Metabolic disturbances and worsening of atherosclerotic lesions in ApoE-/- mice after cola beverages drinking

(Article begins on next page)

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Metabolic disturbances and worsening of atherosclerotic lesions in ApoE$^{-/-}$ mice after cola beverages drinking

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Abstract

Background: Atherosclerosis is a major health burden. Metabolic disorders had been associated with large consumption of soft drinks. The rising incidence of atherosclerosis and metabolic alterations warrants the study of long-term soft drink consumption' effects on metabolism and atherosclerosis in genetic deficiency of apolipoprotein E which typically develops spontaneous atherosclerosis and metabolic alterations.

Methods: ApoE$^{-/-}$ mice were randomized in 3 groups accordingly with free access to: water (W), regular cola (C) or light cola (L). After 8 weeks, 50% of the animals in each group were euthanized (Treatment: W_8, C_8, L_8). The remaining mice (all groups) drank water for 8 weeks and were euthanized (Washout: W_{16}, C_{16}, L_{16}). Body weight and food and drink consumption were periodically measured. Blood was collected (biochemistry). At autopsy, transverse aortic sinus sections were serially cut and stained (histomorphometry); livers and kidneys were processed (microscopy). MANOVA (identification of variance factors) was followed by ANOVA and LSD tests (within-factor differences between levels). Conventionally a p< 0.05 was considered significant.

Results: Treatment increased drinking volumes (vs W_8: 4 fold C_8, p<0.0001; +47% L_8, p<0.02). Only C reduced eating amounts (−54%, p<0.05 vs W_8). I). Compared with W_8: C_8 developed hyperglycemia (+43%, p<0.03) and increased non-HDL cholesterol (+54%, p<0.05); L_8 showed decreased glycemia (−15%, p<0.05 vs W_8) and increased creatinine (2.5 fold, p<0.04), urea (+74, p<0.03) and aspartate-aminotransferase (2.8 fold, p<0.05). Hypercreatininemia was observed in L_{16} (2.7 fold vs W_{16}, p<0.05). Hypertriglyceridemia (+91%, p<0.008) and hyperuremia (+68%, p<0.03) developed over time of study (age). II). Treatment caused plaque area increase (vs W_8: 28% C_8, p<0.02 and 50% L_8, p<0.01; vs W_{16}: 43% C_{16}, p<0.05 and 68% L_{16}, p<0.02) and stenosis (vs W_8: 38% C_8, p<0.04 and 57% L_8, p<0.01; vs W_{16}: 71% C_{16}, p<0.01 and 46% L_{16}, p<0.04). Age also caused plaque area increase (56%, p<0.04). Treatment- and age-effects on plaque enlargement were additive.

Conclusion: Cola beverages caused atherosclerotic lesions' enlargement with metabolic (C) or non metabolic disturbances (L). ApoE$^{-/-}$ mice were particularly sensitive to L treatment. These findings may likely relate to caramel colorant and non-nutritive sweeteners in cola drinks and have potential implications in particularly sensitive individuals.

Keywords: Atherosclerosis, Soft drinks, Glycemia, Urea, Creatinine, Plaque area, Apo-E deficiency

Background

Atherosclerosis is a major health burden in modern society and a leading cause of death worldwide [1]. The rising consumption of soft drinks has been linked to the growing incidence of metabolic syndrome [2,3]. Previously we reported metabolic and cardiovascular alterations associated with cola drinking in normal (eugenic) rats [4,5]. The main components of metabolic syndrome (obesity, diabetes, hypertension) are known risk factors for atherosclerosis. However we have not found information concerning with the possible consequences of long-term cola drinking on atherosclerosis. The incidence of atherosclerosis and metabolic alterations is rising and warranted the study of the effects of long-term soft drink consumption on metabolism and atherosclerosis in genetic deficiency of apolipoprotein E. Apolipoprotein E deficient (ApoE$^{-/-}$) mice are a murine model of spontaneous atherosclerosis and develops metabolic abnormalities [6].

