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Original article

Short-term feeding with high fructose diet impairs bone mineralization in growing rats

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Summary. *Aim:* The objective of this study was to investigate effects of short-term, high fructose intake on mineralization and biomechanical strength of bones in growing rats. *Methodology:* Male Wistar rats (aged 8 weeks) were divided into two experimental groups. Animals were fed with AIN 93G diet or high fructose diet, containing 63 wt% of fructose, for four weeks. Bone calcium and phosphorus content were determined according to PN-EN 15505:2009 norm and AOAC 986.24-1988 method, respectively. The bones biomechanical strength was tested using texture analyzer. *Results:* Feeding rats with high fructose diet resulted in significant ($p<0,05$) decrease of calcium content in femur and tibia. Also phosphorus content was significantly ($p<0,05$) decreased in femoral bone from animals fed high fructose diet. The biomechanical strength of femur and tibia was slightly lower in high fructose group compared to control. *Conclusion:* Short-term high fructose intake impairs calcium and phosphorus bone deposition in growing animals.

Key words: fructose, diet, bone development, calcium, Wistar rats

Introduction

In last few decades there have been global changes in dietary habits revealing i.e. in increased consumption of highly processed food. For this reason in the United States, where High Fructose Corn Syrup (HFCS) is commonly used as a food sweetener, fructose intake significantly rose. In natural sources fructose occurs in fruits, honey and sucrose. Recently, soft drinks, energy drinks, yogurts, jams and ice creams are the main sources of fructose. The consumption of fructose in the American diet is estimated at more than 10% of daily calorie intake (1). Overconsumption of HFCS-sweetened products may contribute to obesity, non-alcoholic fatty liver disease (NAFLD), hyperlipidemia, insulin resistance, inflammation and oxidative damage and renal dysfunction (2-5). Not all of these disorders are directly caused by fructose. However, adverse effect of fructose excess is confirmed by the fact that high fructose diet is often used to induce metabolic syndrome in experimental animals.

Reported negative effects of high fructose intake on liver and kidney function led us to assumption that high fructose diet may affect bone metabolism as these organs are essential in bone metabolic pathways. There are several studies demonstrating that in patients with NAFLD occurs significant decrease in bone mineral density (BMD) (6). Another argument for this hypothesis can be cross-sectional studies, published over a decade ago, where the increased risk of fractures was observed among teenagers regularly consuming soft drinks (7-9). This issue is in the great importance as children and young adults often represent major consumers of HFCS-sweetened products. Normalizing fructose intake to body weight, children are consuming daily about 2 g per kg body weight (10).

In this period, the organism reaches the peak bone mass which play role in a structural strength of the bone in later life. Most authors associates impaired bone metabolism with metabolic syndrome induced by the high fructose diet. In the present work we made attempt to establish the effects of high fructose consumption on bone mineralization and resistance to fractures, without inducing metabolic syndrome. Therefore, we applied the short term feeding to prevent the development of metabolic syndrome in the experimental animals.

Materials and methods

Animals, study design and diets

Eight-weeks-old male Wistar rats (n=20) weighting approximately 100 g were used in the experiment. Animals were housed in collective cages in temperature-controlled (22-24°C) room with 12 h light-dark cycle. Animals had *ad libitum* access to food and water during the experiment.

After seven days acclimatization animals were randomly (using physical generation of numbers) assigned to two groups. For the next four weeks animals received control AIN 93G diet (AIN 93G) or high fructose diet (HF). After four weeks of feeding with the experimental diets, rats were anaesthetized. Then left hind limbs were dissected and cleaned from soft tissue. Both, femur and tibia were weighted and then stored at -20°C for further analysis.

Table 1. Composition of experimental diets (g/kg)

Ingredients	AIN 93G diet	High Fructose diet
Fructose	—	632,486
Corn starch	532,486	—
Casein	200	200
Sucrose	100	—
Soybean oil	70	70
Cellulose	50	50
Mineral mix	35	35
Vitamin mix	10	10
Choline bitartrate	2,5	2,5
t-Butylhydroquinone	0,014	0,014

The composition of experimental diets is shown in the table 1. Both diets had the same energy value. In the experimental group fructose provided 64% of total energy, whereas in AIN 93G starch and sucrose provided respectively 54% and 10% of total energy. Fructose was obtained from Biofan (Poland), cornstarch from Agrotrade (Poland), casein from Kazeina Polska sp. z o.o. (Poland). Remaining diet ingredients were purchased from Sigma-Aldrich (USA).

All procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Union and approved by the 1st Local Ethical Committee on Animal Testing in Cracow.

Bone calcium and phosphorus assay

Bone calcium content was determined by atomic absorption spectrometry (AAS) with flame atomization, using Varian AA240FS atomic absorption spectrometer (Varian, USA). Analysis was performed according to PN-EN 15505:2009 norms. Wet mineralization was carried out by the pressure microwaves method (MarsXPress, CEM, USA) using 65% nitric acid (Suprapur® Merck, Germany) added in amount of 10 ml per 0,5 g of tested sample. The process was conducted in 55 ml volume, teflon container with maximum temperature set at 200°C and 40 min mineralization time. Samples were diluted by addition, 8 ml to 25 ml volumetric flask, of Schinkel solution (cesium chloride and lanthanum chloride, both in 10 g/L concentration; Merck, Germany).

Determination of phosphorus content in rats bones was preformed according to the Association of the Analytical Communities (AOAC) method 986.24-1988 (1997), based on the spectrophotometric method.

Bone biomechanical analysis

Frozen samples were thawed overnight and equilibrated to room temperature (22°C) prior to analysis. The bones were analyzed using a texture analyzer TA.XTplus (Stable Micro Systems LTD, Godalming, Surrey, UK) with a Warner-Bratzler attachment consisted of 3 mm thick steel blade which had a 73°V cut into its lower edge, and was fitted through a 4 m wide slit in platform. The bone sample was placed horizontally on the platform and was cut by the V blade. The assay parameters were: pre-test speed 2,0 mm/s; test-speed 5,0 mm/s; post-test speed 10 mm/s; trigger force 10 G, distance 10 mm.

The bone strength was measured as a force (measured in newtons) necessary to shear the bone. Ten samples from each experimental group were assessed.

Statistical analysis

The collected data were analysed by the one-way analysis of variance ANOVA using Statistica10 (StatSoft, Inc.). Significance of results were considered with $p<0,05$. All values were expressed as means with standard error of mean (SEM).

Results and discussion

Four weeks of feeding rats with high fructose diet resulted in slight, though insignificant reduction in bone-wet weights (figure 1). Femur and tibia weights were decreased by 5% and 8% respectively.

Opposite results obtained Mosavat et al. (11), who observed increase in tibial and femoral wet weights after eight weeks of honey supplementation. Nevertheless, honey contains number of bioactive substances, beyond sugars, which may contribute to bone mass increase.

Wet bone weight reflects bone mass, however it is not relevant indicator of bone strength as it is composed of many components differently affecting its mechanical properties. The extracellular bone matrix consists of mineral and organic phase and water. Mineral phase, providing bone stiffness, is build from calcium and phosphorus in form of insoluble salts, hydroxyapatites. In this phase occurs also a small amount of other minerals like magnesium, bicarbonate and zinc. The organic phase is composed from type I collagen, noncolagenous proteins and lipids. Content of individual components varies depending on age, site, ethnicity, gender and health condition (12, 13). Loss of any of these components may contribute to the reduction of bone mass and to a greater or lesser extent, to increase in its fractures susceptibility.

AAS analysis of calcium and phosphorus content in hind limb bones revealed the significant effect of high fructose diet on mineral deposition (figure 2 and 3). In both, femur and tibia, bone calcium levels were significantly decreased ($p=0,008$ and $p=0,020$, respectively). Phosphorus deposition was significantly reduced in femur ($p=0,030$), while content of this mineral in tibia remained unchanged.

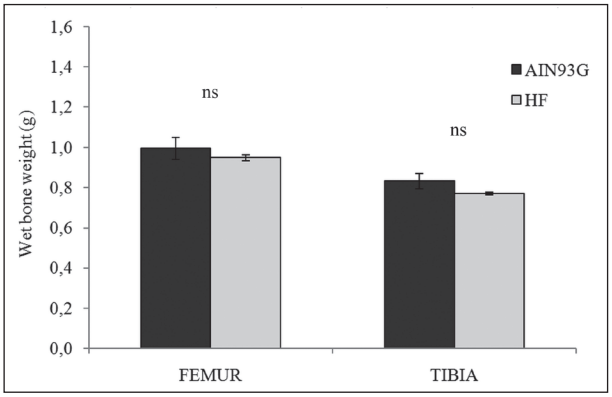

Figure 1. Wet bone weights of rat fed AIN 93G and High Fructose (HF) diets. Each columns represent mean ± SEM (n=10), ns: not significant (p>0,05)

