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EFFECT OF QUERCETIN ON ALVEOLAR BONE STRUCTURE OF RATS SUBJECTED TO COCA-COLA CONSUMPTION: HISTOLOGICAL AND ULTRASTRUCTURAL STUDY

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ABSTRACT

Introduction: Increasing consumption of sweetened carbonated beverages as Coca-Cola has increased dramatically lately. Many detrimental effects have been proven concerning several medical conditions such as obesity, diabetes, inflammation and osteoporosis. Quercetin, is a potent anti-oxidant that is capable of protecting variable organs such as bone against oxidative stress.

Aim: This study aimed at evaluating the effect of Coca-Cola consumption on the structure of the alveolar bone in adult rats as well as the possible protective effect of Quercetin.

Materials and Methods: 30 rats were randomly distributed into three equal groups. Control group I, Coca-Cola group II and Coca-Cola + Quercetin group III. The rats in groups II and III received 2 ml Coca-Cola for three consecutive months. Group (III) received 100mg/kg Quercetin throughout the experimental period. After the experimental period, rats were euthanized and the specimens were dissected and processed for histological, ultrastructural evaluation using SEM, and EDX.

Results: The light microscopic results of Coca-Cola group, revealed irregular bone surface with multiple Hawship's lacunae some of which contain osteoclasts. This was accompanied with widening in PDL thickness and thinning of the bone trabecuae. However, group III, revealed smoother alveolar bone surface accompanied by normal thickness of bone trabeculae with parallel resting lines. These results were confirmed by the SEM and EDX results.

Conclusions: Quercetin has a potential protective effect on Coca-Cola-induced hazards on alveolar bone by relative restoration of the normal bone architecture.

KEYWORDS: QE, Coca-Cola, Alveolar bone.

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INTRODUCTION

The consumption of Soft drinks, such as Coca-Cola, 7-Up, Pepsi and others, has been widely elevated all over the world in the past decades. Epidemiological studies have suggested its associations with many health problems including osteoporotic changes. Many diseases, including dental/bone problems, diabetes mellitus and cardiovascular disease have also been investigated [1].

Experimental studies revealed a correlation between Coca-Cola consumption and disruption in bone metabolism such as reduction in bone mineral content, increased risk of bone fracture and osteoporotic changes at variable ages groups[2]. It was found that Coca-Cola consumption may lead to not only disruption in bone formation and fracture of bone, but also leads to decrease in serum calcium level, in the previous studies. SD are mainly formed of water, sugar, caffeine, phosphoric acid, preservatives, colorings agents and flavors. These contents have hazardous effects on human being wellness. Caffeine in soft drinks is mainly introduced to cause addiction to humans, as it is rapidly absorbed in comparison to other ingredients [1].

A huge content of phosphoric acid is introduced for water sterility as the bacteria is not able to survive in acidic environment. Moreover it is responsible for demineralization of human teeth in 2 days. In addition, different studies reported that these beverages are capable to dissolve nails and limestone [3]. It is well known that the acidic medium may have a bad effect on calcium metabolism as well as accelerating bone resorption. Thus, another possible mechanism effecting bone mineral content is the acid characteristics of Coca-Cola beverages, which might have a negative drawback on calcium level and disrupt bone health by stimulating bone degradation, calcium mobilization and decrease in 25-hydroxy-vitamin-D renal production [2].

Therefore, increased phosphoric acid and caffeine content in Coca-Colas are responsible for the increase in the acidity in the body, which in consequently affect the Ca/P ratio and bone mineral content. Moreover, soft drinks rich in caffeine, phosphoric acid, and fructose leads to interference with Ca absorption contributing to Ca imbalance leading to more Ca loss [4]. Prolonged intake of caffeinated and decaffeinated Coca-Cola drinks leads to bone resorption in humans with hazardous effects on the protein intake. Heavy Coca-Cola consumption in animals and human beings may lead to some metabolic disorders including secondary hyperparathyroidism, hypocalcemia and decrease in 1a, 25-dihydroxyvitamin-D level as well as delay in alveolar bone healing in rats [5].

Interestingly, oxidative stress plays a vital role in the etiology, pathogenesis of various chronic diseases. Accumulation of reactive oxygen species (ROS) may lead to destruction of the cell's lipids inducing peroxidation which in turn harms the bone. It worth mentioning that soft drinks consumption is capable of inducing not only metabolic changes, but also oxidative stress [6]. It was found that different bones respond in a specific manner when exposed to different conditions, such as Coca-Cola consumption. However, few studies have been conducted on evaluate the hazardous effects of Coca-Cola on alveolar bone, a bone region of major interest in dentistry [2].