This is a descriptive study reporting the effects of long-term consumption of regular cola (sucrose sweetened) and light cola (aspartame-acesulfame K sweetened) on a). blood chemistry composition and b). atherosclerotic lesions in the aortic sinus (morphology, morphometry and instability features). The ApoE$^{-/-}$ mouse was used as a model of atherosclerosis and metabolic disorder because of the potential implications of the resulting information on some individuals with personal or familiar history of atherosclerosis or metabolic disorders.

Materials and methods

Animals

Forty-eight Apo E knockout (ApoE$^{-/-}$) mice (25 female, 23 male) on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, Maine). All animals were fed on a standard rodent commercial chow (16%–18% protein, 0.2 g% sodium, Cooperación, Buenos Aires, Argentina) *ad libitum* and housed inside an indoor laboratory facility with a 12 h light/dark cycle. The experiments were approved by The Animal Care Committee of the University of Buenos Aires and were performed in compliance with the ARRIVE guidelines on animal research [7].

Experimental design

ApoE$^{-/-}$ mice (8 week-old) were randomly distributed in 3 groups accordingly with free access to one of the following drinks: water (W), regular cola (C) (sucrose sweetened carbonated drink, Coca-Cola™, Argentina), or light cola (L) (low calorie aspartame–acesulfame K sweetened carbonated drink, Coca-Cola Light™, Argentina). Cola drinks had carbon dioxide content largely removed by vigorous shaking using a stirring plate and placing a magnetic bar in a container filled with the liquid prior to being offered to the animals at room temperature. After 8 weeks, 50% of the animals in each group were euthanized (Treatment: W$_8$, C$_8$, L$_8$). The remaining mice (all groups) drank water for another 8 weeks (i.e.: 16 weeks from beginning of the study) and were then euthanized (Washout: W$_{16}$, C$_{16}$, L$_{16}$). The ratio male/female was 1/1 in all groups except for L$_{16}$ (3/5). The animals were weighed weekly. Food and drink consumption were measured twice a week. Heparinized blood was collected and plasma was separated for biochemical assays. At autopsy, transverse aortic sinus sections were serially cut and stained for histomorphometry and livers and kidneys were processed for microscopic assessment.

According to company specifications, Coca Cola™ is a carbonated water solution containing (in 100 mL): carbohydrate 10.6 g, sodium 7 mg, caffeine 11.5 mg, caramel, phosphoric acid, citric acid, vanilla extract, natural flavourings (orange, lemon, nutmeg, cinnamon, coriander, etc.), lime juice and fluid extract of coca (*Erythroxylon novogranatense*). As far as nutritional information is concerned, the only difference between regular (43 Kcal/100 mL) and light cola (0 kcal) is the replacement of carbohydrates with non-nutritive sweeteners (aspartame 24 mg/100 mL–acesulfame K 16 mg/100 mL).

Sample handling

Animals were sacrificed under anesthesia with sodium pentobarbital and sodium diphenylhydantoin (Euthanyl®). Blood samples were obtained by ventricular puncture and plasma was separated. Commercially available kits were used for plasma analysis and measurement of: glucose using an enzymatic-colorimetric kit (Sigma Chemical Co., St. Louis, MO, kit #315–100) followed by spectrophometry; cholesterol using enzymatic kits (Sigma Chemical Co., St. Louis, MO, kit #402–20) and triglycerides (Sigma Chemical Co., St. Louis, MO, kit #344–20). Lipoproteins were separated by sequential density ultracentrifugation (density ranges: HDL 1.063–1.210 g/mL, HDL < 1.063 g/mL using a TLA-100 rotor (Beckman Instruments, Palo Alto, CA). Liver fragments, sagital sections from both kidneys, the heart and the ascending aorta were dissected and immersed in 10% buffered formaldehyde (Formalin 10% buffered solution, pH= 7.0) at room temperature for at least a 24 h fixation. After dehydration (graded ethanol series of 50%, 70%, and 100%), tissues were embedded in paraffin blocks. Six serial transverse sections (5 μm) were cut through the aorta at the origins of the aortic valve leaflets throughout the entire aortic sinus and stained with hematoxylin-eosin, Heidenhain trichrome (Azan) and orcein for elastic fiber identification.

