Original Article

Harmful effects of chronic consumption of soft drinks on the bone metabolism of laboratory female rats

Suaad Mohammad Joda AL-Hadrawy[1], Shaimaa Mahdi A. Jawad[2]

[1.] Department of Biology, Faculty of Sciences, University of kufa, Iraq.
[2.] Department of Biology, Faculty of Education for Girls, University of Kufa, Iraq.
Corresponding Email: suaadm.alhadrawi@uokufa.edu.iq

Abstract

Consumption of soft drinks can result in depletion and deficiency of calcium, and thus increase the risk of osteoporosis and exposure to fracture. The present study was performed to inspect the consequences of long term usage of soft drink on metabolism and histopathology of bone. Twenty-four female albino white rats (Rattus norvigicus) were randomly divided into six groups each containing four animals: two control groups (1 & 2) were fed with regular pellet; two coca-cola groups (3 & 4) were fed with standard pellet diet and given coca cola (2 ml) once a day and two Seven up (7up) groups (5 & 6) were fed with standard pellet diet and given 7up (2 ml) once a day. The treatment continued for two different timelines i.e. groups 1, 3 and 5 were treated for two weeks, while groups 2, 4 and 6 were treated for four months. Cardiac puncture technique was used for taken blood samples for measurement of calcium, inorganic phosphate, magnesium, Vitamin D3 and bone forming biomarkers including alkaline phosphate and osteocalcin. In addition, level of bone resorption biomarker bone sialoprotein in serum was also measured. Calcium and inorganic phosphate, alkaline phosphate, osteocalcin and bone sialoprotein considerably elevated ($p \leq 0.05$) in rats which were given soft drinks daily for 4 months. Magnesium and Vitamin D3 significantly decreased ($p \leq 0.05$) in rats treated with coca cola for 4 months compared with the control and the other groups. Histopathological study revealed changes in the bone of groups fed with coca cola and 7up for 4 months. It showed many empty lacunae, osteocytes inside indistinct lacunae and numerous resorption cavities of inconstant size in the trabecular plates. In conclusion, chronic consumption of soft drinks has harmful effects on bone health and a significant rise in the concentrations of bone remodeling markers demonstrated a rise in bone -turnover -rate and increased risk of osteoporosis.

Keywords: Soft drinks, Calcium, Magnesium, Osteocalcin, Alkaline phosphate, Bone sialoprotein, Bone histopathology.

Introduction

Soft drinks comprise juice drinks, carbonated drinks, fruit juices, dilutables, energy and sports drinks and bottled waters.[1] The consumption of soft drinks has augmented in the previous two decades. Their side effects on human health are not clear, however, many epidemiological studies have indicated their relationship to osteoporosis, liver, kidney diseases and obesity.[2,3] Soft drinks mostly comprise phosphoric acid, water, sugar in the form of fructose or sucrose, caffeine and additional chemicals for coloring, flavor and preservation.[1,2] These components have adverse effects on health[3] for example; caffeine makes people addictive to soft drinks and it is absorbed quickly as compared to other drinks.[3,4] Many different health complications are connected with consistent intake of Soft drinks.[2] Some studies have indicated the harmful effects of soft drinks on dental health and general health of adolescents and children.[1,5] High content of acids and sugar, which have acidogenic and cariogenic potential, can be the main cause of tooth erosion, obesity and type 2 diabetes. Many studies have indicated that consuming soft drinks causes disruption

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Address for Correspondence: Department of Biology, Faculty of Sciences, University of kufa, Iraq
Email: suaadm.alhadrawi@uokufa.edu.iq

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in bone formation, bone fracture and also disturbs urinary or serum calcium metabolism biomarkers causing hypocalcemia.[6] Moreover, extreme intake of carbonated soft drinks can decrease the consumption of healthy beverages like milk causing deficiency of trace elements such as magnesium and calcium in the body. This deficiency can elevate the danger of exposure to osteoporosis and bone fracture.[7,8] Chronic consumption of all types of soft drinks may cause hip fracture in postmenopausal women.[9] Long-term intake of coca-cola is powerfully related with the progress of osteoporosis demonstrated through the alterations in the expected bone biomarker parameters.[10]

Because female bones are more sensitive or affected by treatments with compounds which affect bone metabolism, hence, the aim of this study is to evaluate the long-term effects of two types of common soft drinks (7-up and coca-cola) on bone metabolism of female albino rats.

