Effect of carbonated soft drinks consumption on the bone of Wistar albino rat: A histomorphometric study

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Abstract: The negative effects of Coca Cola® and Seven-Up® on the bone were studied in adult Winstar albino rats (n=30). The animals were randomly divided according to the type of their drink into 3 equal groups (n=10; 5 males and 5 females); control group maintained on tap water, cola consumption group maintained on Coca Cola and non-cola consumption group maintained on Seven-Up. In cola consumption group both sexes showed a significantly higher thickness in cortical bone than those of control and non-cola consumption groups respectively. The thickness in females of cola consumption group was significantly higher than the males, whereas in non-cola consumption group the thickness was significantly higher in males. Cola and non-cola consumption groups showed a significantly lower trabecular bone percentages in both sexes than those of control group. The trabecular bone percentage in males of cola and non-cola consumption groups was significantly lower than in females. Therefore, CSDs adversely affected the cortical and trabecular bone, with more effect in males, particularly with Cola consumption. The consumption of these beverages would be a risk factor of osteoporosis and must be reduced as much as possible.
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Keywords: Carbonated soft drinks; CSD; cola versus bone; cortical bone; trabecular bone, albino rat, femur

1. Introduction

The consumption of carbonated soft drinks (CSDs) is a popular worldwide habit. The term soft drink refers to a carbonated beverage, that if no adjective is used, indicates a regular or sugar-sweetened drink (Hector et al., 2009). The negative effects of these drinks on the bone health was reported in many studies in animals and humans (Amato, et al., 1998; Fernando, et al. 1999; García-Contreras, et al., 2000; McGartland, et al., 2003; Tucker et al., 2006; Mahmood et al., 2008).

The sugar, used in sweetening of these drinks, had a strong effect of on the bone turnover (Paldanius, et al. 2012). Intake of high fructose, might be used instead sugar to sweeten these drinks, was found to cause depression of calcium balance, particularly with low dietary magnesium, and greater losses of phosphorus, with an adverse impact on bone health (Milne and Nielsen, 2000). CSDs are acidic and it was reported that acid-forming diet increased urinary calcium (Buclin et al., 2001). High dietary acid load produce low-grade, subclinical acidosis (Scialla and Anderson, 2013) and may be detrimental to bone (Mangano et al., 2014). Caffeine, a mildly addictive psycho-active flavor additive in most soft drinks, was found to have no flavor activity but induced a physiological and psychological desire to consume the drink (Keastand and Riddell, 2007) and its high intake (>300 mg/d) caused higher rates of bone loss in elderly postmenopausal women (Rapuri, et al.,2001). Despite the proved detrimental effects of CSDs on the bone metabolism, yet the structural derangements in bone tissue were not thoroughly investigated. Therefore, the main goal of the current study was to evaluate, through histomorphometric analysis, the negative effects of 2 popular brands of CSDs in Egyptian community; Coca Cola® and SEVEN-UP® on the bone tissue of Winstar albino rats.

2. Material and Methods

Experimental animals:

Thirty (30) adult sexually-mature Wistar albino rats weighing 300±20 gram were housed in a temperature-controlled and light-controlled room (12-h light/dark cycle), with free access to laboratory chow diet and tap water. All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Faculty of Medicine; Zagazig University.

Experimental design:

The animals were randomly divided according to the type of their drink into 3 equal groups (n=10; 5 males and 5 females); Group I (Control) maintained on tap water, Group II (Cola consumption) maintained on Coca Cola and Group III (Non-cola consumption) maintained on Seven-Up. The drinking volumes were the same for all groups (50 ml) and were daily-changed. After 30 days, the animals were sacrificed and their femur bones were fixed for 2 days in 10% neutral buffered formaldehyde pH 7.4 (Sterchi, 2013).

Histological study:

Decalcification of bone specimens from the distal part of each femur bone was carried out, using 10% buffered ethylene diamine tetraacetic acid

(EDTA) pH 7.2-7.4, for 2 weeks, during which time the decalcifying solution was daily-changed. The decalcified bone specimens were processed for routine light microscopic examination and stained by hematoxylin and eosin (H&E) (Sterchi, 2013).