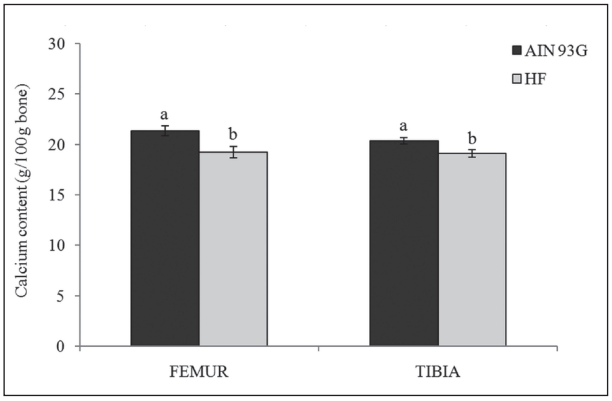

Figure 2. Femur and tibia calcium content of rats fed AIN 93G and High Fructose (HF) diets. Each columns represent mean ± SEM (n=10). The means with different letter were significantly different (p<0,05), ns: not significant (p>0,05).

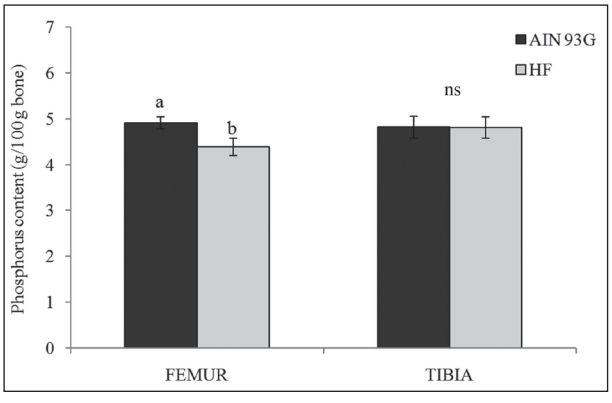

Figure 3. Femur and tibia phosphorus content of rats fed AIN 93G and High Fructose (HF) diets. Each columns represent mean ± SEM (n=10). The means with different letter were significantly different (p<0,05), ns: not significant (p>0,05).

Similarly findings were observed in Tjaderhane and Larmas (14) study. Authors found lower calcium levels in both tibia and femur bone in rats fed high sucrose diet. Also phosphorus tibia content was lower in these animals. Studies on growing rats shown that chronic excessive fructose intake reduce intestinal Ca^{2+} transport. This action was an effect of disruption in the transformation of calcidiol ($25(OH)D_3$) to active form calcitriol ($1,25\text{-}(OH)_2D_3$), which influence lower Ca^{2+} absorption. Impairment of vitamin D metabolism was accompanied by changes in expression of CYP27B1 gene encoding enzymes hydoxylating $25(OH)D_3$ in kidneys. However, authors did not found any differences in total Ca^{2+} weight and percentage content of Ca^{2+} in humerus ash. Only total weight of phosphorus was decreased in fructose group compared to glucose (15). In turn, Bergstra et al. (16) disclose higher calcium concentration in kidneys, in animals fed the fructose diet compared to the glucose group. In both diets sugars provided 77% of total energy. Authors suggested that fructose increased intestinal calcium absorption which is in contradiction with Dourad's findings. There were also few studies on humans examining effects of different sugar consumption on mineral metabolism. However, the results were inconsistent. Some of studies shown that high fructose diet positively affects calcium and phosphorus balance (17). Other researchers revealed negative impact of fructose on calcium and phosphorus metabolism (18, 19).

These contradictory results may be due to different treatment duration. Douard et al. (10) demonstrated that serum levels of $1,25\text{-}(OH)_2D_3$ where not reduced in fructose fed rats in first and second month of experiment. A serum level of this metabolite was decreased only after three months of feeding. Moreover, biochemical analysis did not reveal any changes in Ca^{2+} and phosphorus concentration. Nevertheless, in present study bone calcium and phosphorus levels where decreased after only four weeks of feeding with high fructose diet. Therefore, lower bone mineralization, observed in present study, has other basis than disturbance in vitamin D-related calcium absorption.