Materials and Methods

Study subjects

This study was performed on 24 adult female albino rats (Rattus norvigicus) weighing 180±10g. Rats were obtained from animal house in the Faculty of Science, University of Kufa. The animals were housed in stainless steel cages present in an animal house which was air conditioned at 22- 28 °C. Rats were fed with adequate standard diet (pellets) and water adlibitum and kept under normal laboratory conditions throughout the experimental work. None of the rats had any clinically evident infections. This study was approved by the guidelines of the institutional animal- care and use committee, University of Kufa.

Dose of soft drinks and their rout of administration

The soft drinks used in the present study were "coca-cola" and "7up", product of Al Waha Company for Soft Drinks, Babylon, Iraq. They were purchased from a local supermarket in Al najaf, Iraq. The animals were orally given 2 ml of each soft drink every day for two weeks and 4 months.

Composition of standard pellet diet

The standard pellet diet of experimental rats was prepared from 15% protein, 5% fat and 50% carbohydrate and it was fortified with minerals and vitamins to meet the nutritional requirements of animals.

Experimental design and blood collection

Animals were randomly divided into six groups each having five rats i.e. the control groups 1 and 2 were fed with regular pellet, the coca-cola groups 3 and 4 and 7up groups 5 and 6 were fed with standard pellet diet and 2 ml of coca-cola and 7up respectively through oral gavage daily in the morning. All groups were fed with their respective diet for two weeks and four months. At the end of experiment, each animal was anaesthetized by a mixture of ketamine 0.1 ml and xylazine 0.2 ml and then they were sacrificed. The animals were attached to a container of cork pin and then blood was drawn directly from the heart through the heart puncture technique to get sufficient amount of blood (5 ml). Blood sample was placed in a test tube lacking anticoagulant and left for 30 minutes at room temperature followed by centrifuging it at 6000 rpm for 5 minutes to get serum for the biochemical analysis.

Animal dissection and histological study

The right femur bone was detached and cleaned from the tissues surrounding it. It was then put in 10% neutral buffered formaldehyde (pH 7.4) for two days for fixation.[11] The process of decalcification of bone sample was performed by using 10% buffered ethylenediaminetetraacetic acid (pH 7.2-7.4) for two weeks. The decalcifying solution was changed daily. By using standard methods, the decalcified bone sample was stained by hematoxylin and eosin (H&E) for histological examination.[11]

Laboratory biochemical analysis

Calcium and magnesium concentration in the serum were assayed spectrophotometrically through Modified Arsenazo III method according to procedure provided by the TBA-120FR (AGAPPE DIAGNOSTICS LTD), Dist. Ernakulam, Kerala, India-683 562. Calcium is important ion in technique which is based on the reaction of Ca^{+2} with O- cresolphthalein complex in alkaline solution to form complex violet color which shows at 578nm by using spectrophotometer (EMCLAB Germany). On the other hand, magnesium reacts with xylidyl blue to form a complex in alkaline solution, the absorbance of which is proportional to magnesium in sample.

Inorganic phosphorus in the serum was identified via U.V. method according to procedure provided by the BIOLABO, France. This identification depends on the reaction between ammonium molybdate and sulfuric acid to form phospho molybdic complex.

25-OH vitamin D3 was determined by VIDAS which is an automated multiparametric immunoassay system. This uniquely designed testing device stores the calibration in the analyzer s memory.

Serum alkaline phosphatase was measured by enzymatic colorimetric method which was performed according to the procedure provided by Linear Chemicals, Spain.

Osteocalcin levels and the concentration of Bone Sialoprotein 2 in the serum were identified by using

the enzyme-linked immunosorbent assay (ELISA) method according to the procedure provided by the Elabscience Biotechnology, China, Catalog No: E- EL-H 134396 T and H 059296 T respectively.

Statistical analysis

The data was analyzed by One-way ANOVA (Analysis of Variance) through the use of the SPSS statistics software package (version 25 software). In addition, to conclude a statistically-significant difference, Post-Hoc Tukey Test was used to compare the experimental groups. The findings with $p \leq 0.05$ were considered statistically significant.

