose to HFCS in the food manufacturing industries (Nuttall, 2005) and this shift has led to a higher ratio of consumed fructose to glucose. Fructose is metabolized different than sucrose and fructose has been implemented in the development of several conditions including obesity (Gaby, 2005). It is also of interest to examine the effects of consumption of HFCS and fructose on bone health.

### **3.10 Fructose and/or HFCS and Bone Health**

Several studies have investigated the effect of high fructose and/ or HFCS intake on macromineral homeostasis since a negative effect of these sugars on: calcium, phosphorus and magnesium can adversely impact bone. The effect of

fructose versus glucose on nephrocalcinogenesis (an intratubular deposition of calcium phosphates in the corticomedullary of the kidney) was studied in weanling female Wistar rats (Bergstra et al. 1993). The study was divided into two separate 28-day experiments, with a one week interval between in a cross-over design. In experiment 1, female weanling rats (n=32) were randomly assigned into 4 groups: 1) glucose-normal phosphorus 2) glucose-high phosphorus 3) fructose-normal phosphorus or 4) fructose-high phosphorus. The glucose and fructose concentrations used were ~700 g/kg diet, normal phosphorus was defined as 12.9 mmol/100g diet and high phosphorus was defined as 19.4 mmol/100g diet. In experiment 2, the animals were randomly assigned into glucose-normal magnesium, glucose-low magnesium, fructose-normal magnesium and fructose low magnesium. Glucose and fructose concentrations used were ~700g/kg diet, normal magnesium and low magnesium was defined as 1.6 mmol/100g diet and 0.8 mmol/100g diet, respectively.

The results showed that feeding fructose did not have an effect on urinary calcium, plasma calcium, magnesium and phosphorus compared to glucose. However, fructose consistently induced higher kidney calcium implicating higher nephrocalcinogenesis than glucose, irrespective of the different concentrations of magnesium and phosphorus. Feeding fructose also resulted in significantly higher urinary excretion of magnesium and phosphorus compared to feeding glucose. Intestinal absorption of magnesium and phosphorus were also enhanced by fructose intake. The authors suggested that the increased absorption of magnesium and phosphorus may have accounted for the observation of higher

urinary losses in the fructose fed animals. The authors did not suggest possible mechanisms for the increased absorption of magnesium and phosphorus with fructose consumption. The results of Bergstra et al. (1993) were similar to Holbrook et al. (1989) who observed enhanced magnesium absorption with fructose consumption in human subjects. The basis for the enhanced absorption of magnesium and phosphorus is not known, further work is required to understand the mechanism by which fructose enhances absorption of magnesium and phosphorus.

In a study using human subjects, Ivaturi and Kies (1992) investigated the effect of fructose, HFCS, and sucrose on mineral balance. The project consisted of two 35 day studies. Each study consisted of a 7 day acclimation period and 2 consecutive 14 day experiments that were randomly assigned in a crossover design. The subjects in the study consisted of healthy males and females (n=24). Data on the distribution of the subjects by sex and age were not provided. In the first study, subjects received supplements of 60 g/d of either sucrose or fructose. In the second study, subjects received 60g/d of either sucrose or HFCS. Complete urine and fecal collections were obtained from each subject and were pooled into 7 day lots for mineral assessment. Diet records were obtained to determine mineral intakes. Ivaturi and Kies (1992) observed negative balances for calcium, magnesium and phosphorus for subjects consuming fructose supplements ( $p < 0.05$ ). Higher fecal losses of these minerals rather than urinary losses accounted for the negative balances of these minerals. The authors suggested that this observation may be an indication that fructose has a general negative effect of

reducing mineral absorption. In contrast, Bergstra et al. (1989) showed that the absorption of magnesium and phosphorus was enhanced with fructose intake. The different findings in these studies may be due to the use of animals rather than humans subjects. Ivaturi and Kies (1992) also observed that HFCS resulted in positive mineral balances when compared to fructose which can be explained by the presence of other substances such as glucose and higher saccharides. The higher saccharides present in HFCS are in the form of glucose polymers. Wood et al. (1986) reported glucose polymers enhance calcium absorption in humans.

Milne and Nielsen (2000) investigated the interaction between fructose and magnesium on macromineral homeostasis. The subjects in the study were healthy males aged between 22 and 40 years. The subjects were kept in a metabolic unit for approximately 6 months. The study consisted of a 16 day equilibration diet and four 42-day dietary periods. The study was a randomized, double blind, 2x2 factorial design. The 4 treatment groups consisted of: 1) Starch-low magnesium, 2) Starch-high magnesium 3) Fructose-low magnesium, and 4) Fructose-high magnesium. Fructose and starch were supplied at 20% of total energy per day. Fructose was supplied mainly as high fructose corn syrup as contained in commercial sodas. Magnesium was varied at 170 mg/2500 kcal (low) and 370 mg/2500 kcal (high). Urine and fecal samples were collected and compiled into 6-day aliquots that were used to determine the calcium, phosphorus and magnesium content. Blood samples were also collected after the subjects had fasted for 10 h. Fasting blood samples were used to analyze for serum magnesium, calcium, parathyroid hormone, osteocalcin, and alkaline phosphatase

concentrations. Milne and Nielsen (2000) observed greater losses of both calcium and phosphorus with high fructose intake mostly through fecal excretion and the greatest loss occurred during the high fructose-low magnesium intake period. In contrast, Ivaturi and Kies (1992) found that mineral balance was not affected by HFCS. It should be noted however, that the Ivaturi and Kies (1992) compared HFCS to sucrose; whereas, Milne and Nielsen (2000) compared HFCS to starch. The composition and metabolism of starch and sucrose are different. Starch is composed of glucose molecules only; whereas, sucrose is composed of glucose and fructose molecules. Fructose is metabolized differently from glucose and thus, starch and sucrose can have different effects on mineral balance. In addition, Milne and Nielsen (2000) use the term fructose loosely, in their study they use HFCS which is a mixture of fructose and glucose, the most common form used in beverages being: 45% glucose and 55% fructose (Buck, 2001). HFCS also contains higher saccharides in the form of glucose polymers which may be metabolized differently from fructose, a monosaccharide.

Milne and Nielsen (2000) observed that urinary magnesium was not affected by elevated dietary fructose but fecal magnesium was reduced with high fructose intake levels for both high and low magnesium compared to starch. The authors suggested that fructose enhanced the absorption of magnesium. In support, Holbrook et al. (1989) observed higher magnesium retention in humans fed fructose compared to those fed starch. Milne and Nielsen (2000) hypothesized that fructose may chelate magnesium in the intestines forming a compound that is stable and can be readily absorbed by the epithelial cell. The

formation of this fructose-magnesium complex has been demonstrated *in vitro* by Charley et al. (1963), however whether this process can occur *in vivo* needs to be established.

To our knowledge, few studies have examined the direct effect of fructose or HFCS on bone. Milne and Nielsen (2000) observed elevated levels of serum alkaline phosphatase with high fructose intake ( $p < 0.05$ ). Alkaline phosphatase is a marker of bone formation and may indicate an increase in bone formation. However, alkaline phosphatase is also produced by the liver and the intestines and thus, it is not a specific marker for bone formation. Milne and Nielsen (2000) did not observe any significant differences in the osteocalcin level, a specific marker of bone formation, among treatment groups.

In summary, the negative mineral balance observed with fructose consumption provides potential evidence that high fructose intake might impact bone health. However it is difficult to conclude from Milne and Nielsen (2000) the impact on bone since the study did not measure any changes in bone characteristics. More studies are needed to determine the effect of high fructose and/or HFCS on bone mineralization and strength and potential mechanisms of action (mineral balance and bone turnover markers).

