ORIGINAL ARTICLE

Cola Beverages Accelerate Growth of the Atherosclerotic Plaque in ApoE-/- Mice

ENRIQUETA M. SERAFINI, MATILDE E. OTERO-LOSADA, GABRIEL CAO, GASTÓN RODRÍGUEZ-GRANILLO; JIMENA AGUILERA, ANGÉLICA MÜLLER, GRACIELA OTTAVIANO, FRANCISCO AZZATO, JOSÉ MILEI[MTSAC,]

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Address for reprints:
Dr. José Milei
(ININCA) Instituto de Investigaciones Cardiológicas
UBA - CONICET
Marcelo T. de Alvear 2270
(C1122AAJ) CABA - Argentina
Tel./Fax +54 +11 4508-3836
e-mail: ininca@fmed.uba.ar

ABSTRACT

Objectives
Unhealthy eating habits during childhood and youth have been suggested as predisposing factors for atherosclerotic complications later in life. The growing consumption of cola beverages in recent decades has been associated with the development of obesity and increased incidence of atherosclerosis and cardiovascular disease. We also know that there is a correspondence between the consumption of these beverages and the different stages of life, being higher in children, adolescents and young adults.

Objective
This study evaluates the effect of cola beverage consumption on atherosclerosis.

Methods
ApoE-/- mice (8 weeks old) were randomized into 3 groups according to free access to water (W), sucrose sweetened carbonated cola beverage (C) or aspartame-acesulfame K sweetened carbonated 'light' cola beverage (L). At 8 weeks, cola beverages were switched to water. Mice were sequentially euthanized: before treatment (8 week old mice) and after treatment discontinuation (16, 20, 24, and 30 week old mice). The ascending aorta and the liver were removed. The ratio between the aortic plaque area and the media layer thickness (plaque/media-ratio) was calculated. Hepatic inflammation was assessed according to the NASH scale.

Results
Plaque/media-ratio varied according to the type of beverage treatment ($F_{2,54} = 3.433$, $p < 0.04$) and age ($F_{4,54} = 5.009$, $p < 0.03$), and was higher in the C and L groups ($p < 0.05$ at 16 and 20 weeks, $p < 0.01$ at 24 and 30 weeks). Hepatic parenchymal inflammation ($F_{2,9} = 13.29$, $p < 0.002$) and portal inflammation ($F_{2,9} = 6.30$, $p < 0.02$) increased fivefold and twofold in contrast to steatosis and hepatocellular damage which remained unchanged throughout the study. The W group (natural evolution of atherosclerosis) evidenced acceleration of plaque growth in parallel with a rapid increase in hepatic inflammation around week 20 of age.

Conclusions
Cola beverage consumption in 8-16 week old ApoE-/- mice accelerated atherosclerosis progression Data suggest that, in this murine model, sustained cola consumption at early stages may aggravate atherosclerosis progression later in life.

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Key words > Atherosclerosis - Carbonated Beverages - Apolipoprotein E

INTRODUCTION
Atherosclerosis is the leading cause of death worldwide (1) and its risk increases with age (2). Unhealthy eating habits during childhood and youth have been suggested as predisposing factors for atherosclerotic complications later in life (3, 4). Several longitudinal

Cardiological Research Institute. Universidad de Buenos Aires. National Scientific and Technological Research Council (ININCA.UBA.CONICET). Buenos Aires, Argentina
"Prof. Dr. Alberto C. Taquini"
[MTSAC] Full Member of the Argentine Society of Cardiology
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studies have shown increased cardiovascular risk in the adulthood of obese children (5.6), added to the fact that exposure to cardiovascular risk factors at early ages can contribute to the development of atherosclerosis (7). The growing consumption of cola beverages in recent decades has been associated with the development of obesity and increased incidence of atherosclerosis and cardiovascular disease. At the same time we know that there is a correspondence between the consumption of these beverages and different stages of life, being higher in children, adolescents and young adults. We have recently observed the development of metabolic syndrome after prolonged consumption of cola beverages (i.e. treatment) in rats (8, 9). These studies showed the development of hypertension, hyperglycemia, weight gain, dyslipidemia and echocardiographic alterations, whereas histopathological findings were not consistent and were more related to the aging process than to treatment (8, 9). We also noted that ApoE-/- C57BL/6J mice are particularly sensitive to damage derived from cola beverage consumption. Sucrose sweetened cola beverages (C) caused arterial disease associated with hyperglycemia (10). Consumption of C or L indistinctly produced increased plaque area (28 % C, 50 % L) and aortic stenosis (38 % C, 57% L) (10). Paradoxically, after discontinuing cola beverage consumption, lesions worsened (plaque area increased by 43 % in C and 68 % in L and stenosis by 71 % in C and 46 % in L). A likely explanation is that the recovery period was insufficient to allow the observation of any reversal of arterial damage considering that the determinations were carried out only at the end of treatment, and the timing sequence of the posterior evolution was not assessed. Age was associated with increased atherosclerotic lesions (56%).