Results

Influence of soft drinks on minerals and Vitamin D3

The intake of coca-cola and 7up for four months prompted significant rise ($P < 0.05$) in the serum calcium and inorganic phosphorus. In addition, there was a significant decrease ($P < 0.05$) in serum magnesium and vitamin D_3 in female rats which were given cold drinks for two weeks. Results are shown in table 1.

Influence of soft drinks on biochemical marker for bone remodeling

Figure 1 shows an increase in bone forming biomarkers alkaline phosphate and osteocalcin in the groups of female rats treated with coca-cola and 7up for 4 months and 2 weeks. When compared with other groups also, the data indicated that there was a significant increase in bone resorption biomarker (bone sialoprotein) in the experimental groups treated with cold drinks for 4 months.

Table 1: Serum calcium, inorganic phosphate, magnesiun and vitamin D3 in groups 1 to 6.

Parameters Study Group		Calcium (mg/dl)	Inorganic phosphate(mg/dl)	Magnesium (mg/dl)	$VitD_3$ (ng/ml)
	Control (1) (N= 4)	9.550 ± 0.30	3.400 ± 0.30	2.730 ± 0.36	50.940 ± 3.27
2 weeks	**Coca cola (2) (N=4)**	10.440 ± 0.28	4.040 ± 0.62	2.690 ± 0.21	43.640 ±2.95
	Seven up (3) (N=5)	9.940 ± 0.13	3.920 ± 0.53	2.260 ± 0.32	49.660 ± 2.57
	Control (4) (N=4)	9.260 ±. 0.36	3.320 ± 0 26	2.580 ± 0.30	54.140 ± 4.17
4 Months	**Coca cola (5) (N=4)**	13.320 ± 0.28*	5.260 ± 0.25*	1.358 ± 0.28*	31.980 ± 2.12*
	Seven up (6) (N=4)	12.700 ± 0.63*	4.960 ± 0.13	1.320 ± 0.24*	33.840 ±1.54*
	Significant	0.003	0.007	0.000	0.000

P < 0.05; the findings are exposed as mean ± SE (n=4)

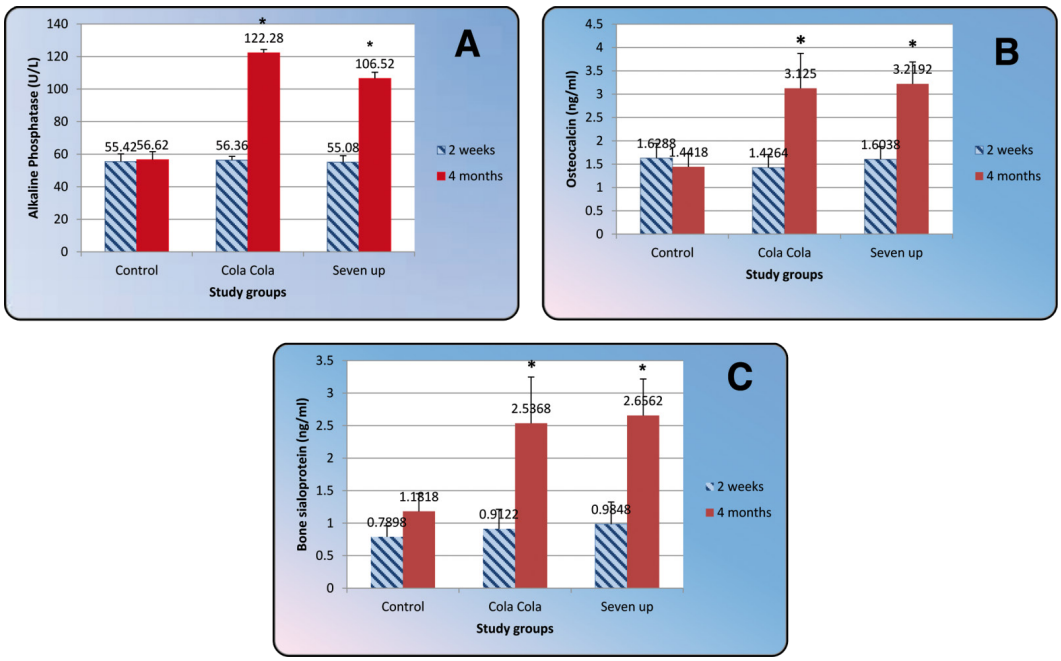

Figure 1. Alkaline phosphate (A), Osteocalcin (B) and Bone sialoprotein (C) levels in serum of control and experimental female rats. *P < 0.05; the findings are exposed as mean ± SE (n=4)