The discovery of natural compounds that target the immune responses of the host offers a promising improvement in the clinical outcomes. Quercetin (QE), is a plant-extracted polyphenol, that is highly safe with numerous benefits such as effective anti-oxidant and anti-inflammatory effects. QE showed to be effective in treating several diseases including rheumatoid arthritis and neuroinflammation [7].

Quercetin is a one of the phytochemicals that belong to the flavonoids, thus it has an anti-oxidant

effect and the capability to compromise ROS in the body. QE is found in apples. It was reported that QE has several positive effects including improving insulin-stimulated glucose uptake, as well as acting as a chelating agent [8].

Moreover, it is considered the most powerful scavenger for ROS among all the flavonoids. Its anti-oxidant properties may be due to its metal chelation abilities, scavenging of radicals, enzyme inhibition. In addition, QE is suggested to act as an internal antioxidant shield due to its antioxidant capacity [9]. Previous investigations approved the beneficial effect of QE on bone by affecting bone quality through improving bone mineral density (BMD), trabecular and cortical bone microstructure, bone strength and bone histomorphometric parameters in animals with osteopenia, osteolysis and bone defects.

The Osteoblasts proliferation and differentiation, is namely regulated by certain transcription factors as osterix, Runx-2, BMP-2 and bone markers (alkaline phosphatase (ALP) and bone sialoprotein. It was also found that QE is capable of increasing the activity of ALP and expression of Runx-2 significantly. Moreover it has been documented that QE stimulated mineralization, Runx-2, BMP-2 and osteocalcin in rat osteoblasts and bone marrow cells [10].

Osteoclastogenesis is a multistep process that is mediated by osteoblasts and osteocytes. These cells are responsible for the RANK/RANKL/OPG system. RANKL is responsible for RANK activation which, subsequently leads to osteoclast differentiation and maturation. On the contrary, OPG, inhibits osteoclast differentiation by blocking the RANK–RANKL interaction. QE influences the OPG and RANKL expression where the OPG level was increased and the RANKL level was decreased), resulting in an increase in OPG/RANKL ratio in the presence of QE [11]. That's why QE was used to this study to counter act the effect of cola drinking on alveolar bone.

MATERIALS AND METHODS

Animal housing

This study was performed in agreement with the ethical guidelines of research on experimental animals at Faculty of Dentistry, Alexandria University (IRB No:00010556-IORG 0008839). 30 adult male albino with weight range 200-250 g with age 3-7 months were used in the current study. The animals were kept at well ventilated clean cages with constant controlled climate (at Institute of Medical Research, Alexandria University, Egypt). All rats were supplied with regular diet with free consumption of food and water throughout the whole experimental period which lasted for 3 consecutive months [1].

Sample size calculations

Sample size was calculated using Power Analysis and Sample Size Software (PASS 2020) "NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/pass". A minimal total hypothesized sample size of 30 rats (10 per group) is needed to evaluate the effect of Coca-Cola consumption on the structure of the alveolar bone in adult rats as well as the possible protective effect of QE; taking into consideration 95% confidence level and 80% power using Chi Square-test [12, 13].

Materials

1. Coca-cola

Supplied from supermarket

2. Quercetin

Supplied from Sigma-Aldrich Company in the form of powder that was dissolved in distilled water and administered by orally.

Random allocation

Rats were divided randomly by computer-assisted software into 3 equal groups. The random allocation was performed using computer-formed

random sequence of numbers to assign treatment status to reduce the possibility of error in which all personnel who performed the tests were unaware of the treatment assignment.

Group I (n=10) Control group: animals of this group received regular diet and water ad libtum throughout the experimental period.

Group II (n=10) Coca cola group: animals of this group were given 2ml cola beverage orally for the 3 months experimental period (1).

Group III (n=10) Coca-cola + Quercetin group: animals of this group received QE by oral gavage with a dose of 100mg/kg/day + 2ml cola beverage throughout the 3 months of the experimental period [14].

Histological examination

After the 3 months duration of the period of the experiment, the animals underwent euthanization. For histopathological examination, sections from the mandibles were immersed in 10% NBF overnight, then decalcified, washed, dehydrated and embedded in paraffin. Serial sections of 5um thickness were performed and stained with Haematoxylin and Eosin stain. Then the histolopathological features of alveolar bone in different groups were examined [15].