The following study investigates the direct effects of fructose, HFCS, sucrose and glucose on bone. Moreover, the potential mechanisms for carbohydrate-induced alterations in bone characteristics are explored.

## **4.0 Materials and Methods**

### **4.1 Animal and Diets**

All animal procedures were conducted in accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol. Immature (28 days old) female Sprague-Dawley rats (n=44) were purchased from Taconic Farms (Rockville, MD). Upon arrival, animals were individually housed in metabolic cages in a room kept at 21°C with a 12 h light/dark cycle. All animals were given *ad libitum* access to deionized distilled water (ddH<sub>2</sub>O) and AIN-93G diet (Harlan Teklad, WI). Deionized distilled water was used because it does not contain any minerals. AIN-93G was used because it is a standard purified diet that meets the National Research Council (1995) nutrient requirements for growing rats and has a precisely defined carbohydrate source and sucrose concentration (Table 1) and is also has precisely defined levels of minerals (Table 2). After a 7-d acclimation, animals were randomly assigned n=8-9 rats/group to drink ddH<sub>2</sub>O or ddH<sub>2</sub>O containing 13% w/v sugar because this is the sugar content of most soft drinks. Sugars were added as: glucose, sucrose, fructose or HFCS-55 (Cornsweet®55, Archer Daniels Midland, IL). HFCS-55 was chosen because it is the form of HFCS commonly added to sweetened beverages and soft drinks (Bray et al., 2004).

Sucrose, fructose and HFCS-55 were used in this study because these sugars are the most popular sweeteners used in foods and beverages. These

sweeteners were compared against glucose and no sugar. The treatment groups were as follows:

<b>Treatment Group</b>	<b>Animal Number</b>
1. ddH <sub>2</sub> O (control)	n=8
2. ddH <sub>2</sub> O + 13% w/v glucose	n=9
3. ddH <sub>2</sub> O + 13% w/v sucrose	n=9
4. ddH <sub>2</sub> O + 13% v/v HFCS-55	n=9
5. ddH <sub>2</sub> O + 13% w/v fructose	<u>n=9</u>
<b>Total</b>	<b>n=44</b>

Rats were given their assigned sugar-sweetened beverage throughout the 50-d experiment. Food and beverage intake were measured weekly and replaced with fresh diet and the assigned sugar beverage. Body weight was also measured weekly.

Table 1. Ingredients of the AIN-93G Purified Diet

<b>Ingredients</b>	<b>g/kg</b>
Casein	200.0
L-Cystine	3.0
Corn Starch	397.486
Maltodextrin	132
Sucrose	100
Soybean oil	70
Cellulose	50
Mineral Mix <sup>§,*</sup>	35
Vitamin Mix <sup>§</sup>	10
Choline Bitartrate	2.5
TBHQ, antioxidant	0.014

<sup>§</sup>Prepared according to AIN-93 formulation (Reeves et al. 1993)

\*See Table 2

Table 2. Ingredients of the AIN-93G Mineral Mix<sup>§</sup>

<b>Ingredients</b>	<b>g/kg</b>
Calcium Carbonate	357.00
Potassium Phosphate (monobasic)	196.00
Potassium Citrate	70.78
Sodium Chloride	74.00
Potassium Sulfate	46.60
Magnesium Oxide	24.30
Ferric Citrate	6.06
Zinc Carbonate	1.65
Manganous Carbonate	0.63
Cupric Carbonate	0.31
Pottasium Iodate	0.01
Sodium Selenate	0.0103
Ammonium Paramolybdate	0.008
Sodium Metasilicate nonahydrate	1.45
Chromium Potassium Sulfite, dedecahydrate	0.275
Lithium Chloride	0.0174
Boric Acid	0.0815
Sodium Flouride	0.0635
Nickle Carbonate	0.0318
Ammonium Vanadate	0.0066
Sucrose, fine ground	220.716

<sup>§</sup>Reeves et al. (1993)

## **4.2 Urinary Calcium, Phosphorus and Creatinine**

Baseline urine samples (7-d acclimation) were collected. During the experiment, weekly urine volume and samples were collected. Ascorbic acid (0.1%) was added to the urine collection tubes as a preservative and 1 ml mineral oil to prevent evaporation. Collected urine samples were centrifuged at 1,500 g for 10 min at 4<sup>0</sup>C. Following centrifugation, urine samples were aliquoted into fresh tubes and kept at -20<sup>0</sup>C until assayed. Urinary calcium and phosphorus concentrations were determined by inductively coupled plasma optical emission spectrometry (model P400, Perkin Elmer, Shelton, CN). Urinary concentration of creatinine was measured using a commercially available enzyme immunoassay (EIA) kit (Quidel Corp, CA). If significant differences were found in the creatinine values among the treatment groups, then the urinary calcium and phosphorus values were corrected by dividing by the obtained creatinine values.

## **4.3 Fecal Calcium and Phosphorus**

Baseline fecal samples were collected (7-d acclimation). During the experimental period weekly fecal weights and samples were collected and stored at -20<sup>0</sup>C until assayed. The fecal matter was freeze-dried for 48 h and then transferred into a muffle furnace at 600<sup>0</sup>C for 18-24 h. Fecal total mineral content was determined by the weight of the fecal ash.

To determine fecal calcium (Ca) and phosphorus (P), all glassware was washed overnight in a solution of 10% (v/v) HCl in ddH<sub>2</sub>O prior to use. Ashed samples were dissolved in 2 mL of 70% nitric acid. The acidified samples were

neutralized in 5 mL of ddH<sub>2</sub>O and filtered through Whatman no. 1 paper.

Samples were diluted to volume with ddH<sub>2</sub>O in a 50 mL volumetric flask. Fecal Ca and P concentrations were determined by inductively coupled plasma optical emission spectrometry (model P400, Perkin Elmer, Shelton, CN).

#### **4.4. Bone Morphology**

At the end of the 50-d experiment, rats were euthanized by CO<sub>2</sub> inhalation. Both right and left femurs and tibiae were collected. The bones were defleshed with care being taken not to damage the periosteum. Each bone was individually wrapped in saline-soaked gauze and stored at -20°C until analysis. For analysis, each bone was brought to room temperature and the bones were weighed. Bone length, width and depth were determined using a vernier caliper.

#### **4.5 Bone Mineral Density and Content**

The bones were packed on dry ice and shipped overnight to Dr. Marybeth Brown at the University of Missouri-Columbia. Dr Brown determined bone mineral density (BMD), bone mineral content (BMC) and area of the whole, distal, diaphysis and proximal region of both the tibia and femur using dual energy X-ray absorptiometry (DEXA). The femur and tibia were placed in a dish of deionized water and scanned using DEXA equipped with a small animal high resolution collimator (Hologic Delphi A, Bedford, MA). The CV for total BMD and BMC was 0.12%. BMD, BMC and area of the distal, diaphysis and proximal

regions of the tibia and femur were determined as shown in (Figure 3). Following completion of DEXA scans, the bones were packed on dry ice (-20°C) and returned by overnight shipping.