Morphological and morphometric study of atheroma lesions

Features of plaque instability [8] were evaluated according with the presence of 3 or more of the following criteria: thickness of the fibrous cap (thin fibrous cap was defined as 3 or fewer cell layers), size of the necrotic core (a large necrotic core was defined as occupying more than 1/3 of the volume of the plaque), intraplaque hemorrhage (defined as the presence of red blood cells independent of microvessels) and the deposition of cholesterol crystals. Results were computed as binary outcomes and the frequency for each group was determined. Each of six serial cross sections was analysed using a software-coupled (Image Pro Plus for Windows, v3) Nikon Eclipse E400 microscope and data were averaged. In addition, plaque area, intimal layer and the media layer length were measured and stenosis percentage was calculated.

Statistical analysis

Data were overall analyzed by multiple analysis of variance test (MANOVA) to identify sources of variation. Main effects and interactions were opened, followed by one way ANOVA and post hoc tests (LSD, least significant difference) to detect differences between levels (experimental groups) within each source of variation (within factor). Conventionally a p< 0.05 was considered significant (SPSS™ version 17.0 software).

Results
Blood chemistry

Cola beverages treatment increased drinking volumes (C_8: 4 fold, p<0.0001 and L_8: +47%, p<0.02 vs W_8). Only regular cola reduced food consumption (−54% C_8, p<0.05 vs W_8) (Table 1). Compared with W_8: C_8 developed hyperglycemia (+43%, p<0.03) and higher non-HDL cholesterol levels (+54%, p<0.05) so that hyperglycemia accounted for 83% of non-HDL cholesterol increase (p< 0.001); L_8 had lower glycemia (−15%, <0.05) and increased levels of creatinine (2.5 fold, p<0.04), urea (+74, p<0.03) and-aspartate aminotransferase (AST) (2.8 fold, p<0.05). These changes reversed after treatment discontinuation except for persistent hypercreatininemia as found in L_{16} (2.7 fold vs W_{16}, p<0.05). Over time of study (mice age

effect) hypertriglyceridemia (+91%, $F_{1,46}$=7.69, p<0.008) and hyperuremia (+68%, $F_{1,46}$=5.01, p<0.03) developed (Figure 1).

Morphological and morphometric study of atheroma lesions

All 3 groups of mice developed qualitatively similar atherosclerotic lesions, but showed quantitative differences according with the group. The overall picture was represented by larger lesions in cola beverages treated mice (see differences below). As shown in Figure 2, the aortic plaques extended from the endothelium to the internal elastic membrane, bulging into the arterial lumen. Along with this, luminal caliber was reduced with thickening or thinning and even disruption of the fibrous cap. Extensive atrophy of the aortic media was observed, being replaced with plaque components, consisting of huge acellular necrotic xanthomas shaped into fibro-fatty nodules of cholesterol crystals surrounded by dense or loose connective tissue. Internal and external elastic membranes were disrupted and discontinued or even disappeared at the site of plaque expansion. In some sections, the ostia to the coronary arteries were mildly or largely obstructed by the plaque. Aortic leaflets were thickened and their free borders often presented linear calcifications and/or loose fibrotic excrescences (Figure 2).

Treatment caused irreversible increase in plaque area (Figure 3) (vs W_8: 28%, p<0.02 in C_8 and 50%, p<0.01 in L_8; vs W_{16}: 43%, p<0.05 in C_{16} and 68%, p<0.02 in L_{16}) and stenosis (vs W_8: 38%, p<0.04 in C_8 and 57%, p<0.01 in L_8; vs W_{16}: 71%, p<0.01 in C_{16} and 46%, p<0.04 in L_{16}). Age was overall associated with plaque area increase (56%, $F_{2,42}$=3.48, p<0.04). Treatment and age had additive effects on plaque area and did not interact with each other ($F_{Treatment*Age\ 2,42}$=2.17, N.S.).