Scanning electron microscope examination (SEM)

The specimens, were preserved in gluteraldehyde and prepared for SEM to study the surface characterization of different groups [16].

Energy dispersive X-ray microanalysis (EDX)

The mandibles were washed, dehydrated and air-dried and he specimen surfaces of the 3 groups were subjected to EDX system for Ca and P elemental analysis [17].

Statistical analysis

EDX results were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Normality was tested by the **Shapiro-Wilk test**. All quantitative variables showed normal distribution; thus, means and standard deviation (SD) were calculated **One way ANOVA test** was performed for comparing the three studied groups. [18].

RESULTS

Histological findings

Group I (Control group)

In the control group, light microscopic sections of the alveolar bone revealed normal bone histology. This was illustrated by having a smooth, regular and uniform surface interrupted by Volkman's canals at variable areas. The bone surface was lined by a continuous layer of osteoblast. The resting lines ran parallel to each other and deeply stained indicating continuous bone formation. Also the osteocytes appeared normal in shape. The bone marrow spaces present between the thick bone trabeculae appeared cellular. PDL fiber bundles revealed uniform thickness and was noticed between the alveolar bone proper and root cementum at different locations along the root length (Figure 1A & B).

Group II (Coca-Cola group)

However, the light microscopic results of this group demonstrated bone resorption in the form of a generalized irregular alveolar bone surface. The alveolar bone surface showed resorptive lacunae with or without osteoclasts along with loss of osteoblasts lining. Thin bone trabeculae accompanied with excessive widening of the marrow spaces were also observed. The histological sections of this group also showed some widening in the osteocyte lacunae indicating lacunar resorption. Disorganization and thickening in the PDL fibers were noticed at different locations along the root length especially at the apical region (Figure 1 C & D).

Group III (Coca-Cola + Quercetin group)

The administration of QE to the rats as a preventive measure demonstrated a recognizable