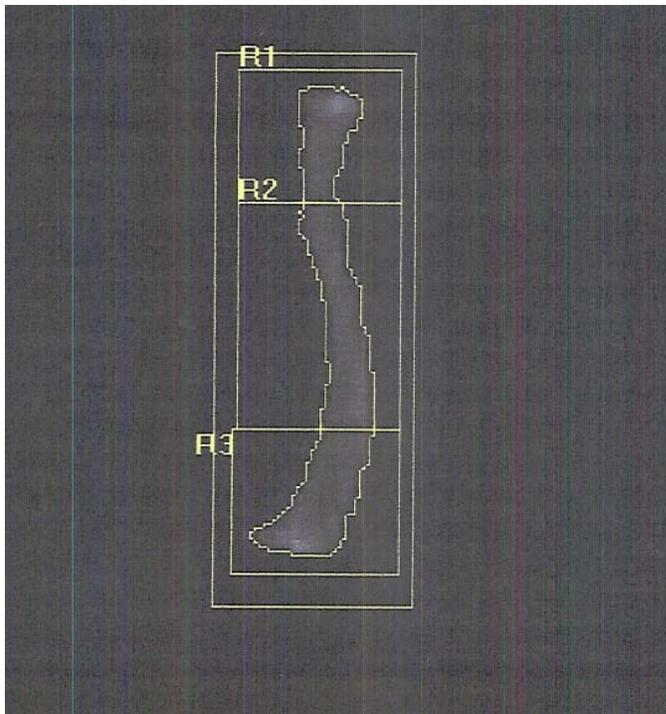


Figure 3. DEXA bone scan for whole bone and bone sections. R= region: R1=proximal, R2=diaphysis, R3=distal.

#### 4.6 Bone Biomechanics

Bone strength parameters were assessed using a TA.HDi Texture Analyzer (Texture Technologies Corp, NY) outfitted with a three-point bending apparatus. Femora and tibiae were placed on supports (1 mm width at tip) and bent until broken by lowering a centrally placed blade (1 mm width) at constant crosshead speed (0.1 mm/s). The load-deflection data collected by a PC interfaced with the TA.HDi Texture Analyzer was used to determine bone biomechanical measurements of peak force, bending failure energy, ultimate stiffness, ultimate bending stress (UBS, N/mm<sup>2</sup>) and Young's modulus (N/mm<sup>2</sup>) as described in Yuan and Kitts (1992). The bone biomechanical forces were identified as:

- 1) Peak force (N): the maximum force obtained during the bending procedure resulting in the initiation of the bone breaking
- 2) Bending failure energy (N/s): the area under the time-force deformation curve
- 3) Ultimate stiffness (N/s): the slope of the time-force deformation curve
- 4) Ultimate bending stress (UBS, N/mm<sup>2</sup>): a normalized, calculated force that takes into consideration the size of the bone calculated as:

$$\text{UBS} = \frac{8 \times \text{peak force} \times L \times a_1}{\pi ((a_1^3 a_2) - (b_1^3 b_2))}$$

Where L is the distance between the supporting points (17 mm for tibia and 10 mm for femur); a<sub>1</sub>= the outer diameter in the direction of the load, a<sub>2</sub>= the outer

diameter at right angles to  $a_1$ ,  $b_1$ =the inner diameter in the load direction and  $b_2$ =the inner diameter at right angles to  $b_1$ .

5) Young's Modulus (N/mm<sup>2</sup>): a normalized calculated stiffness that takes into consideration the size of the bone calculated as:

$$\text{Young's Modulus} = \frac{4 \times L^3 \times \text{ultimate stiffness (slope)}}{3 \times \pi ((a_1^3 a_2) - (b_1^3 b_2))}$$

Where L=the distance between the two edges in the three-point bending (17 mm for the tibia and 10 mm for the femur),  $a_1$ = the outer diameter in the direction of the load,  $a_2$ = the outer diameter at right angles to  $a_1$ ,  $b_1$ =the inner diameter in the load direction and  $b_2$ =the inner diameter at right angles to  $b_1$ .

#### 4.7 Bone Calcium and Phosphorus

Following bone biomechanical testing, the bone fragments were dried at 110°C for 48 h to determine dry weights. Bones were then ashed at 600°C in a muffle furnace for 18-24 h. Total bone mineral content was determined by the weight of the bone ash. To determine bone Ca and P, all glassware was washed overnight in a solution of 10% (v/v) HCl in ddH<sub>2</sub>O prior to use. Ashed samples were dissolved in 2 mL of 70% nitric acid. The acidified samples were neutralized in 5 mL of ddH<sub>2</sub>O and filtered through Whatman no. 1 paper. Samples were diluted to volume with ddH<sub>2</sub>O in a 50 mL volumetric flask. Femur and tibia Ca and P concentrations were determined by inductively coupled plasma optical emission spectrometry (model P400, Perkin Elmer, Shelton, CN).

## **4.8. Bone Turnover Markers**

### **1) Osteocalcin**

Trunk blood was collected immediately after the animals were euthanized by CO<sub>2</sub> inhalation. Blood was allowed to clot then placed on ice for 10 min. Blood samples were then centrifuged at 1,500 g for 10 min at 4°C to separate serum. Serum samples were collected and stored at -80°C until assayed.

Serum osteocalcin was measured as a marker of bone formation. Serum osteocalcin was measured by a commercially available enzyme-linked immunassay (EIA) rat osteocalcin kit (Biomedical Technologies, Stoughton, MA). The osteocalcin assay is a competitive enzyme immunoassay in a microtiter stripwell utilizing a monoclonal anti-osteocalcin antibody coated on the stripwell. The osteocalcin in the samples or the standards compete with the conjugated osteocalcin for the monoclonal anti-osteocalcin antibody coated in the stripwells. Optical density (OD) was then measured at 450 nm using a microplate reader (Spectramax Plus, (Molecular Devices, Sunnyvale, Ca).

### **2) Alkaline Phosphatase**

Serum alkaline phosphatase (ALP) was determined by enzymatic reactions that produced a color changes using a vet 16 rotor Hemagen Analyt (Hemagen Diagnostics, Inc. Columbia, MD). The rotors were read using the Hemagen

Analyst automated spectrophotometer (Hemagen Diagnostics, Inc. Columbia, MD).

### **3) Deoxypyridinoline (DPD)**

Urinary deoxypyridinoline (DPD) crosslinks were measured as a marker of bone resorption using commercially available rat DPD EIA kit (Quidel Corp, CA). The DPD assay is a competitive enzyme immunoassay in a microtiter stripwell utilizing a monoclonal anti-DPD antibody coated on the stripwell. The DPD in the samples and the standards competed with the conjugated DPD for the monoclonal anti-DPD antibody coated in the stripwells. Optical density (OD) was then measured at 405 nm using a microplate reader Spectramax Plus (Molecular Devices, Sunnyvale, Ca).

### **4.9 Kidney Function Biochemical Parameters**

Serum biochemical variables: alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein, albumin (ALB), creatinine, Ca, P and BUN/creatinine ratio were determined by enzymatic reactions that produced a color changes using a vet 16 rotor Hemagen Analyst (Hemagen Diagnostics, Inc. Columbia, MD). The rotors were read using the Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics, Inc. Columbia, MD).

#### **4.10 Statistical Analysis**

A one-way ANOVA was used to determine differences in bone variables, bone turnover markers, urinary calcium, urinary phosphorus excretion, fecal calcium and fecal phosphorus excretion due to the consumption of beverages containing different sugars. Post-hoc multiple comparison tests were performed using Tukey's test with treatment differences considered significant at  $p < 0.05$ . All statistical analyses was done using the statistical software SigmaStat 3.1 (Systat Software Inc, San Jose, CA).

## **5.0 RESULTS**

### **5.1 Bone Morphology**

There were no significant differences in the femoral morphology between rats fed non-sugar beverage (Control) and rats fed sugar-containing beverages. Among the sugar-sweetened beverages, the glucose-fed animals had significantly ( $p=0.03$ ) lower femur weights compared to fructose-fed animals (Table 3). There were no differences in tibial weight, length, width and depth among the treatment groups (Table 3).