Most authors considers impaired bone metabolism as a result of metabolic syndrome induced by excess fructose intake. In our rats metabolic syndrome did not develop but Periodic Acid-Schiff (PAS) staining shown excessive glycogen storage in liver (20). Human patients with glycogen storage diseases often are at high risk of osteoporosis. Glycogen storage diseases are group of genetic disorders resulting in excessive glycogen accumulation in hepatocytes leading to many metabolic disorders. Higher risk of osteoporosis in this group may have few causes. Firstly, management of this disease requires dietary restriction of lactose and galactose, found in dairy products, which are one of the main sources of calcium and cholecalciferol. Vitamin D deficiency is frequent in patients with glycogen storage disease (21, 22). However, in present study dietary restriction could not be cause of reduced bone mineralization as calcium and vitamin D levels where equal in both experimental groups and met rodent's requirements. Secondly in some types of glycogen storage disease appear neutrophils and monocytes deficiencies, some patients develop Crohn disease. In such abnormal conditions, absorption of minerals and vitamins may be impaired, which in turn, leads to reduction in pool of calcium and phosphorus required for bone mineralization (23). Other possible link between excess glycogen storage and low bone mineral density may be lactic acidosis. One of the defence mechanism in case of low system pH is mobilization of calcium from bone tissue. Confirmation of this may be fact that in patients with glycogen storage disease type 1 appears hypercalcaemia as a result of prolonged lactic acidosis (24).

The biomechanical strength of femur and tibia bone was insignificantly lower in animals fed the high fructose diet compared to rats in the control group (figure 4). Average force required to femur and tibia frac-

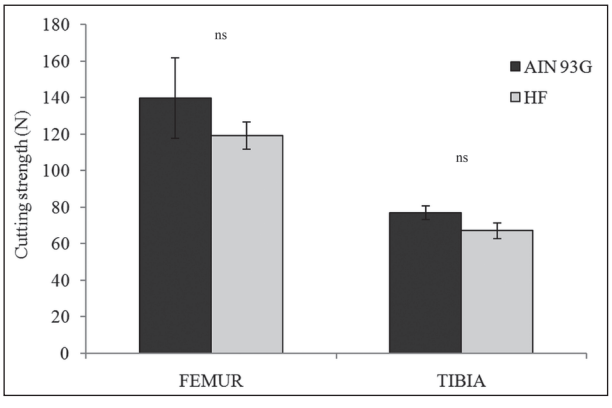

Figure 4. Average strength required to femur and tibia fracture in rats fed AIN 93G and High Fructose (HF) diets. Each columns represent mean ± SEM (n=10), ns: not significant (p>0,05)

ture in the high fructose rats was reduced by 15% and 13%, respectively.

The biomechanical strength of bone depends on their material and geometric proprieties (25). Therefore the lower resistance to fracture is, at least in part, result of reduced bone mineralization.

Studies on rats shown that fructose-induced metabolic syndrome contribute to negative changes in bone microarchitecture and decreased bone repair. Feeding animals with fructose rich diet decreased osteogenic potential of marrow stromal cells and reduced expression of runt-related transcription factor 2 (RUNX2), which is essential for osteoblasts differentiation. Additionally rats fed fructose rich diet had increased peroxisome proliferator-activated receptors γ (PPARγ) expression (26). PPARγ regulates not only lipid and carbohydrates metabolism but also posses antiosteoblastic activity. Activation of PPARγ is associated with bone loss and increased fracture risk (27). Impact of high fructose diet on PPARγ upregulation was reported by Prakash et al. (28). Felice's et al. (26) studies are involving impaired bone metabolism with metabolic syndrome induced by fructose rich diet. Under our study's conditions, in rats fed high fructose diet, metabolic syndrome did not appear because of short treatment duration (20). As so, lower bone strength seems to be attributed to reduced bone mineralization.

Studies conducted by Bass et al. (29) shown that feeding rats with high fructose diet resulted in stronger bones and enhanced their microarchitecture in comparison to animals fed high glucose diet. However, authors did not use control diet (properly balanced) in this study. Therefore these results cannot indicate positive effect of high fructose intake on bone strength. Similar findings came from Tsanzi's et al. (30) research. Their results also show that glucose is more harmful for bone mineralization than fructose. However, despite this adverse effect authors did not notice changes in bone strength.

Conclusion

In conclusion, in the present study we demonstrated adverse effect of short-term high fructose consumption on bone mineralization in growing rats. Four weeks of feeding with high fructose diet were sufficient to reduce calcium and phosphorus depositions in bones, despite the fact that in these animals metabolic syndrome has not developed. Reduced bone mineralization resulted in mild, though insignificant reduction in biomechanical strength of bones. Therefore our results indicate that high fructose diet can negatively affect bone metabolism not only by inducing metabolic syndrome. This issue should be in the great concern as an overconsumption of fructose-rich products often occurs in children's and adolescents group, which may be reflected in skeletal system diseases in adulthood.

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