Plaque instability features showed no variation related to group (inset table in Figure 3).

Liver and kidney morphology

Treatment with cola beverages had no effect on the morphology or the aging process of these organs. Accordingly all mice regardless of the group showed a mild degree of liver steatosis and scattered foci of acinar inflammation, whereas only a slight chronic interstitial

Table 1 Body weight and nutritional data of ApoE-/- mice after cola beverages treatment and after washout

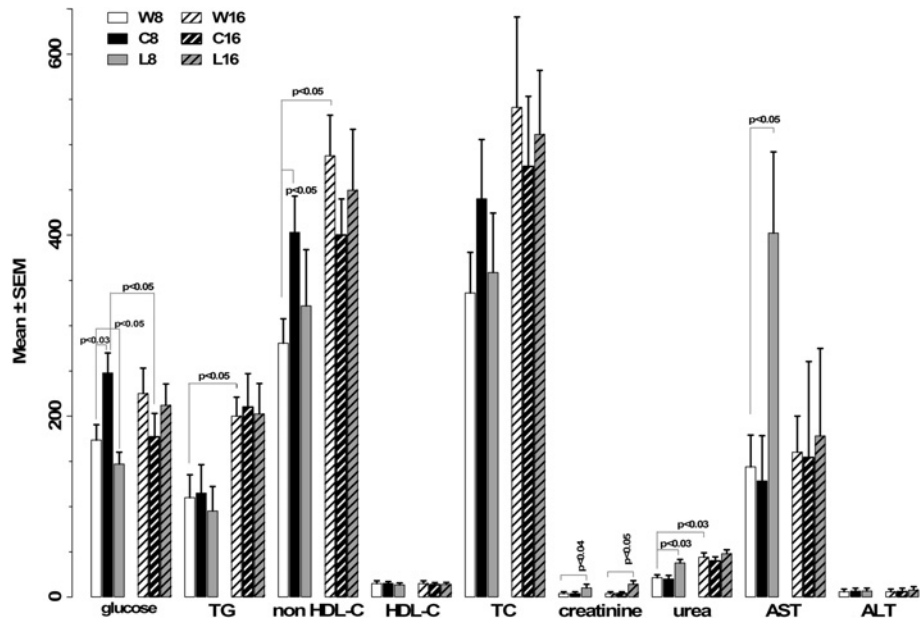| Group	W_8	C_8	L_8	W_{16}	C_{16}	L_{16}
BW (g)	21.4 ± 2.2	23.7 ± 3.0	20.6 ± 1.9	23.2 ± 2.0	24.5 ± 3.1	22.9 ± 2.8
Solid intake (g)	3.7 ± 0.3	1.7 ± 0.2	3.8 ± 0.3	3.8 ± 0.4	3.3 ± 0.3	4.1 ± 0.4
Liquid intake (mL)	4.9 ± 1.1	21.1 ± 1.6	7.2 ± 0.7	4.7 ± 0.4	4.5 ± 0.4	5.9 ± 0.9
Solid intake/BW (g/g)	0.17±0.02	0.07±0.01	0.18±0.02	0.16 ± 0.02	0.18±0.02	0.18±0.02
Liquid intake/BW (mL/g)	0.23±0.02	0.89±0.06	0.35±0.03	0.20 ± 0.03	0.18±0.02	0.26±0.03