### **5.2 Bone Area and Bone Mineral Content**

#### Femur

There were no differences in femoral area among the treatment groups (Figure 4A). There was no significant difference in the femoral total BMC between rats fed non-sugar containing beverages and rats fed sugar beverages. Among the sugar-sweetened beverages, the glucose-fed animals had significantly lower total BMC than animals fed sucrose ( $p=0.03$ ) and fructose ( $p=0.02$ ), but did not differ from animals fed HFCS-55 (Figure 4B). According to Table 4, there were no differences in the distal femoral BMC among any of the treatment groups but the proximal femur, BMC was significantly lower ( $p=0.02$ ) in the glucose-fed animals compared to fructose-fed animal. For the femoral diaphysis, BMC was significantly lower ( $p=0.03$ ) in glucose-fed rats than sucrose-fed animals but not different from the other treatment groups.

#### Tibia

There were no differences in bone area among the treatment groups for the tibia

(Figure 5A). There were no differences in tibia total BMC between rats fed non-sugar beverages and rats fed sugar-sweetened beverages. Among rats consuming sugar-sweetened beverages, glucose-fed animals had lower ( $p=0.045$ ) tibial BMC compared to fructose-fed animals but it was not different from the other treatment groups (Figure 5B). There were no differences in the tibia proximal, diaphysis and distal BMC among the treatment groups (Table 5).

### **5.3 Bone Mineral Density (BMD)**

#### Femur

##### *Whole bone*

As shown in Figure 4C, there was no significant difference in the femoral total BMD between rats fed non-sugar beverages and rats fed sugar beverages. Among the sugar-sweetened beverages, the glucose-fed animals had significantly lower ( $p=0.005$ ) total BMD compared to all the other sugar beverage groups.

##### *Bone sites*

As shown in Table 4, there was no significant difference in the BMD of any of the femoral bone sites between rats fed non-sugar beverages and rats fed sugar beverages. Among the sugar-sweetened beverages, BMD in the proximal femur was lower ( $p=0.02$ ) in the glucose-fed animals compared to sucrose, fructose-and HFCS fed animals. BMD in the distal femur was lower ( $p=0.01$ ) for glucose-fed animals compared to sucrose and fructose-fed animal. There were no differences in the BMD in the femur diaphysis among any of the treatment groups.

## Tibia

### *Whole bone*

As shown in Figure 5C, there was no significant difference in the tibia total BMD between rats fed non-sugar beverages and rats fed sugar beverages. Among the sugar-sweetened beverages, the glucose-fed animals had lower ( $p=0.001$ ) tibia total BMD compared to all the other sugar-containing beverage groups.

### *Bone sites*

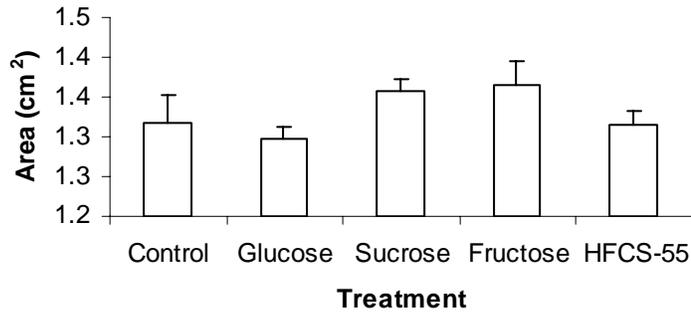
As shown in Table 5, there was no significant difference in the BMD of any of the tibial bone sites between rats fed non-sugar containing beverages and rats fed sugar beverages. Among the sugar-sweetened beverages, BMD in the diaphysis was lower ( $p=0.01$ ) in the glucose-fed rats than that of sucrose-fed animals. Glucose-fed animals also had lower ( $p=0.001$ ) BMD in the distal tibia compared to the other sugar treatment groups. No differences were detected in the BMD of the proximal tibia among the treatment groups.

Table 3. Final body mass and bone morphology in rats fed different sugar-sweetened beverages.<sup>§</sup>

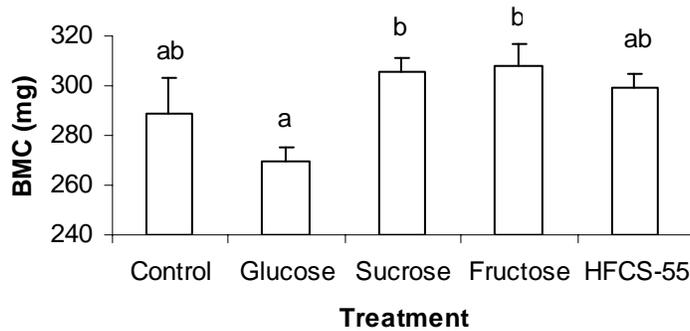
Treatment	Control	Glucose	Sucrose	Fructose	HFCS-55	p-value
n	8	9	9	9	9	
Final Body Mass (g)	256 ± 5 <sup>a</sup>	256 ± 3 <sup>a</sup>	274 ± 3 <sup>ab</sup>	276 ± 5 <sup>ab</sup>	283 ± 10 <sup>b</sup>	0.05
<u>Femur</u>						
Length (mm)	28.04 ± 0.23	28.25 ± 0.28	28.47 ± 0.18	28.34 ± 0.34	29.92 ± 0.25	0.49
Width (mm)	3.64 ± 0.06	3.59 ± 0.06	3.70 ± 0.07	3.72 ± 0.07	3.64 ± 0.05	0.64
Depth (mm)	2.96 ± 0.05	2.93 ± 0.04	3.04 ± 0.03	3.06 ± 0.07	2.98 ± 0.06	0.20
Weight (mg)	572 ± 17 <sup>ab</sup>	539 ± 9 <sup>a</sup>	584 ± 9 <sup>ab</sup>	593 ± 16 <sup>b</sup>	565 ± 9 <sup>ab</sup>	0.03
<u>Tibia</u>						
Length (mm)	34.88 ± 0.35	34.88 ± 0.22	35.23 ± 0.15	35.13 ± 0.24	34.98 ± 0.20	0.78
Width (mm)	3.80 ± 0.04	3.78 ± 0.04	3.91 ± 0.05	3.88 ± 0.07	3.78 ± 0.07	0.28
Depth (mm)	2.93 ± 0.05	2.72 ± 0.07	2.83 ± 0.08	2.87 ± 0.09	2.83 ± 0.09	0.35
Weight (mg)	417 ± 13	396 ± 8	434 ± 7	439 ± 13	420 ± 13	0.07

<sup>§</sup>Values are expressed as the mean ± SE of n=8-9 bones/group. Different letters a, b, within the same row indicate significant differences at P<0.05 by one-way ANOVA followed by Tukey's test. HFCS=High fructose corn syrup

A)



B)



C)