Influence of soft drinks on bone histopathology

Both control groups (Fig. 2 A, D) and experimental groups of female rats which were fed for two weeks (Fig. 2 B, C), revealed a normal histological structure of bone with a normal external surface covered with fibrous periosteum and a normal thin cellular endosteum layer. Also, the bone matrix exposed osteocytes inside their lacunae, normal blood vessels and haversian canal. While the groups of female rats which were fed for a period of 4 months (Fig. 2 E, F) showed that the bone matrix contained osteocytes which were irregularly oriented inside unclear lacunae. Moreover, many resorption cavities of inconstant size were also observed.

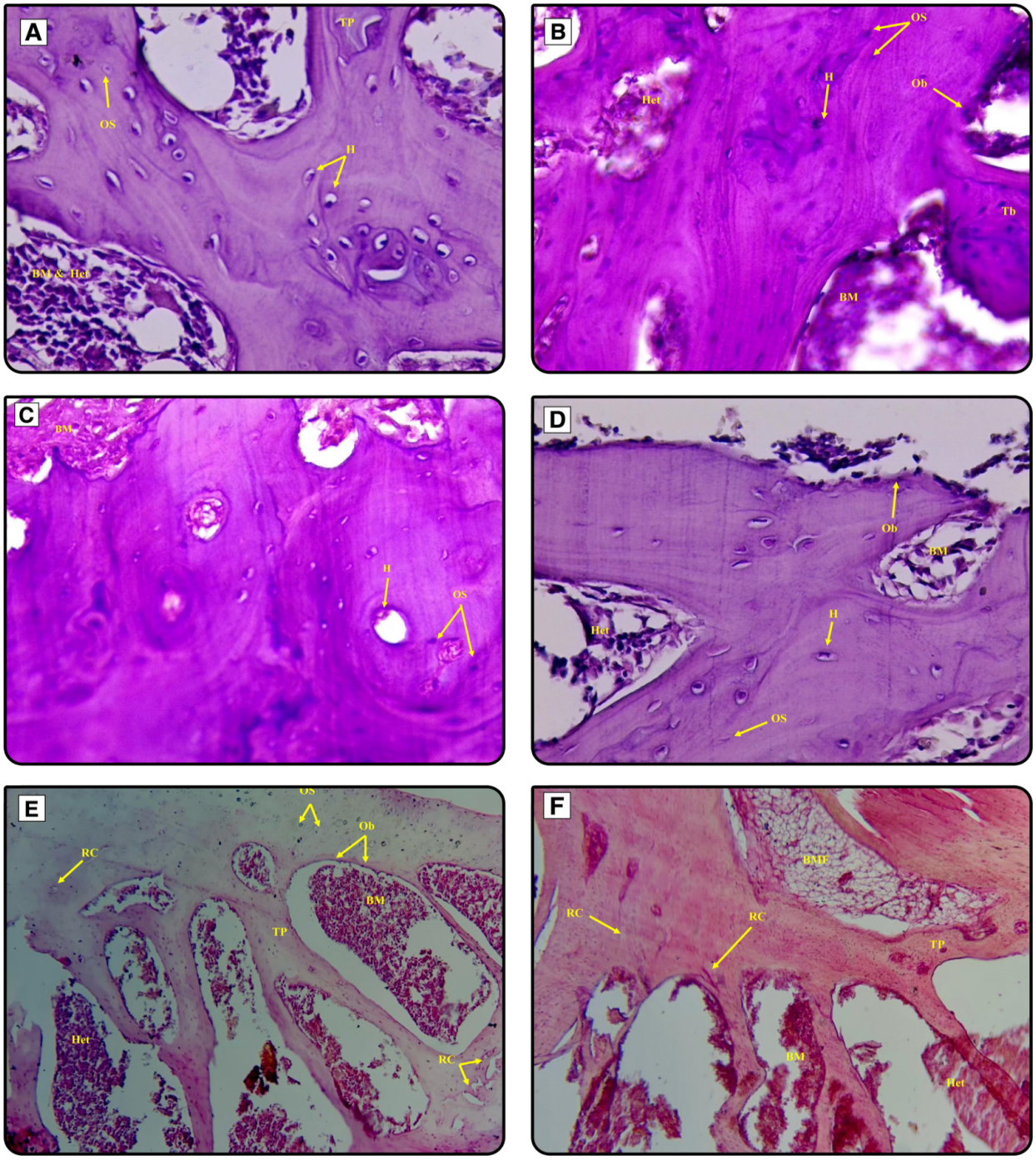

Figure 2: Photomicrograph of the epiphysis of femur bone show: the normal histological structure of (H) Haversian canal, (Os) osteocytes, (BM) bone marrow, (Ob) osteoblast in control groups (A and D) and in groups of females rats treated with coca-cola (2 ml) (B) and 7up (2 ml) (C) for 2 weeks. The bone in females rats treated withcoca-cola (2 ml) (E) and 7up (2 ml) (F) for 4 months show the presence of a resorption cavities (RC) in the trabecular plates (TP) (Specimens stained with H and E, magnification ×40, ×10).

Discussion

In the current study, the results revealed that the concentration of bone minerals in blood including calcium and inorganic phosphate was significantly elevated in female rats which were given coca-cola and 7up for 4 months. Conversely, the levels of vitamin D were significantly decreased in female rats which given these cold drinks for 4 months. Most of the studies on the association between soft drink consumption and bone health were conducted on laboratory animals and on children. In a study on animals, the results showed a decrease in bone mineral density (BMD) and an increase in calcium in urine (hypercalciuria) when they were given glucose-sweetened beverage and coca-cola.[12] Moreover, in adolescents, increased soda intake has been found associated with decreased BMD, though the relationship was extra dependable in girls than boys.[9,13]