Microscopic examination and histometric study:

The distal femoral diaphysis and metaphysis were used to study the cortical and the cancellous varieties of bone respectively. The nomenclature and symbols used in bone histomorphometry are the same as those described in the report of the American Society for Bone and Mineral Research Committee (Parfitt, et al., 1987). Two parameters were quantified in all bone specimens; the cortical thickness and the trabecular bone volume respectively. The Cortical bone thickness was obtained as the length of a perpendicular line drawn from just underneath the periosteum to the endosteum, below the lowest point of the epiphyseal plate (Surve, et al., 2001). The trabecular bone volume (BV/TV%) was expressed as percentage of the area occupied by the trabeculae vs the total bone area (Takano-Yamamoto and Rodan, 1990).

The parameters were quantified using the public domain image-processing software "Image J 1.49v/Java 1.6.0_244 (64-bit)" (National Institutes of Health, USA) in five (5) non-overlapping fields per each slide in 5 slides form each animal. The image analyzer was calibrated for measurements before use to automatically convert the image pixels into actual micrometer units and data were presented as mean ± standard deviation.

Statistical analysis:

Two-paired student's t-test was used for quantitative differences between males and females in all experimental groups. One way analysis of variance (ANOVA) with post-hoc Tukey test was used to determine a statistically-significant difference, when comparing the experimental groups. A significant or highly significant difference was present with *p*-values less than 0.05 or 0.01 respectively.

3. Results

A-Cortical bone:

Group I: both sexes revealed a normal bone structure with a fibrous periosteum covering the external surface and a thin cellular endosteum with a smooth outline lining the bone surface facing the marrow cavity. The bone matrix revealed osteocytes within their lacunae, remodeling or cement lines, Haversian canal and blood vessels (Fig.1 a&b). The mean thickness in group I was 514.278 ± 19.166 and 634.798±38.063 microns in females and males respectively and the higher thickness in males was highly significant [t=14.1(48), p=(<0.0001)](Table 1).

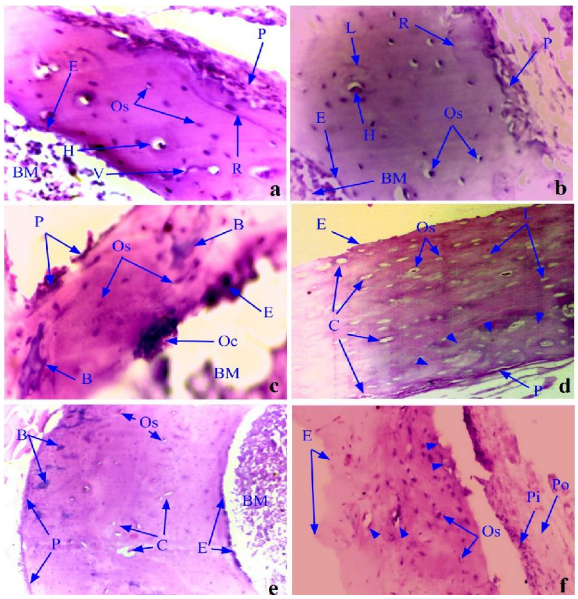

Figure 1. Compact (cortical) bone in group I male (a) & female (b), group II male (c) & female (d), group III male (e) & female (f): (P) periosteum, (E) endosteum, (BM) bone marrow space, (Os) osteocytes, (R) reversal line, (H) Haversian canal, (V) blood vessels, (Oc) osteoclasts, (B) basophilic-stained areas, (L) empty lacunae, (C) resorption cavities, (arrow heads) lightly-stained basophilic area (shown in d), (Pi) inner cellular periosteum, (Po) outer fibrous periosteum, (arrow heads) resorption cavities (shown in f). [H&E x400]

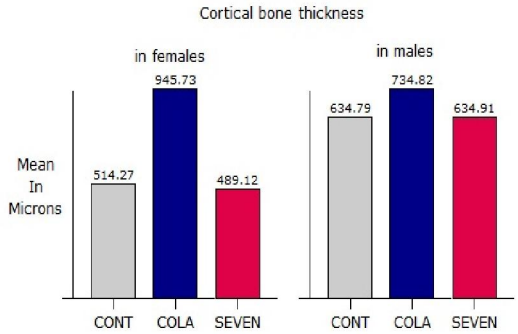

Figure 2. Figure 2.Histogram of the mean cortical bone thickness (in µm) in females and males of all groups; CONT (Control or Group I), COLA (Cola consumption or Group II), SEVEN (Non-cola consumption or Group III).