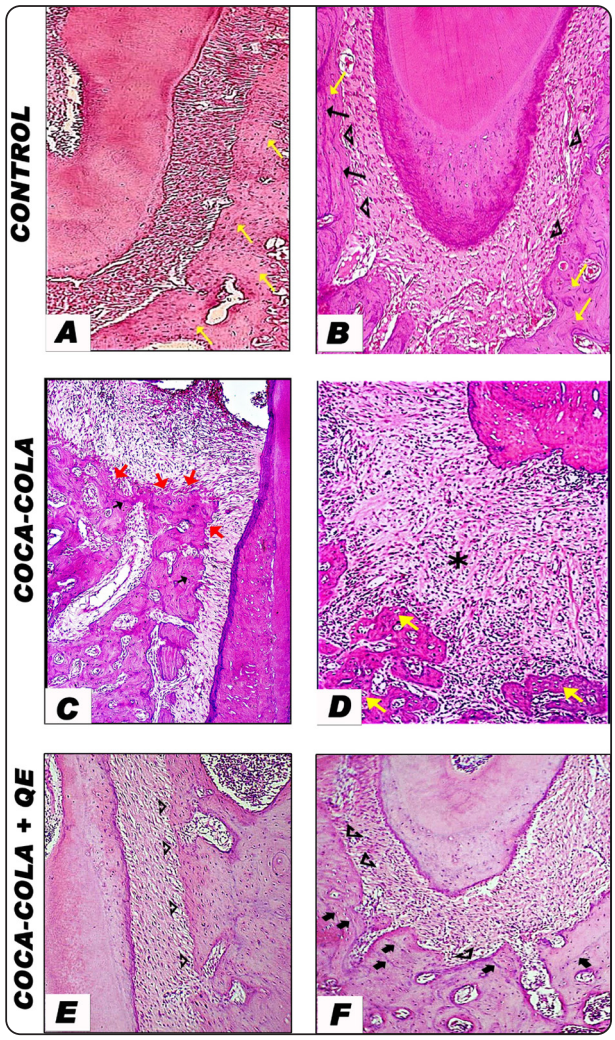

Fig. (1): Light micrograph (LM) (A) (Control group) showing regular alveolar bone surface along the root length with normal thickness of bone trabeculae and marrow spaces. Note regular multiple osteocytes (yellow arrows) (H&E X100). (B): (Control group) showing smooth alveolar bone surface at the apical region with continuous osteoblasts lining (arrow heads). Regular parallel resting lines can be seen (black arrows). Numerous osteocytes in lacunae (yellow arrows) (H&E X 100). (C): (Coca-Cola group) showing irregular alveolar bone surface with multiple resorption lacunae some of which contain osteoclasts (red arrows) at crest and middle region. Note thinning of alveolar bone trabeculae and multiple reversal lines (black arrows). (H&E X100). (D): (Coca-Cola group) at the apical region showing resorbed alveolar bone surface with extensive widening and disorganization in the PDL (black star). Note multiple osteosytes with widened lacunae (yellow arrows) (H&E X 100). (E): (Coca-Cola + QE group) showing relatively regular alveolar bone surface. Regular shaped osteocytes are clear. Relative preservation of the osteoblasts cell lining can be noticed (arrow heads). (H&E X100). (F): (Coca-Cola + QE group) at the apical region showing regular smooth alveolar bone surface lined by osteoblasts (arrow heads) and relative normal PDL thickness. Parallel resting lines are obvious (black arrows). (H&E X 100)

improvement to some extent in the histological structure of the alveolar bone. The alveolar bone preserved its regular smooth surface with a noticeable decrease in the resorptive lacunae and osteoclasts as well, accompanied by relative preservation of the osteoblast lining. Thickened regular bone trabeculae with normal cellular marrow spaces and regular shaped osteocytes were clearly detected. Relative normal thickness and orientation of the PDL could be clearly seen (Figure 1 E & F).

Scanning electron microscope examination (SEM)

Group I (Control group)

Scanning electron microscopic illustrated a generalized smooth bone surface topography with variable nutritive canals sizes showing regular intact borders in the buccal cortical plate of bone (Figure 2A).

Group II (Coca-cola group)

Rough irregular alveolar bone surface revealing multiple resorptive pits and craters in some areas in the mandibular region of the surface topography of this group. Tiny nutritive canals were also seen on the irregular bone surface (Figure 2B).

Group III (Coca-cola + Quercetin group)

Surface alveolar bone topography of this group revealed relatively smooth and homogenous surface with numerous nutritive canals showing well

defined regular borders compared to the previous group (Figure 2C).

Energy dispersive X-ray microanalysis (EDX)

Ca and P levels of the control and study groups were tabulated in table (1). There was a statistical significant decrease in calcium and phosphorus levels in Coca-Cola group (group II) in comparison to control group and Coca-Cola + Quercetin group, where (P1 for calcium= <0.001) and (P1 for phosphorus = <0.001) and (P3 for calcium= <0.001) and (P3 for phosphorus= <0.001). While, in comparison between control group and Coca-cola + Quercetin group, phosphorus level showed no significant difference (P2 for phosphorus= 0.552). Whereas, value of calcium level showed much increase in the Coca-cola + Quercetin group, however it still revealed significant difference with the control group (P2 for calcium =0.001).