Figure 1 Blood chemistry in ApoE^{-/-} mice after cola beverages treatment and after washout. Data are expressed in mg/100 mL except for enzymes (AST and ASL) activity which is expressed in units/L. An enzyme unit is defined as the amount of enzyme catalyzing the transamination of 1 μmol substrate, aspartate or alanine respectively, in 1 min at 20–25°C/68–77°F.
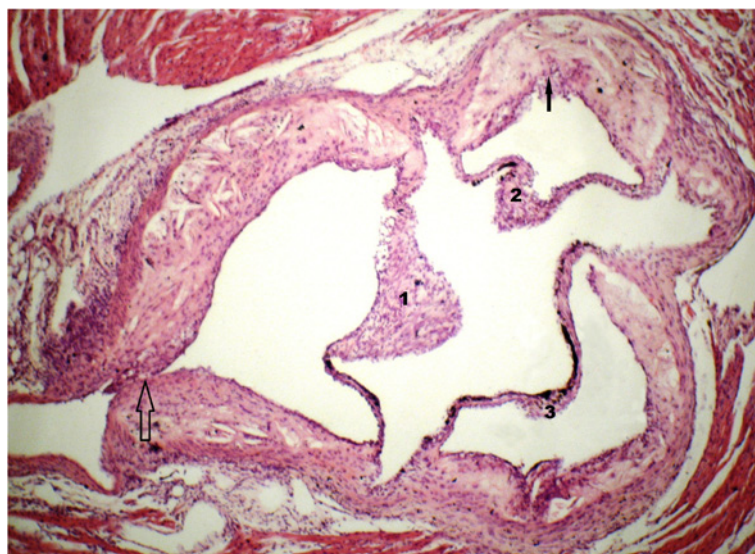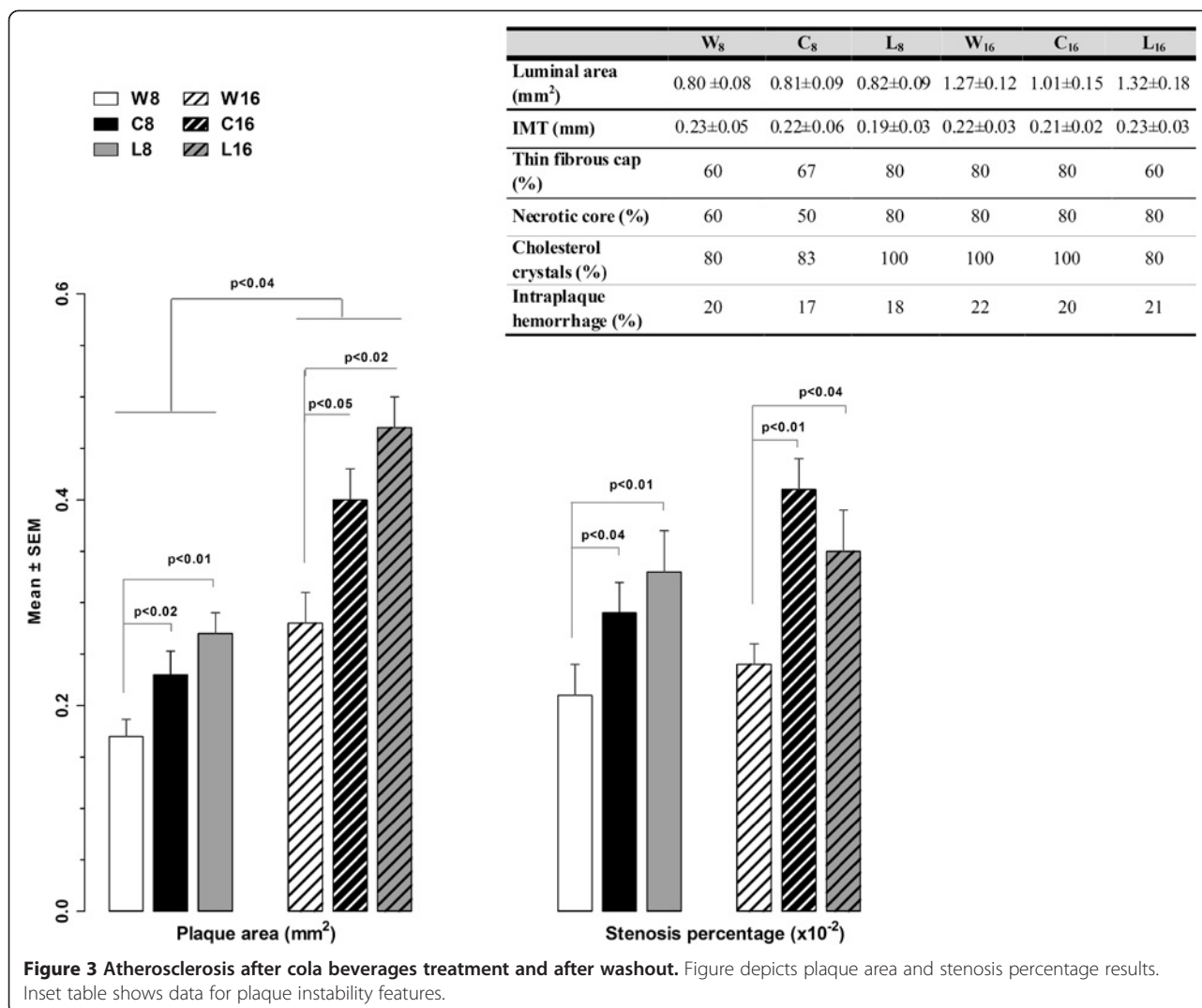