The aim of this study was to explore the impact of cola beverage consumption (i.e. treatment) in the progression of arterial damage in ApoE-/- mice (mouse model of atherosclerosis) (11) at different times after treatment discontinuation. The hypothesis tested was that consumption of cola beverages early in life can affect the development and progression of atherosclerosis in adulthood.

Since the literature suggests the involvement of hepatic inflammatory processes in the development and progression of atherosclerosis, it was of interest to assess possible changes in the liver in response to cola beverage consumption in this mouse model. Specifically, the temporal association between hepatic damage and atherosclerotic lesions was assessed. Experimental evidence shows that ApoE-/- deficiency is related to the hepatic expression of proinflammatory mediators (12) and to aging accelerators (13).

Despite the widespread use of ApoE-/- mice for multiple purposes, the relationship between atherosclerotic and hepatic damage is not yet clear (14).

METHODS

The tests described in this study were approved by the Institutional Animal Care and Use Committee of the Universidad de Buenos Aires (IACUC) and were conducted in accordance with the recommendations of the Weatherall report (15).

A batch of sixty C57BL/6J ApoE-deficient mice (ApoE-/-), (Jackson Laboratory, Bar Harbor , Maine) were fed ad libitum with commercial standard rodent chow (16-18 % protein, 0.2 g % sodium, Cooperación, Buenos Aires, Argentina) and were housed in a vivarium with a 12/12-hour light-dark cycle. At eight weeks of age ApoE-/-mice were randomized into 3 groups (n = 20 each). Each group had free access to one of the following beverages at room temperature: water (W), common cola beverage (C) (sucrose -sweetened carbonated cola beverage, Coca -Cola™, Argentina), and light cola beverage (L) (aspartame-acesulfame K sweetened carbonated light cola beverage, Coca -Cola Light™, Argentina) for 8 weeks. Carbon dioxide was removed by vigorous shaking until its total elimination.

After 8 weeks, colas were replaced by water in the C and L groups. Four mice per group were sequentially euthanized under anesthesia with sodium pentobarbital - diphenylhydantoin sodium solution (Euthanyl™): before treatment (8 weeks old: W8, C8 and L8) at the end of treatment (16 weeks old: W16, C16 and L16) and after discontinuation (20 weeks old: W20, C20 and L20, 24 weeks old: W24, C24 and L24, and 30 weeks old: W30, C30 and L30).

Tissue was removed from the ascending aorta and liver, dissected and immersed in buffered 10% formaldehyde solution (10% formalin buffer solution, pH = 7.0) at room temperature for a period of 24 hours. After dehydration (solutions of increasing concentration of ethyl alcohol at 50%, 70 %, 100%), tissues were included in paraffin blocks. Six 5 μm serial transverse sections were obtained from the aorta at the origin of the aortic valve leaflets and throughout the entire aortic sinus and stained with hematoxylin-eosin, Masson trichrome and orcein for elastic fiber identification. Each of the sections was evaluated using a Nikon Eclipse E400 microscope coupled to a program (Image Pro plus for Windows, v3) to analyze and average data. The plaque area, the intima layer and the media thickness were measured. The ratio between plaque area and media layer thickness (plaque/media-ratio) was calculated to estimate the degree of arterial remodeling (15). Liver sections 4 μm thick were processed for microscopy and the degree of parenchymal inflammation was determined according to the non-alcoholic steatohepatitis scale (NASH) (16), whose score ranges from 0 to 4 (0 = lowest, 4 = highest) and includes steatosis, parenchymal inflammation, hepatocellular injury, portal inflammation and fibrosis.

The data obtained were subjected to multivariate analysis of variance (MANOVA). The factorial ANOVA model was used to identify the factors responsible for result variations. Subsequently, post hoc Dunnett's test allowed evaluating differences among experimental groups of the same age throughout the study. The limit of statistical significance was conventionally set at $p < 0.05$ (SPSS™ software version 17.0).