Group II: males revealed a loss of the periosteum with interrupted parts and an irregularly thickened endosteal surface with attached osteoclasts. The bone matrix contained irregularly-oriented osteocytes within indistinct lacunae and multiple basophilic-stained areas of irregular outline (Fig.1c), whereas females revealed a thin fibrous periosteum and an

endosteum of smooth outline. The bone matrix contained few osteocytes within indistinct lacunae, numerous empty lacunae and multiple resorption cavities of variable size. Occasionally, lightly-stained basophilic areas of irregular outline containing osteocytes of irregular orientation were observed particularly in subperiosteal region (Fig.1d). The mean thickness was 945.73 ± 73.12 and 734.82 ± 64.65 microns in females and males respectively and the higher thickness in females was highly significant [t= 10.8 (48), p= (< 0.0001)] (Table 1).

Table 1. Cortical bone thickness in the studied groups (in µm) with t-test and P value (Group I, Control; Group II, Cola consumption; Group III, Non-cola consumption; SD, standard deviation; S, significant; HS, highly significant & NS, non-significant)

	Range	Mean	SD	*t*- test (P value)
1-Group I:				
Females	469.326 - 543.016	514.278	19.166	14.14 (< 0.0001; HS)
Males	552.125 - 695.781	634.798	38.063	
2- Group II:				
Females	864.422 - 1199.547	945.730	73.123	10.80 (< 0.0001; HS)
Males	590.933 - 822.484	734.823	64.651	
3- Group III:				
Females	398.898 – 603.523	489.1283	65.08154	6.25461 (< 0.0001; HS)
Males	429.524 – 800.578	634.9151	96.67879	

Group III: males revealed a very thin periosteum and a smooth regular endosteum. The bone matrix contained osteocytes within their lacunae and multiple small basophilic-stained areas in the subperiosteal region with occasional resorption cavities (Fig.1e), whereas females revealed a thickened periosteum formed of inner cellular and outer fibrous portions, occasionally detached from the bone surface and the endosteum was absent. The subendosteal bone matrix showed a smooth irregular wavy surface and appeared paler in staining with few or no osteocytes. The subperiosteal bone matrix showed a more dense staining with the osteocytes within their arranged parallel to the bone surface and occasional resorption cavities (Fig.1f). The mean thickness in group III was 489.1283 ± 65.08154 and 634.9151 ± 96.67879 microns in females and males respectively and the higher thickness in males was highly significant [t= 6.25 (48), p= (< 0.0001)] (Table 1).

One-way ANOVA revealed a statistically significant difference in cortical thickness between all groups in males [F (2, 72)=16.68, p=.0001] and in females [F (2, 72)=496.57, p=.0001]. Post hoc analyses indicated that the mean cortical thickness of males and females in group II showed a significantly higher thickness than that of males and females in groups I and III respectively. The slight differences in thickness between groups I and III were not statistically significant in both sexes (Fig. 2 and table 2).

Table 2. The difference in cortical bone thickness in all groups using one way analysis of variance with post hoc Tukey test (Group I, Control; Group II, Cola consumption; Group III, Non-cola consumption; M, mean; SD, standard deviation; S, significant; HS, highly significant & NS,non-significant)

Group	Compared group	Difference between means	P- Value	Significance
1- Females:				
Group I (M= 734.82 ± SD=64.65)	Group II (M= 734.82 ± SD=64.65) Group III (M= 734.82 ± SD=64.65)	-431.452 25.1499	<0.01 >0.05	HS NS
Group II (M= 734.82 ± SD=64.65)	Group III (M= 734.82 ± SD=64.65)	456.6021	<0.01	HS
2- Males:				
Group I (M= 634.79 ± SD=38.06)	Group II (M= 734.82 ± SD=64.65) Group III (M= 634.91 ± SD=96.68)	-100.0253 -0.1173	<0.01 >0.05	HS NS
Group II (M= 734.82 ± SD=64.65)	Group III (M= 634.91 ± SD=96.68)	99.908	<0.01	HS

B- Cancellous bone:

Group I: both sexes revealed a normal trabecular pattern, with branched trabecular plates which were separated by wide irregular bone marrow spaces filled by a red hemopoietic tissue. The trabecular plates in males, had a surface rim of osteoblasts, minor resorption pits, remodeling or cement lines and a lamellar matrix between the osteocytes (Fig. 3; a1 & a2), whereas in females, the trabeculae were thicker and more interconnected with a lamellar matrix between osteocytes and multiple resorption cavities (Fig. 3; b1 & b2). The mean trabecular bone percent in group I was 29 ± 3 and 17.1 ± 2.6 % in females and males respectively and the higher percentage in females was highly significant [t= 6.7 (7.8), p= (< 0.0002)] (Table 3).