Figure 2 Aortic plaque histology from an ApoE^{-/-} mouse after cola beverage treatment. The image corresponds to one of six serial sections obtained where the aortic sinus becomes the ascending aorta. The aortic plaque extends from the lumen to the internal elastic membrane. Along with this, luminal caliber is reduced with thickening or thinning and even disruption (solid arrow) of the fibrous cap. Extensive atrophy of the aortic media is observed, being replaced with plaque components, consisting of huge acellular necrotic xanthomas shaped into fibro-fatty nodules of cholesterol crystals and dense or loose connective tissue. Internal and external elastic membranes are disrupted and discontinued at the site of plaque expansion. The arrow identifies the ostia to a coronary artery which is largely obstructed by the plaque (empty arrow). Aortic leaflets are thickened and their free borders show linear calcifications and loose fibrotic excrescences (1–3). Hematoxylin and eosin x 200.

	W$_8$	C$_8$	L$_8$	W$_{16}$	C$_{16}$	L$_{16}$
Luminal area (mm^2)	0.80 ±0.08	0.81±0.09	0.82±0.09	1.27±0.12	1.01±0.15	1.32±0.18
IMT (mm)	0.23±0.05	0.22±0.06	0.19±0.03	0.22±0.03	0.21±0.02	0.23±0.03
Thin fibrous cap (%)	60	67	80	80	80	60
Necrotic core (%)	60	50	80	80	80	80
Cholesterol crystals (%)	80	83	100	100	100	80
Intraplaque hemorrhage (%)	20	17	18	22	20	21

Figure 3 Atherosclerosis after cola beverages treatment and after washout. Figure depicts plaque area and stenosis percentage results. Inset table shows data for plaque instability features.

inflammatory infiltration at the level of the transition between the outer and internal medullar zones were observed in the kidneys (not shown).

Discussion

In this paper, whereas predictable results were observed after the administration of regular cola to ApoE$^{-/-}$ mice, light cola consumption revealed unexpected observations. Neither regular cola nor light cola drinking modified body weight. Interestingly, solid food intake decreased in the regular cola group, likely as the result of drinking large volumes of regular cola which provided excess caloric intake. We reported the same nutritional behavior in eugenic rats after drinking regular cola for a long period [4,5].

Blood chemistry

Regular cola drinking (C, sucrose sweetened) resulted in hyperglycemia which largely accounted for the observed increase in the non-HDL cholesterol fraction. These changes reversed after the washout period. Differently, light cola drinking (L, aspartame–acesulfame K sweetened) induced a mild decrease in glycemia, with hypercreatininemia, hyperuremia and increase in AST, all of which reversed after washout except for hypercreatininemia. Acesulfame K and other non-nutritive sweeteners (but not aspartame) have been reported to activate enteroendocrine sweet taste receptors and release incretins which stimulate pancreatic insulin secretion [9-11]. Reasonably, this mechanism might help to understand the decrease in glycemia observed in the L group. Likewise, phenylalanine, 40% of metabolilzed aspartame [12], acting synergically with solid food and stimulating insulin release [13] might participate in the decrease in glycemia in L group.