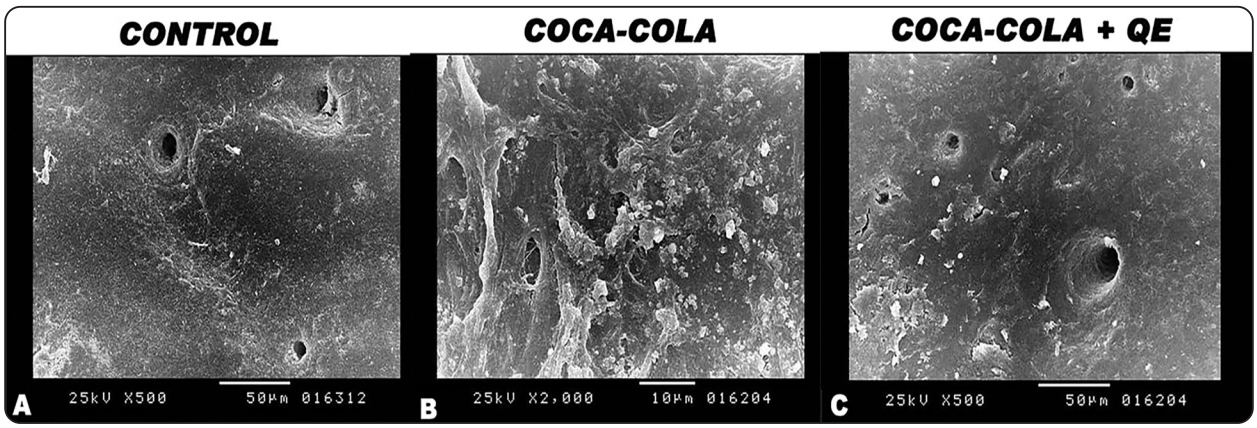

Fig. (2): Scanning electron micrograph (SEM) of buccal cortical plate (A) (Control group) showing regular bone surface with multiple nutritive canals (X500). (B) (Coca-Cola group) showing rough irregular bone surface with multiple areas of shallow depressions and craters. Note in different sizes ill-defined nutritive canals (X2000). (C) (Coca-Cola + QE group) showing relatively regular bone surface with multiple well-defined nutritive canals (X500).

TABLE (1): Comparison between the three studied groups according to Calcium and Phosphorus

	Control (n = 10)	**Cola (n = 10)**	**Quercetin (n = 10)**	**F**	**p**
Calcium (Ca)					
Min. – Max.	28.17 – 31.01	17.02 – 20.77	26.20 – 28.92	266.131*	<0.001*
Mean ± SD.	29.38 ± 1.01	18.42 ± 1.35	27.34 ± 1.0		
Sig. bet. grps	p_1<0.001*, p_2=0.001*, p_3<0.001*				
Phosphorus (P)					
Min. – Max.	13.05 – 17.47	10.09 – 12.42	13.70 –14.75	46.451*	<0.001*
Mean ± SD.	14.54 ± 1.37	10.91 ± 0.75	14.10 ± 0.32		
Sig. bet. grps.	p_1<0.001*, p_2=0.552, p_3<0.001*				

SD: Standard deviation
F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the studied groups ***p1: p value for comparing between Control and Cola***
p2: p value for comparing between Control and Quercetin ***p3: p value for comparing between Cola and Quercetin***
****: Statistically significant at p ≤ 0.05***

DISCUSSION

There has been considerable interest about the overall health effects of sodas and their potential to cause medical illness. The most prominent medical issues that are discussed are the effect of sodas on bone, diabetes, obesity, cardiovascular and neurologic systems [19].

The present study was conducted to examine alveolar bone deterioration due to high long term Coca-Cola consumption and to investigate the expected prophylactic effect of Quercetin. The 3 consecutive months duration of the experiment was done in approval with previous studies that proved that this duration of Coca-Cola intake caused a significant increase in the oxidative stress biomarkers and an imbalance in the oxidant/antioxidant system[1,6]. Pervious investigations used QE for this duration and revealed an efficient antioxidant effect [20].

In the current study, the LM examination of the Coca-Cola group of the alveolar bone surface revealed multiple resorptive lacunae with multiple osteoclasts and discontinuity of the osteoblasts lining. This was also accompanied by decrease in osteocytes and their lacunae within the bone matrix. These results were in accordance with the results of a previous study done on the effect of cola intake on the femur of rats where it demonestrated numerous empty lacunae and resorption cavities with increase in osteoclasts activity. It also revealed few numbers of osteocytes with indistinct lacunae and in irregular orientation [21]. However, in certain regions as in the apical areas of the alveolar bone, as was clearly demonstrated in the results of the current work, osteocytes illustrated irregular appearance with widening in their lacunae indicating lacunar resorption. This was explained by the predominance of lytic acidic lysosomes in mature osteocytes, and the irregular outline of the lacunar wall in calcium-demanding conditions. The osteocytic-osteolysis process has powerful effects on the bone physiology, where it affects the mechanosensation of osteocytes, alter the bone turnover and it also can free calcium from the bone matrix [22].

Although there are few research on the effect of Coca-Cola on bone, theories explaining the hazardous effect of excessive Coca-Cola drinks on bone have been attributed to their constituents. It was suggested that the phosphoric acid forms an acid medium which leads to an increases in the bone resorption. Another theory assumed that the phosphoric acid leads to an increase in the serum phosphate level, slightly lower serum calcium level as well as elevation of serum parathyroid hormone that leads to increased bone resorption [19].