Group II: males revealed a loss of the classic trabecular pattern with trabeculae appearing as bone stumps of variable size scattered in a very wide bone marrow space filled by a red hemopoietic tissue. The trabecular stumps showed few irregularly-oriented osteocytes, small basophilic-stained areas, remodeling lines and occasional resorption cavities (Fig. 3; c1 & c2). Females showed a preserved trabecular pattern with some trabeculae in the form of bone stumps. The trabecular plates were thin with few irregularly-oriented osteocytes, osteoclasts within their resorption lacunae, remodeling lines, small basophilic-stained areas and resorption cavities (Fig. 3; d1 & d2). The mean trabecular bone percent in group II was 15.33 ± 4.67 and 8.75 ± 3 % in females and males respectively and the higher percentage in females was statistically significant [t= 2.66 (8), p= (< 0.0288)] (Table 3).

Group III: males revealed a loss of the trabecular pattern with trabecular plates forming isolated non-connected bone bars or bone stumps scattered in a wide bone marrow space. The trabeculae showed few surface osteoblasts, few irregularly-oriented osteocytes, small basophilic-stained areas and remodeling lines (Fig. 3; e1 & e2). Females showed a preserved trabecular pattern, but trabecular plates were irregularly interconnected and revealed few surface osteoblasts, clausters of osteocytes close to small basophilic-stained areas, remodeling lines and resorption cavities (Fig. 3; f1 & f2). The mean trabecular bone percent in group III was 21.6 ± 3.14 and 10.9 ± 1.97 % in females and males respectively and the higher percentage in females was statistically significant [t= 6.44 (8), p= (< 0.0002)] (Table 3).