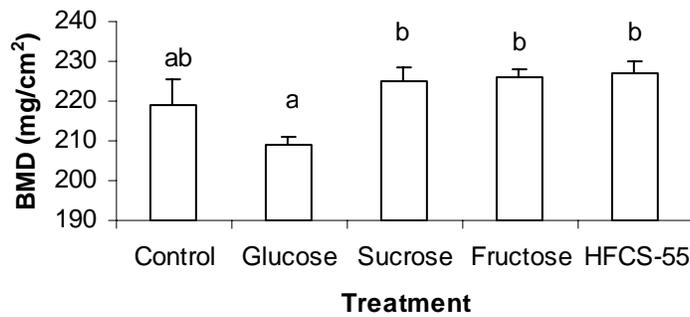
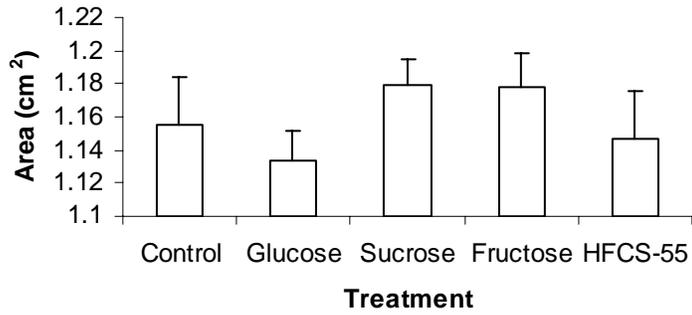
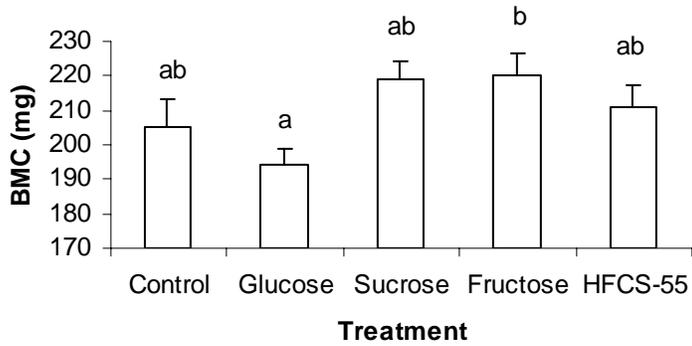


Figure 4. Femur total bone area, mineral content and mineral density. Values are the means  $\pm$ SE of n=8-9 rats/group. Different letters a, b indicate significant differences at  $p < 0.05$  by one-way ANOVA followed by Tukey's test. BMC=bone mineral density, BMD= bone mineral density.

A)



B)



C)

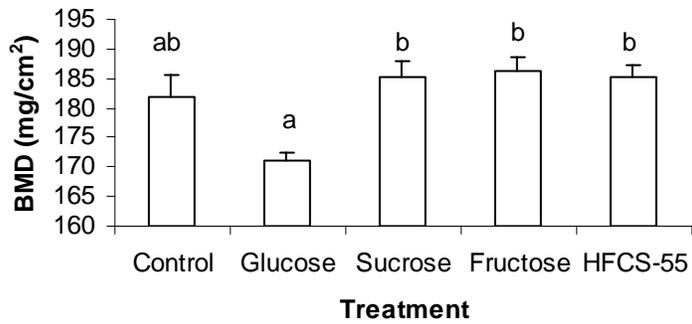


Figure 5. Tibia total bone area, mineral content and mineral density. Values are the means  $\pm$ SE of n=8-9 rats/group. Different letters a, b indicate significant differences at  $p < 0.05$  by one-way ANOVA followed by Tukey's test. BMC=bone mineral density, BMD= bone mineral density.

Table 4. Femoral area, mineral content and mineral density at different sites<sup>§</sup>

Section	Control	Glucose	Sucrose	Fructose	HFCS-55	p-value
<u>Area (cm<sup>2</sup>)</u>						
Proximal	0.311 ± 0.012	0.304 ± 0.007	0.293 ± 0.008	0.326 ± 0.012	0.300 ± 0.007	0.15
Diaphysis	0.614 ± 0.031	0.623 ± 0.014	0.686 ± 0.014	0.668 ± 0.018	0.629 ± 0.018	0.06
Distal	0.421 ± 0.037	0.392 ± 0.008	0.407 ± 0.007	0.397 ± 0.009	0.409 ± 0.040	0.66
<u>BMC (mg)</u>						
Proximal	64 ± 3 <sup>ab</sup>	51 ± 6 <sup>a</sup>	62 ± 3 <sup>ab</sup>	69 ± 3 <sup>b</sup>	63 ± 2 <sup>ab</sup>	0.02
Diaphysis	131 ± 7 <sup>a</sup>	131 ± 4 <sup>a</sup>	149 ± 4 <sup>b</sup>	148 ± 5 <sup>ab</sup>	141 ± 5 <sup>ab</sup>	0.03
Distal	103 ± 9	91 ± 3	100 ± 3	100 ± 3	102 ± 3	0.25
<u>BMD (mg/cm<sup>2</sup>)</u>						
Proximal	204 ± 6 <sup>ab</sup>	186 ± 4 <sup>a</sup>	211 ± 5 <sup>b</sup>	211 ± 2 <sup>b</sup>	212 ± 5 <sup>b</sup>	0.02
Diaphysis	216 ± 9	207 ± 2	217 ± 3	219 ± 3	222 ± 3	0.12
Distal	239 ± 6 <sup>ab</sup>	232 ± 2 <sup>a</sup>	248 ± 4 <sup>b</sup>	249 ± 4 <sup>b</sup>	246 ± 3 <sup>ab</sup>	0.01

<sup>§</sup> Values are expressed as mean ± SE of n=8-9 bones/group. Different letters a, b within the same row indicate significant differences at p<0.05 by one-way ANOVA followed by Tukey's test. BMC= Bone mineral content, BMD= Bone mineral density, HFCS=High fructose corn syrup.

Table 5. Tibia area, mineral content and mineral density at different sites<sup>§</sup>

Section	Control	Glucose	Sucrose	Fructose	HFCS-55	p-value
<u>Area (cm<sup>2</sup>)</u>						
Proximal	0.259 ± 0.008	0.253 ± 0.007	0.268 ± 0.012	0.249 ± 0.013	0.237 ± 0.011	0.29
Diaphysis	0.563 ± 0.032	0.561 ± 0.019	0.583 ± 0.023	0.581 ± 0.032	0.601 ± 0.031	0.84
Distal	0.354 ± 0.013	0.334 ± 0.016	0.348 ± 0.016	0.367 ± 0.015	0.367 ± 0.013	0.21
<u>BMC (mg)</u>						
Proximal	49 ± 2	44 ± 2	45 ± 6	49 ± 2	42 ± 5	0.57
Diaphysis	94 ± 5	93 ± 4	103 ± 4	104 ± 6	106 ± 5	0.28
Distal	71 ± 3	61 ± 4	71 ± 4	76 ± 3	69 ± 3	0.13
<u>BMD (mg/cm<sup>2</sup>)</u>						
Proximal	185 ± 4	177 ± 2	191 ± 3	194 ± 6	187 ± 5	0.07
Diaphysis	165 ± 3 <sup>ab</sup>	162 ± 1 <sup>a</sup>	172 ± 3 <sup>b</sup>	171 ± 3 <sup>ab</sup>	170 ± 2 <sup>ab</sup>	0.01
Distal	196 ± 6 <sup>ab</sup>	182 ± 3 <sup>a</sup>	204 ± 3 <sup>b</sup>	206 ± 2 <sup>b</sup>	211 ± 2 <sup>b</sup>	0.001

<sup>§</sup> Values are expressed as mean ± SE of n=8-9 bones/group. Different letters a, b within the same row indicate significant differences at p<0.05 by one-way ANOVA followed by Tukey's test. BMC= Bone mineral content, BMD= Bone mineral density, HFCS=High fructose corn syrup.

#### **5.4 Bone Calcium and Phosphorus**

There were no differences in total bone Ca among treatment groups for the femur or tibia (Table 6). There were no differences in the amount of P in both tibia and femurs of animals among the treatment groups.

#### **5.5 Bone Biomechanical Strength**

There were no significant differences in bone strength as determined by peak force, ultimate stiffness, bending failure, ultimate bending stress and Young's modulus among treatment groups for the femur (Table 7) and the tibia (Table 8).

Table 6. Bone ash, calcium and phosphorus of rats fed different sweetened beverages.