Many mechanisms have been proposed to explain the possible association between soft drink intake and bone health. First, diet high in phosphorous and low in calcium such as soft drinks, causes a deficiency of calcium in the serum, stimulates the parathyroid hormone, thus causing bone resorption.[14] In addition, increased consumption of phosphorus stimulates the secretion of fibroblast growth factor-23 from the bone, which has been observed to decrease renal stimulation of 25-hydroxyvitamin D.[15] Second, caffeine is a component of many soft drinks and is considered a risk factor for osteoporosis by interfering with calcium absorption and excretion.[4] Third, sugars are a foremost component in soft drinks and have been recognized as an essential factor in the bone density and macromineral homeostasis in men[16], middle-aged women[17] and children.[18]

Regarding the effect of caffeine in soft drinks, it is one of the compounds whose daily consumption is equivalent to 120 mg.[19] This ingredient is found in many foods (chocolate and cocoa) and beverages (soft drinks, energy drinks, tea and coffee).[20] When caffeine reaches its target organs, its biological action occurs through several mechanisms including the adenosine receptor antagonism.[21] Also, caffeine interacts with other receptors, such us vitamin D receptors[22] and the epidermal growth factor receptor (EGFR).[23] In addition, caffeine elevates intracellular cyclic adenosine monophosphate concentration (cAMP).[24] Furthermore, in many body tissues, intake of caffeine elevates mRNA expression of mitogen inducible gene-6 (Mig-6)[23] and declines the mRNA expression of TGF-ß.[25] Additionally, this alkaloid molecule not only increases the excretion of Ca^{2+} and B vitamins but also negatively affects iron absorption. Many studies have also proven that in adults, elevated and chronic consumption of caffeine can cause many health problems like hypercalciuria , osteoporosis, hip fracture, nervousness, increased diuresis, insomnia and gastrointestinal disturbances.[22] Besides, numerous in vivo and in vitro studies have reported excessive caffeine consumption to be detrimental to the musculo-skeletal-system, comprising the hyaline cartilage.[26]

High content of phosphoric acid in soft drinks has been suggested as one of the linking mechanisms between these drinks and exposure to fracture. Extreme consumption of phosphoric acid upsets the balance of calcium/ phosphoric acid ratio and of acid/bases in the body. This disturbance causes a decrease in bone density, osteoporosis and bone fractures.[27,28]