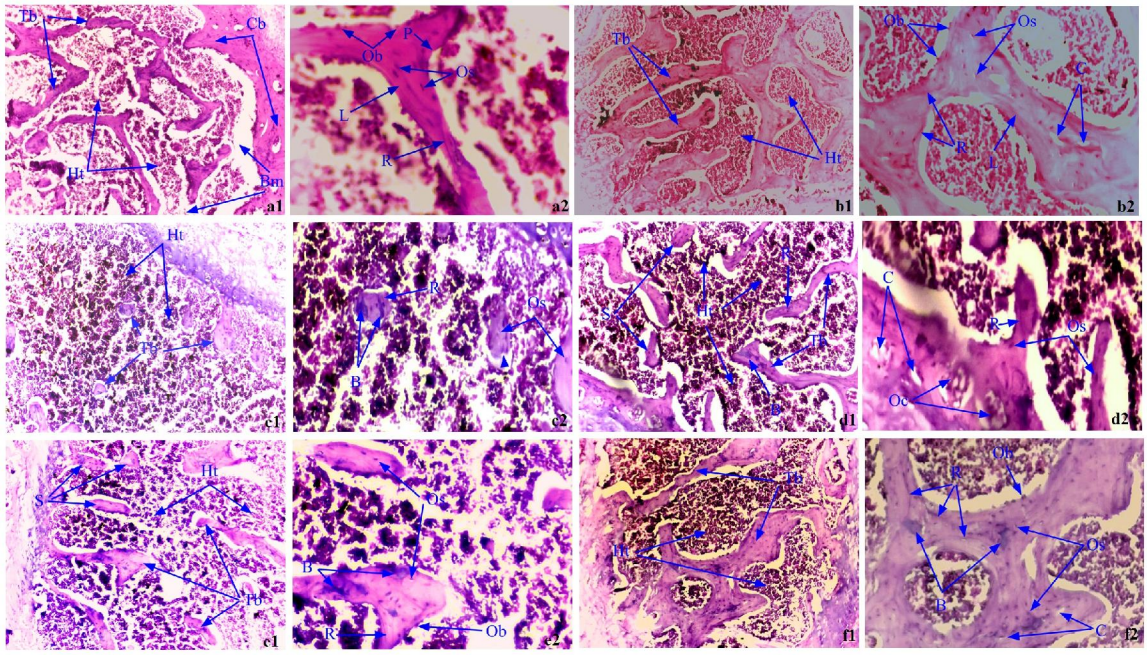

Figure 3: Cancellous (trabecular) bone in group I male (a1& a2) & female (b1& b2), group II male (c1& c2) & female (d1 & d2), group III male (e1 & e2) & female (f1 & f2); (Tb) trabecular plates, (Bm) bone marrow spaces, (Ht) hemopoietic tissue, (Ob) osteoblasts, (Os) osteocytes, (P) resorption pits, (L) lamellar pattern, (R) remodeling line, (C) resorption cavities, (B) basophilic-stained areas, (R) remodeling line, (arrow head) resorption cavity, (S) bone stumps, (Oc) osteoclasts. [H&E a1, b1, c1, d1, e1, f1 x100 and a2, b2, c2, d2, e2, f2 x 400]

Table 3. Trabecular bone volume in the studied groups (in %) with t-test and P value (Group I, Control; Group II, Cola consumption; Group III, Non-cola consumption; SD, standard deviation; S, significant; HS, highly significant & NS, non-significant)

	Range	Mean	SD	*t*- test (P value)
1-Group I:				
Females	26.6- 34	29	3	(6.7028 < 0.0002; HS)
Males	13.3 – 19.7	17.1	2.6	
2- Group II:				
Females	11.35 – 22.76	15.33	4.67	(2.6600 < 0.0288; S)
Males	4.35 – 12.08	8.75	3	
3- Group III:				
Females	16.2 – 24.11	21.6	3.14	(6.4398 < 0.0002; HS)
Males	8.96 – 13.68	10.9	1.97	

One-way ANOVA revealed a statistically significant difference in trabecular bone percentages between all groups in males [F (2, 12) = 14.25, p=.0006] and in females [F (2, 12) = 17.36, p=.0002]. Post hoc analyses indicated that the trabecular bone percentages of males and females in group II and of males and females in group III were significantly lower than that in males and females of group I. The differences in the trabecular bone percentages between groups II and III were statistically significant in females, but not significant in males (Fig. 4 and table 4).

4. Discussion

In the present study, both types of CSDs had induced histological changes in the cortical and trabecular bones. The cortical bone revealed numerous empty lacunae and resorption cavities within bone matrix, with loss of osteocytes or their presence in few numbers within indistinct lacunae and in irregular orientation. The associated abnormalities in the periosteum, the endosteum with activation of osteoclasts suggested that repairing these defects would be difficult. On the other hand, the trabecular bone revealed thinning of trabeculae or their subtotal loss leaving only bone stamps, reduced connectivity and osteoclast activation. The aforementioned histological changes were indicating destructive action of CSDs that would cause bone loss. In Accordance, Parfitt (1984) reported that a rapid bone loss occurs through excessive deepening of osteoclastic resorption cavities, whereas the slow bone loss is due to incomplete refilling of resorption cavities by osteoblasts. The same author added that during bone loss, rapid or slow, is going on endosteal surface, bone is being simultaneously, but slowly, added on the periosteal surface, by slight overfilling of shallow resorption cavities and this periosteal gain partly offset the weakness resulting from endosteal loss, but is not directly compensatory. Moreover, the documented histological changes causing bone loss in this study were probably similar to those described in studies on osteoporosis. According to Lane et al. (2003), osteoporosis was defined as a syndrome of excessive skeletal fragility resulting from both the loss of trabecular bone mass and trabecular bone connectivity. Moreover, Weber et al. (2004) in the ovariectomized rats, noted a reduction in cortical bone area due to expansion of the marrow cavity and found that both vitamin D analogs; $1\alpha(OH)D_2$ and $1\alpha(OH)D_3$, prevent cortical bone loss in these rats. In addition, Orlic et al. (2007) observed up regulation of genes involved in bone resorption in ovariectomized animals, at 21 days following ovariectomy.