Moreover, Caffeine, as an important ingredient especially in the Coca-Cola soft drink, has a major participation in the negative impact on bone. Caffeine has several hazardous effects that it may lead to bone resorption. The most important one is the pharmacological effect by the non-specific antagonism of adenosine receptors. Adenosine is responsible for regulating bone metabolism, where some in vitro studies suggested that stimulation of adenosine A2A and A2B receptors induces bone formation by not only by suppressing osteoclast differentiation and function but also by activating osteoblasts. Therefore, the blocking of adenosine A2 receptors by caffeine will promote bone resorption and impair bone formation. Caffeine also affects bone through alteration of both calcium metabolism and vitamin D responses as well as other mechanisms. Clinical studies, investigating the impact of caffeine consumption on bone metabolism suggests that there is a potential link between caffeine intake and the reduction in bone mineral density leading to increasing the risk of bone fracture [23]. Both caffeine and phosphoric acid elevates the urinary calcium excretion, and this may be the reason for reduction in BMD.

These histological results were further supported by the SEM results which prevailed rough surface bone topography in the Coca-Cola group with

multiples craters and resorptive pits in comparison to the control and the Coca-Cola + Quercetin groups. Furthermore, the calcium and phosphorus levels documented by the energy dispersive X-ray microanalysis of the Coca-Cola group showed a significant decrease compared to the other two groups. These EDX results are supported by previous study that explained that excessive soft drink consumption have been found to be associated with low BMD and bone fractures. Decrease in bone minerals (calcium and phosphorus) accompanied with an increase in their levels in serum and urine plays a major role in the genesis of Coca-Cola-associated bone loss [(4)]. Moreover, Garcia et al. recorded subsequent hypocalcaemia and loss of femoral BMD in ovaryectomized rats received cola[(24)]. The decreased results of the BMD represented by the low levels of Ca and P in the current work is also in consistent with a previous study that was performed on the femur of Wistar rats subjected to Coca-Cola consumption. This later study revealed that all BMD and concentration of the rats with cola intake were less than those of the control group. This was explained by the presence of high phosphate and caffeine content in these drinks together with the very low pH leading to an increase in the acidc load in the body. Meanwhile the decrease in the intake of milk and milk containing drinks when cola drinks are consumed in large amounts [(25)].

Flavonoids, are present in some vegetables and fruits and they are considered as a semi-essential nutrients for human beings. Quercetin is an abundant flavonol-type flavonoid. However, rare studies have been conducted to investigate the anti-inflammatory relation of Quercetin and bone resorption induced by long term cola intake. Thus, QE has been used in this study to examine its preventive effect on the alveolar bone of rats with high Coca-Cola soft drink consumption. The results showed significant promising effect of the QE in comparison to the Coca-Cola group. A relatively smooth alveolar bone surface with a continuous osteoblast lining and very few resorption lacunae were detected. Moreover, thick bone trabeculae with regular osteocytes in lacunae were clearly seen. It has been speculated lately in a previous study that Quercetin has a stimulating effect on osteoblasts thus increasing bone formation locally [(26)]. Our results are also in accordance with a recent review that was conducted to uncover the effect of QE on bone and its mechanism of action. QE was found to be capable of inhibiting the RANKL mediated osteoclastogenesis, osteoblast apoptosis, inflammatory response and oxidative stress. Meanwhile, it promotes osteogenesis, angiogenesis, antioxidant expression, and osteoclasts apoptosis. The overall action reveals quercetin positive effect on bone. Moreover, QE administered orally by oral gavage for 2 weeks elevated bone mineral density, Ca and P content in the femur. All of which are in consistent with our present study [(27)]. The positive effect of QE on the femur bone of rats with induced osteoporosis has been studied previously. It proved the improvement caused by QE treatment on the cortical bone histology, BMD, bone weight, length as well as calcium and phosphorus levels in the femur. It further revealed the Quercetin ability in suppressing bone resorption by interfering with the differentiation and activation of osteoclasts. Moreover induction of mature osteoclasts apoptosis [(28)].

CONCLUSION

Quercetin has a principal and major role in preventing the harmful damaging effects of Coca-Cola high consumption on alveolar bone. Thus, the hazardous effect of Coca-Cola on alveolar bone and the effective prophylactic effect of QE must be taken in consideration.

Conflict of interest

The authors declare that they have no conflict of interest.

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RECOMMENDATIONS

According to the results of the present study, we recommend the following:

1. More studies should be conducted to detect the effect of different soft drinks on alveolar bone and teeth supporting tissues
2. Further investigations should be administered to examine the efficacy of soft drinks and Quercetin on different time intervals.
3. More studies should be executed to study the detrimental effects of Coca-Cola on enamel and different tissues of the tooth.

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