Treatment	Ash <sup>§</sup> (mg)	Calcium <sup>§</sup> (mg/g)	Phosphorus <sup>§</sup> (mg/g)
<u>Femur</u>			
Control	268 ± 9.1	32.9 ± 1.1	101.3 ± 2.0
Glucose	246 ± 5.4	36.2 ± 1.9	99.8 ± 2.5
Sucrose	265 ± 5.7	32.2 ± 0.7	103.0 ± 0.7
Fructose	278 ± 6.5	32.2 ± 0.7	104.0 ± 0.7
HFCS-55	271 ± 4.0	31.2 ± 0.6	100.1 ± 2.5
<u>Tibia</u>			
Control	197 ± 6.5	86.9 ± 9.7	100.8 ± 1.0
Glucose	179 ± 4.6	83.6 ± 8.0	98.7 ± 0.8
Sucrose	199 ± 3.8	77.5 ± 2.0	100.4 ± 0.8
Fructose	206 ± 4.2	79.1 ± 0.98	107.7 ± 0.5
HFCS-55	202 ± 3.4	78.4 ± 0.94	107.7 ± 1.0

<sup>§</sup> Values are expressed as the mean ± SE of n=8-9 bones/group. Ca=Calcium, P=Phosphorus, HFCS= High fructose corn syrup. There were no significant differences among the treatment groups at p<0.05.

Table 7. Biomechanical measurements of femur strength in rats fed different sugar-sweetened beverages.<sup>§</sup>

Treatment	n	Peak Force (N)	Ultimate Stiffness (N/s)	Bending Failure (N/s)	Ultimate Bending Stress (N/mm <sup>2</sup> )	Young's Modulus (N/mm <sup>2</sup> )
Control	8	161.0 ± 16.4	1630.5 ± 113.2	9.4 ± 1.6	197.8 ± 20.9	2266.5 ± 266.2
Glucose	9	155.3 ± 9.5	1313.9 ± 167.3	9.4 ± 1.2	215.8 ± 12.9	2900.8 ± 208.9
Sucrose	9	177.0 ± 6.9	1447.1 ± 35.5	12.2 ± 1.1	226.2 ± 8.3	2762.5 ± 203.4
Fructose	9	155.1 ± 10.5	1227.5 ± 176.5	9.3 ± 1.3	197.1 ± 15.4	2304.1 ± 230.1
HFCS-55	9	155.2 ± 10.2	1321.5 ± 183.9	8.3 ± 1.1	203.7 ± 15.8	2535.3 ± 268.5

<sup>§</sup>Values are expressed as the mean ± SE of n=8-9 bones/group. HFCS= High fructose corn syrup. There were no significant differences among the treatment groups at p<0.05.

Table 8. Biomechanical measurements of tibia strength in rats fed different sugar-sweetened beverages.<sup>§</sup>

Treatment	n	Peak Force (N)	Ultimate Stiffness (N/s)	Bending Failure (N/s)	Ultimate Bending Stress (N/mm <sup>2</sup> )	Young's Modulus (N/mm <sup>2</sup> )
Control	8	78.2 ± 2.9	506.5 ± 34.2	7.5 ± 0.8	131.3 ± 8.1	3771.6 ± 542.6
Glucose	9	68.7 ± 2.6	462.2 ± 17.5	5.6 ± 0.6	143.0 ± 11.0	5909.4 ± 1165.1
Sucrose	9	76.4 ± 2.3	470.9 ± 21.7	7.6 ± 0.8	134.4 ± 5.9	4106.5 ± 383.4
Fructose	9	82.2 ± 5.2	516.1 ± 47.7	7.9 ± 0.7	138.0 ± 7.3	3968.8 ± 432.5
HFCS-55	9	76.8 ± 2.5	527.8 ± 21.6	6.5 ± 0.4	131.7 ± 5.2	3877.0 ± 447.0

<sup>§</sup>Values are expressed as the mean ± SE of n=8-9 bones/group. HFCS= High fructose corn syrup. There were no significant differences among the treatment groups at p<0.05.

## 5.6 Mineral Balance

Glucose fed animals had a higher ( $p < 0.001$ ) beverage intake compared to all the other groups (Table 9). Glucose fed animals had lower ( $p < 0.001$ ) food intake when compared to all the other treatment groups except sucrose.

### Calcium Balance

Considering the final week, animals fed the non-sugar beverage had significantly higher ( $p = 0.001$ ) Ca consumption than all the other treatment groups. Among the groups fed sugar-sweetened beverages, glucose-fed animals had lower Ca intake compared to fructose-fed animals ( $p < 0.001$ ) and HFCS-55 fed animals ( $p = 0.001$ ) but did not differ from animals consuming sucrose (Table 10). Despite having the lowest Ca consumption, glucose fed animals had highest Ca absorption rate and lowest fecal Ca excretion.

There were no significant differences in urinary calcium excretion among the treatment groups. Retention of Ca was lower ( $p < 0.001$ ) in glucose-fed animals compared to all the other groups (Table 10).

### Phosphorus Balance

Considering the final week, animals consuming the non-sugar beverage had higher P intake ( $p = 0.001$ ) compared to all the other groups (Table 11). Absorption of P was significantly lower in animals fed non-sugar beverage compared to glucose and sucrose-fed animals ( $p < 0.001$ ) but not fructose and HFCS-55 fed animals. Fecal P excretion was significantly higher ( $p < 0.001$ ) in animals fed non-sugar beverage compared to sugar treatment groups but there were no differences in urinary P excretion. Retention of P was significantly lower ( $p < 0.05$ ) in animals fed non-sugar beverage

compared to fructose-fed animals but did not differ from glucose, sucrose and HFCS-55 fed animals.

Among the animals consuming sugar-sweetened beverages, fructose-fed animals had significantly higher P intake compared to glucose ( $p < 0.001$ ) and sucrose ( $p = 0.01$ ) but not HFCS-55 (Table 11). There were no differences in P absorption among the animals fed sugar-sweetened beverages. Glucose fed animals had significantly lower ( $p = 0.02$ ) fecal P excretion compared to fructose-fed animals. HFCS-55 fed animals had significantly higher ( $p < 0.05$ ) urinary P excretion compared to fructose-fed animals but not glucose and sucrose-fed animals. No differences were detected in P retention among animals fed sugar-sweetened beverages.

Table 9. Total food and beverage intake.<sup>§</sup>

Treatment	Total Food Intake (g)	Total Beverage Intake (g)
Control	970.69 ± 27.28 <sup>d</sup>	1892.31 ± 198.89 <sup>a</sup>
Glucose	597.23 ± 27.90 <sup>a</sup>	5153.70 ± 356.16 <sup>c</sup>
Sucrose	692.29 ± 34.36 <sup>ab</sup>	3333.03 ± 307.12 <sup>b</sup>
Fructose	878.13 ± 16.34 <sup>cd</sup>	1767.68 ± 240.29 <sup>a</sup>
HFCS-55	791.25 ± 31.56 <sup>bc</sup>	2825.63 ± 234.36 <sup>ab</sup>

<sup>§</sup>Values are expressed as mean ± SE of n=8/9. Different letter a, b, c and d in the same column indicate significant differences at p<0.05 by one-way ANOVA followed by Tukey's Test. HFCS= high fructose corn syrup.