Reduced creatinine clearance has been reported in 8 week-old (young) ApoE$^{-/-}$ mice [14] indicating some degree of vulnerability in glomerular filtration. Oxidative

stress is associated with either high uremia levels or methanol ocurrence in blood [15]. In L mice uremic toxicity and increased oxidative stress may correlate with a pro-inflammatory condition. However liver pathology was not detectable by optic microscopy in this study. A temporal delay between biochemical changes and morphological alterations may partly contribute to explain the lack of correlation.

Over time of study hypertriglyceridemia and hyperuremia developed in all groups irrespective of drink treatment likely as a result of the aging process which is typically accelerated in ApoE$^{-/-}$ mice.

Arterial pathology

Cola drinking resulted in enlargement of atherosclerotic lesions and increased stenosis. Time over study (aging) was *per se* associated with both increased plaque area and stenosis. After washout further worsening of atherosclerotic lesions (i.e. enlargement of plaque area and increase in stenosis degree) observed in mice that had consumed colas was interpreted as a result of aging.

Recently, safrole-2',3'-oxide (main component of sassafras oil in nutmeg) which is found in cola beverages, has been reported to aggravate atherosclerosis in ApoE$^{-/-}$ mice [16].

It is known that lipid profile and atherosclerosis are influenced by gender in mice, particularly in ApoE$^{-/-}$ mice [17-19]. However, the influence of gender on atherosclerosis and the protective effect of estrogens are not yet clear [20-22]. We observed only a trend towards increased total cholesterol levels in males compared to females. One likely explanation is that the number of animals per gender was too small.

Possible mechanisms responsible for the effects of cola drinking on aortic sinus plaque

Atherosclerosis in ApoE$^{-/-}$ mice can be affected by several factors. Defective insulin secretion, smaller islet mass and islet inflammation have been found in atherosclerosis-susceptible B6.ApoE$^{-/-}$ mice compared to atherosclerosis-resistant BALB.ApoE$^{-/-}$ mice (C57BL/6 and BALB/cJ respectively) [23]. Present finding of hyperglycemia after chronic regular cola drinking may be consistent with such possibility, i.e: reduced beta cell mass and insulin secretion which have not been evaluated in this study. We have observed islet mass reduction with hyperglycemia after chronic regular cola drinking in rats (unpublished observations). Genetic deficit or pharmacological blockade of angiotensin receptor 1 (AT$_1$R) attenuates atherosclerosis and improves endothelial function in experimentally induced diabetes in ApoE$^{-/-}$ mice via peroxisome proliferator-activated receptor γ (PPARγ) pathway [24,25]. We have found no reports relating cola drinking with ATR1 or the PPARγ pathway.

Caramel colorant, contained in both regular and light cola beverages. is a source of advanced glycation end products (AGEs) [3,4] promoting a proatherogenic pro-oxidative status [26].

Uremia and even mild renal dysfunction have been reported to cause a dramatic increase in plaque size and aggressive morphology (foam cell rich soft plaques) in ApoE$^{-/-}$ mice [27]. High uremia, which has been associated with a prooxidative and proinflamatory condition, was presently observed after chronic light cola drinking [15]. In addition, the generation of ROS by methanol originated by aspartame cleavage offers a reasonable mechanism underlying the effects of light cola drinking. Also unmetabolized aspartame, 10–15% of ingested aspartame [28], modifies the intestinal environment and triggers inflammatory (pro-atherogenic) processes [11].

In this study light cola drinking caused hyperuremia and atherosclerosis in 16 weeks-old mice (8 weeks-old mice at the beginning of the study + 8 week-treatment) while these conditions typically develop in aged ApoE-/- mice [29-31]. Accordingly, it follows that long term L consumption might precipitate aging mechanisms in arterial vessels in susceptible hosts.

Conclusions

ApoE$^{-/-}$ mice were particularly sensitive to the effects of light cola drinking. Increase in AST, uremia and creatininemia suggest functional interference at one or more levels (liver, kidney, muscle) and warrants future research.

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