In the present study, the worst effect on bone tissue was associated with consumption of Coca Cola, particularly in trabecular bone of male animals. This finding was probably due to absence of the protective effects of estrogenic hormones. In agreement, Lean et al. (2003) described that estrogen causes suppression of reactive oxygen species (ROS) which promote osteoclastic bone resorption and that its deficiency causes bone loss by lowering thiol antioxidants in osteoclasts which directly sensitizes osteoclasts to osteoclastogenic signals and entrains ROS-enhanced expression of cytokines that promote osteoclastic bone resorption. In addition, estrogen deficiency stimulates osteoclast formation both by increasing the production of osteoclastogenic cytokines as tumor necrosis factor –alpha (TNF-a) and receptor activator of nuclear factor –kappaB ligand (RANKL) production by activated T cells and increasing the number of osteoclast precursors (D'Amelio, et al., 2008).

In the present study, the marked negative effect on bone tissue with consumption of Coca Cola would be probably attributed to an ingredient in Coca Cola, but not in Seven Up, therefore, caffeine and phosphoric acid were accused in this respect. Concerning caffeine, Wink et al. (1996) found that caffeine induced significant histological changes in the bones of growing rats, such as fewer osteocytes, impaired structural remodeling with disrupted swollen

mitochondria of osteoblasts and osteocytes. Moreover, Rapuri, et al. (2001) reported that its high intake (>300 mg/d) caused higher rates of bone loss in elderly postmenopausal women. On contrary, Heaney (2002) described that here was no evidence that caffeine has any harmful effect on bone status. Concerning phosphoric acid, *Amato, et al.* (1998) reported that it had induced significant hypercalciuria and hyperphosphaturia, with more severe derangement of calcium and phosphate metabolism in the immature animals. In addition, Fernando et al. (1999) suggested that consumption of soft drinks with phosphoric acid should be considered as an independent risk factor for hypocalcemia in postmenopausal women. In contrast, Fenton, et al. (2009) described that no evidence that higher phosphate intakes were detrimental to bone health. However, the present study suggested that if the potential ability of either caffeine or phosphoric acid is not enough to adversely affect bone tissue individually, yet their combination in Coca Cola drinks would augment their negative consequences.

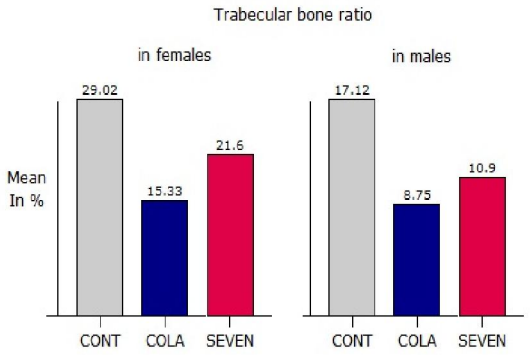

Figure 4: Histogram of the mean trabecular bone ratio (in %) in females and males of all groups; CONT (Control or Group I), COLA (Cola consumption or Group II) and SEVEN (Non-cola consumption or Group III).

Table 4. The difference in trabecular bone ratio in all groups using one way analysis of variance with post hoc Tukey test (Group I, Control; Group II, Cola consumption; Group III, Non-cola consumption; M, mean; SD, standard deviation; S, significant; HS, highly significant & NS, non-significant)

Group	Compared group	Difference between means	P- Value	Significance
1- Females:				
Group I (M= 29.02 ± SD= 2.97)	Group II (M= 15.33 ± SD=4.67) Group III (M= 21.6 ± SD=3.14)	13.69 7.42	<0.01 <0.05	HS S
Group II (M= 15.33 ± SD=4.67)	Group III (M= 21.6 ± SD=3.14)	-6.27	<0.05	S
2- Males:				
Group I (M= 17.12 ± SD=2.64)	Group II (M= 8.75± SD=2.99) Group III (M= 10.90 ± SD=1.97)	8.37 6.22	<0.01 <0.01	HS HS
Group II (M= 8.75± SD=3)	Group III (M= 10.90 ± SD=1.97)	-2.15	>0.05	NS

Conclusion: CSDs adversely affected the cortical and trabecular bone, with more effect in males, particularly with Cola consumption. The pronounced negative effects associated with Cola consumption, are probably due to a combined adverse effect of both caffeine and phosphoric acid. The presence of marked bone changes in males are probably attributed to lack of protective effect of female hormones. Finally, consumption of CSDs would be a risk factor of osteoporosis and must be reduced as much as possible.

Disclosure:

All authors state that they have no conflicts of interest.

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