Table 10. Calcium intake, excretion, absorption and retention in the experimental groups.<sup>§</sup>

Group	Ca intake (mg/d)	Fecal Ca Excretion (mg/d)	Urinary Ca excretion (mg/d)	Ca absorption* rate (%)	Calcium retention† (mg/d)
<u>Baseline</u>					
Control	70.49 ± 2.81	12.23 ± 0.57	0.07 ± 0.03	86.61 ± 0.65	58.19 ± 2.46
Glucose	70.98 ± 3.13	13.08 ± 0.94	0.08 ± 0.02	81.14 ± 1.66	57.84 ± 3.67
Sucrose	72.02 ± 3.01	10.89 ± 0.73	0.06 ± 0.02	84.55 ± 1.36	61.06 ± 3.37
Fructose	70.34 ± 3.06	11.50 ± 0.72	0.03 ± 0.01	83.58 ± 0.93	58.81 ± 2.86
HFCS-55	73.49 ± 3.46	13.13 ± 1.15	0.09 ± 0.04	82.33 ± 0.98	58.63 ± 2.72
<u>Final week</u>					
Control	79.06 ± 2.94 <sup>d</sup>	24.12 ± 1.64 <sup>d</sup>	0.28 ± 0.09	69.58 ± 1.41 <sup>a</sup>	66.56 ± 2.48 <sup>d</sup>
Glucose	37.33 ± 3.14 <sup>a</sup>	7.77 ± 1.54 <sup>a</sup>	0.98 ± 0.43	80.44 ± 2.17 <sup>c</sup>	23.28 ± 3.76 <sup>a</sup>
Sucrose	48.50 ± 2.94 <sup>ab</sup>	11.06 ± 1.85 <sup>ab</sup>	1.42 ± 0.54	77.89 ± 2.76 <sup>b</sup>	37.16 ± 3.01 <sup>b</sup>
Fructose	62.49 ± 2.65 <sup>c</sup>	17.55 ± 1.36 <sup>c</sup>	0.22 ± 0.12	72.39 ± 1.36 <sup>ab</sup>	50.78 ± 2.58 <sup>c</sup>
HFCS-55	55.00 ± 3.00 <sup>bc</sup>	14.90 ± 1.26 <sup>bc</sup>	0.99 ± 0.22	72.96 ± 1.81 <sup>ab</sup>	40.92 ± 2.81 <sup>bc</sup>

<sup>§</sup>Values are expressed as the mean ± SE of n=8-9 rats/group. Different letters a, b, c, and d within the same column indicate significant differences at p< 0.05 by one-way ANOVA followed by Tukey's test. Ca= Calcium, HFCS=High fructose corn syrup.

\*Calcium absorption rate (%) = (Calcium intake-fecal Ca excretion)/Ca intake x 100.

†Calcium retention = Total Ca Intake- (Fecal Ca excretion + Urinary Ca excretion).

Table 11. Phosphorus intake, excretion, absorption and retention in the experimental groups.<sup>§</sup>

Group	P intake (mg/d)	Fecal P Excretion (mg/d)	Urinary P excretion (mg/d)	P absorption* rate (%)	P retention <sup>†</sup> (mg/d)
<u>Baseline</u>					
Control	22.25 ± 0.89	8.29 ± 0.67	0.01 ± 0.002	62.49 ± 3.13	13.95 ± 0.98
Glucose	22.40 ± 0.99	9.48 ± 1.04	0.02 ± 0.003	56.39 ± 6.35	12.91 ± 1.77
Sucrose	22.74 ± 0.95	7.44 ± 0.43	0.01 ± 0.003	66.86 ± 2.48	15.23 ± 1.04
Fructose	22.21 ± 0.96	7.78 ± 0.72	0.01 ± 0.003	65.33 ± 2.72	14.42 ± 0.81
HFCS-55	23.20 ± 1.09	9.21 ± 1.00	0.01 ± 0.04	60.92 ± 2.81	13.91 ± 0.70
<u>Final week</u>					
Control	24.96 ± 0.93 <sup>d</sup>	17.84 ± 1.47 <sup>c</sup>	1.78 ± 0.36 <sup>ab</sup>	28.91 ± 1.53 <sup>a</sup>	5.35 ± 1.12 <sup>a</sup>
Glucose	11.78 ± 0.99 <sup>a</sup>	4.22 ± 0.94 <sup>a</sup>	1.61 ± 0.57 <sup>ab</sup>	66.84 ± 4.39 <sup>b</sup>	6.42 ± 0.89 <sup>ab</sup>
Sucrose	15.31 ± 2.78 <sup>ab</sup>	5.81 ± 1.54 <sup>ab</sup>	2.02 ± 0.71 <sup>ab</sup>	64.59 ± 7.21 <sup>b</sup>	7.82 ± 1.23 <sup>ab</sup>
Fructose	19.73 ± 0.84 <sup>c</sup>	10.07 ± 1.16 <sup>b</sup>	0.84 ± 0.28 <sup>a</sup>	50.34 ± 4.31 <sup>ab</sup>	9.93 ± 1.06 <sup>b</sup>
HFCS-55	17.36 ± 0.93 <sup>bc</sup>	8.71 ± 0.90 <sup>ab</sup>	3.08 ± 0.80 <sup>b</sup>	49.58 ± 5.17 <sup>ab</sup>	5.48 ± 1.43 <sup>ab</sup>

<sup>§</sup>Values are expressed as the mean ± SE of n=8-9 rats/group. Different letters a, b, c, and d within the same column indicate significant differences at P < 0.05 by one-way ANOVA followed by Tukey's test. P= Phosphorus, HFCS=High fructose corn syrup.

\*P absorption rate (%) = (P intake-fecal P excretion)/P intake x 100.

<sup>†</sup>P retention = Total P Intake- (Fecal P excretion + Urinary P excretion)

## **5.7 Biochemical Variables**

### **Kidney Function**

As shown in Table 12, there were no differences in the serum total protein, albumin (ALB), blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, serum calcium and phosphorus among the treatment groups.

### **Born Turnover Markers**

There were no differences in the urinary DPD (expressed as DPD/creatinine), serum osteocalcin and serum ALP among the treatment groups (Table 12).

Table 12. Serum biochemical variables and bone markers.<sup>§</sup>

Variable	Control	Glucose	Sucrose	Fructose	HFCS-55
Serum Ca (mg/dL)	9.44 ± 0.58	9.12 ± 0.69	8.39 ± 0.72	8.32 ± 0.66	8.54 ± 0.52
Serum P (mg/dL)	7.43 ± 0.60	8.23 ± 0.62	7.04 ± 0.56	7.43 ± 0.50	7.20 ± 0.54
BUN (mg/dL)	15.49 ± 1.39	11.79 ± 1.48	11.27 ± 1.23	13.12 ± 1.95	12.71 ± 1.10
Albumin (g/dL)	4.04 ± 0.25	3.89 ± 0.26	3.64 ± 0.31	3.57 ± 0.29	3.71 ± 0.21
Creatinine (mg/dL)	0.50 ± 0.07	0.78 ± 0.09	0.54 ± 0.08	0.56 ± 0.09	0.61 ± 0.06
Total Protein (g/dL)	5.71 ± 0.33	5.41 ± 0.45	5.70 ± 0.47	5.26 ± 0.46	5.18 ± 0.36
BUN/Creatinine	36 ± 6	17 ± 3	27 ± 8	24 ± 4	22 ± 2
ALP (U/L)	66.63 ± 7.45	79.22 ± 7.95	86.89 ± 12.81	68.78 ± 8.98	59.22 ± 6.38
Osteocalcin (ng/ml)	1.88 ± 0.29	1.52 ± 0.20	1.81 ± 0.33	2.09 ± 0.41	1.39 ± 0.12
Urinary DPD (nmol/mmol creatinine)	166.69 ± 53.6	80.37 ± 25.2	105.8 ± 46.3	107.56 ± 36.7	197.61 ± 66.7

<sup>§</sup>Values are expressed as mean ± SE of n=8/9 rats group. Ca= Calcium, P=Phosphorus, BUN= Blood urea nitrogen, ALB=Albumin,

ALP=Alkaline phosphatase, DPD= deoxypyridinoline.