A large prospective cohort study confirmed the association of extreme intake of soft drinks with an elevated danger of bone fracture. This study also showed that daily intake of soft drinks was related with an increased risk of bone fracture independent of lifestyle and sociodemographic features.[8] Some studies have also reported increased risk of hip fracture in postmenopausal women with increased consumption of soft drinks.[9,29]

Numerous studies have revealed that high intake of soft drinks causes overweight and obesity.[30] Lipids are involved in the metabolism of bone active hormones and can disturb the regulation of the bone metabolism.[31] Augmented lipid content in muscular tissue leads to additional reductions, which can raise the risk of bone fracture in different parts.[32]

The present study demonstrated a significant decrease in magnesium concentration among groups of female rats which have been given coca-cola and 7up for four months. Hypomagnesaemia i.e. magnesium deficiency is related with numerous diseases including osteoporosis, coronary heart disease, several neurological disorders and diabetes.[33] This is because magnesium plays an important role in many physiological activities i.e. in potassium and calcium ion transport, energy metabolism, DNA replication and repair, cell signaling and genome stability.[34] Many beverages, such as soft drinks, which comprise high phosphoric acid content, contribute to hypomagnesaemia due to their capability to bind magnesium, therefore, harmfully impacting its availability and absorption.[34,35]

In accordance with other studies, results of the current study also demonstrated a significant increase in the bone forming markers (alkane phosphatase and calcitonin) and bone resorption marker (bone sialoprotein). The increases in alkaline phosphatase is due to the caffeine content in the soft drinks.[36] Caffeine disturbs the biosynthesis process of alkaline phosphatase and prevents the creation of a proficient extracellular matrix which is important for bone remolding process.[3] The disturbance of equilibrium between new bone formation and bone resorption causes bone damage which leads to osteoporosis.[37,38] Consequently, bone formation and

resorption biomarkers rise as a compensatory mechanism to fill the higher number of resorption cavities.[39]

In the present study, the histological examination of female rats treated with coca-cola and 7up for 4 months showed many resorption cavities of inconstant size in bone matrix. Moreover, osteocytes which were irregularly oriented inside unclear lacunae were also noticed. This result might be due to the high concentration of sugar in the soft drinks. Numerous studies have also indicated the effect of sugar on bone health for example; a group of researchers studied the effect of dietary glucose and fructose on the strength, microarchitecture and formation of bone in an animal model.[40] Their results depicted that the area of the trabecular distal femur bone had lower bone surface and higher bone volume fraction in rats fed with fructose diet as compared to those fed with glucose diet. These results indicated that consumption high fructose diet by the experimental rats for 12 weeks had a greater bone mass with a greater microarchitecture as compared to those fed with elevated glucose diet.[40] Another study depicted the adverse consequence of elevated fructose diet on the structure of the bone skeleton of 8-week old laboratory rats.[41] In a different study, a group of scientists evaluated the effects of fructose rich diet on the bone tissue regeneration and long bone histomorphometry.[42] They observed 20% reduction in the osteocytic density of the femoral trabecular bone and a 30% decrease in osteoclast covered (TRAP-positive) bone surface in the animal group fed with fructose rich diet. Another study also assessed the influence of fructose rich diet prompted metabolic syndrome on –redevelopment of bone tissue and proved that fructose concurrently reduced reossification, significantly decreased osteocytic density and osteoclastic action in the lesion site, which inferred a reduction in bone turnover and remodeling.[42]

A study reported the effect of two types of soft drinks on the histological structure of the bones of lab animals. Its results showed that soft drinks prompted histological alterations in the trabecular and cortical bones and exposed many empty lacunae.[43] These negative effects might be due to the presence of phosphoric acid and caffeine in soft drinks. Many studies have found that caffeine prompted important histological alterations in the bones of experimental rats, such as reduced structural remodeling and osteocytes. Another study reported that consumption of caffeine (>300 mg/d) caused a greater rate of bone injury in postmenopausal women.[4]

Conclusion

Excessive use of soft drinks caused clear changes in bone minerals and vitamin D_3 in the serum. In addition, significant increase in alkaline phosphatase, osteocalcin and bone sialoprotien depicted the effect of cold drinks on bone formation and resorption. This study also showed the harmful effects of soft drinks on histological structure of the bone. Therefore, avoiding excessive consumption of soft drinks, especially by women and children is highly recommended.

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