## 6.0 DISCUSSION

Feeding sugar-sweetened beverage to immature rats for 8 weeks had no significant effect on femur and tibia growth, mineralization, Ca, P or strength compared to animals consuming deionized distilled water (ddH<sub>2</sub>O) without sugar. In contrast, other studies have reported that rats fed diets high in sugar had significant reductions in bone mineralization and strength compared to animals fed diets low in added sugars (Li et al., 1990; Zernicke et al., 1995; Tjaderhane and Larmas, 1998). Absence of significant reductions in bone mineralization and strength in animals fed sugar-sweetened beverages compared to those fed non-sugar beverage in our study may be due to a shorter study duration (8 weeks) compared to Li et al. (1990) and Zernicke et al (1995) whose studies were 10 weeks and 2 years long, respectively.

On the other hand, Tjaderhane and Larmas study (1998) reported reduced bone mass and strength after a shorter duration than the current study. The amount of sweetener used in their study (~43%) was ~23% higher than the combination of sugar in solid diet and beverage used in our study. Most studies reporting reduced bone mineralization and strength with high-sugar intake used high levels of simple carbohydrates and added sugars to solid diets. Such high sugar levels in the diet may lead to gastrointestinal problems that can result in increased calcium losses. Whether this was the mechanism responsible for the bone changes observed in the Li et al. (1990), Zernicke et al. (1995) and Tjaderhane and Larmas (1998) studies could not be confirmed since fecal and urinary mineral losses were not determined. The amount of sweetener used in the

current study was more physiologically relevant. In the present study, sugars were added at a level comparable to the typical sugar content of soft drinks.

Several studies have observed a decrease in BMD or BMC in subjects with high intakes of soft drinks (Wyshak and Frisch, 1994; Wyshak, 2000; Whiting et al., 2001). However, it should be noted that these studies were population studies in which a causal relationship could not be determined. In an animal study, Garcia-Conteras et al. (2000) observed decreased BMD in rats consuming soft drinks compared to those consuming tap water. Despite similar levels of sugar to that in soft drinks, our study showed sugar beverages had no effect on BMD compared to rats fed non-sugar beverages. Other ingredients such as phosphoric acid and caffeine in the soft drinks may have contributed to bone loss rather than sugar (Fitzpatrick and Heaney, 2003).

Although we did not observe differences in bone properties between the animals fed non-sugar and sugar-sweetened beverages, there were significant differences depending on the type of sweeteners used. It is important to examine the effect of different sugars on bone because the type of sugar used to sweeten beverages has changed resulting in a lower glucose and higher fructose intake (Elliot et al., 2002). In the current study, both the tibia and the femur BMD of glucose-fed animals were significantly reduced compared to the other sweeteners. A possible mechanism for reduced bone mass with sugar beverage consumption is reduced Ca intake. In our study, the solid diet used was the AIN-93G diet which provides adequate mineral intake with the desired Ca: P ratio of 2:1. In our study, glucose-fed animals had reduced Ca intake; however, accompanying decreased

Ca intake was an increase in Ca absorption. Similarly, Zheng et al. (1985) and Younoszai et al. (1985) observed that feeding glucose increased intestinal Ca absorption in normal and diabetic rats. In human subjects, Norman et al. (1980) and Monnier et al. (1978) observed that glucose consumption resulted in a two-fold and a 21% increase in intestinal calcium absorption, respectively. Despite increased Ca absorption in glucose-fed rats, Ca retention was decreased which may explain the reduced BMD.

Another possible mechanism for reduced BMD may be the direct effects of glucose on bone. Terada et al. (1998) observed that high glucose concentrations inhibited osteoblast proliferation and this in turn, may impair bone formation resulting in bone loss. However, in this study, there were no differences in bone formation as indicated by no reduction in serum osteocalcin and ALP in the glucose-fed rats.

Differences between the sugar treatment groups were most pronounced between glucose and fructose. Glucose-fed rats had reduced BMD and BMC compared to fructose-fed animals. In addition, glucose-fed animals had reduced BMD and BMC in the proximal femur. BMC and BMD loss in the proximal region is important because if the bone is weakened, the proximal end of the femur near the hip joint is prone to fragility fractures. Despite the reduced total BMD and BMC in glucose compared to fructose-fed animals, no differences in bone strength were detected. Although reduced bone mass in glucose-fed rats had no effect on bone strength in young growing animals, fragility fractures due to reduced bone mass may manifest later in adulthood. The age of our rats at the end

of the study was 3 months which is considered young adulthood when bone growth is still rapid. At about 6 months, rats are mature and bone growth slows (Kalu et al, 1991). To study the impact of the sugar consumption by immature animals on bone at adulthood requires a study done over a long period of time (at least a year). However, other researchers have observed decreases in bone strength in animals younger or about the same age as animals in our study (Tjaderhane and Larmas, 1998; Li et al., 1990). This discrepancy may be explained by the high doses of sugars used by Tjaderhane and Larmas (1998) and Li et al. (1990) compared to the current study.

Fructose appeared to have positive effects on bone compared to glucose. Femoral weight, total BMC and total BMD were significantly higher in fructose-fed animals compared to glucose-fed animals. Similarly, tibia total BMC and total BMD were higher in fructose-fed animals compared to glucose-fed animals. Tibial P content was also significantly higher in the fructose compared to glucose-fed animals. Milne and Nielsen (2000), using human subjects, observed elevated levels of alkaline phosphatase (bone formation marker) with high fructose intake suggesting that fructose might have a positive impact on bone. In our study, fructose had no effect on alkaline phosphatase. It should be noted that alkaline phosphatase is not specific to bone, it is also produced in the intestines and the liver, and thus it may not be an accurate indicator of bone formation. We did not observe any significant differences in osteocalcin (a marker specific to bone) among the treatment groups.

The higher BMD and BMC in fructose-fed animals may be due to higher Ca intake and retention compared to glucose-fed animals. Contrary to our findings, Milne and Nielsen (2000) observed negative calcium balances with high fructose intake in humans. Milne and Nielsen (2000) also observed that the effects of high fructose intake on Ca balance were more marked when dietary magnesium was low, thus the observed results could be a result of an interaction between fructose and magnesium. The diet used in our study provided adequate amount of magnesium to meet rat requirements (Reeves, 1993).

HFCS-55 was one of the sweeteners included in our study. It is important to determine the effect of HFCS-55 on bone because the type of sugar used to sweeten beverages has changed from sucrose to HFCS-55 (Bray et al., 2004). HFCS-55 leads to a higher fructose: glucose ratio compared to sucrose. In our study, there were no differences in femoral or tibial morphometry, BMC, BMD, Ca and P content, and strength between HFCS-55 and sucrose-fed animals. We also observed that there were no differences in Ca intake, absorption and retention between HFCS-55 and sucrose fed animals. Our observations are in agreement with observations by Ivaturi and Kies (1992) who also observed that HFCS did not appear to negatively affect mineral balance compared to sucrose in human subjects.

## **7.0 SUMMARY AND CONCLUSION**

There were no differences in bone growth, mineralization and strength between rats consuming sugar at the level present in most soft drinks and non-sugar beverages. However, the displacement of Ca rich beverages such as milk by increased consumption of sugar beverages may still compromise bone health. Although there were no differences in bone mineralization and strength between the animals consuming no sugar beverage and those consuming sugar-sweetened beverages, there were significant differences between glucose and fructose. Fructose has been linked to several conditions including obesity and the increase in fructose consumption (with soft drinks contributing the most with the use of HFCS-55) has raised concern. Glucose appears to have more detrimental effects on bone compared to fructose. In light of our study observations, consumption of sports drinks which are sweetened with glucose and use of HFCS-42 which has a higher ratio of glucose: to fructose raises concern. In terms of bone, changes in the glucose: fructose ratio that increases glucose may need to be the focus rather than the current attention being given to increased fructose levels.

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