RESULTS

From a qualitative point of view, at the time of autopsy focal accumulations of lipid-laden macrophages were observed in 16 week old mice (Figure 1). Twenty and 24 week old mice showed globular clusters of grouped macrophages covered by a thin fibrous layer (Figure 2). At week 30, large acellular necrotic xanthomas forming a fibro - fatty nodule extending from

the lumen to the internal elastic lamina were found. The luminal caliber was greatly reduced due to the thinning and loss of the fibrous layer. Interruption of the internal elastic lamina with extensive atrophy of the media layer, which was replaced by plaque components (Figure 3), was observed. The plaque/media ratio varied with treatment ($F_{2,54} = 3.433$, $p < 0.04$) and mice age ($F_{4,54} = 5.009$, $p < 0.03$) and was higher in the C and L groups compared with mice of the same age in the W group ($p < 0.05$ in 16 and 20 week old mice, $p < 0.01$ in 24 and 30 week old mice) (Figure 4). The ApoE-/- mice that never consumed cola beverages (group W) spontaneously developed accelerated changes in the growth of atherosclerotic plaque in parallel with a rapid increase in hepatic inflammation at around 20 weeks of age (Figure 5). Hepatic parenchymal inflammation ($F_{2,9} = 13.29$, $p < 0.002$) and portal inflammation ($F_{2,9} = 6.30$, $p < 0.02$) varied with time (i.e. mice age) increasing fivefold and twofold, respectively ($p < 0.01$ and $p < 0.03$) between weeks 20 and 30, in contrast with steatosis and hepatocellular damage that remained unchanged throughout the study (Figure 5).

DISCUSSION

Accelerated growth of the atherosclerotic plaque was higher in C and L groups compared with the W group throughout the study. At an earlier stage of this research we observed the paradoxical worsening of atherosclerosis after discontinuation of cola beverage

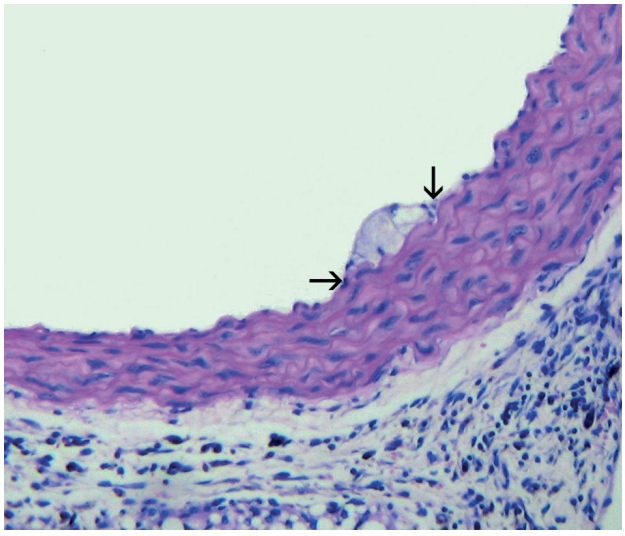

Fig. 1. Histological image corresponding to the aortic wall of a 16 week old mouse. An accumulation of macrophages containing intracytoplasmic lipid microvacuoles (foam cells) is observed immediately below the vascular endothelium (between arrows). H & E, 20 ×.

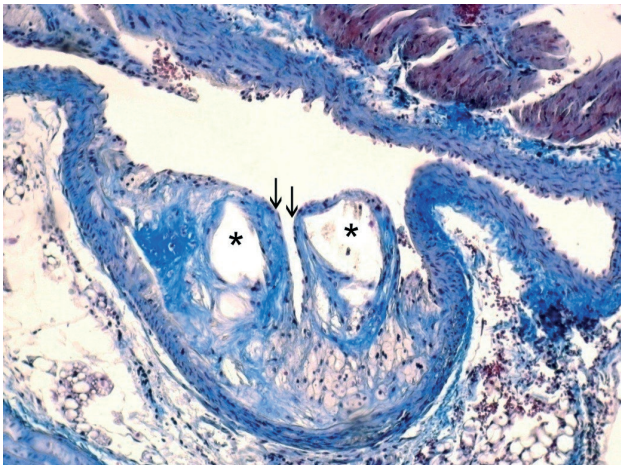

Fig. 3. Representative histological image of an aortic intimal injury corresponding to a 30 week old mouse, characterized by large acellular, lipidic accumulations (asterisks) with xanthomatous appearance, contained in fibrous tissue (arrows), in dense areas, constituting nodular formations protruding into the lumen. Masson trichrome, 10 ×.

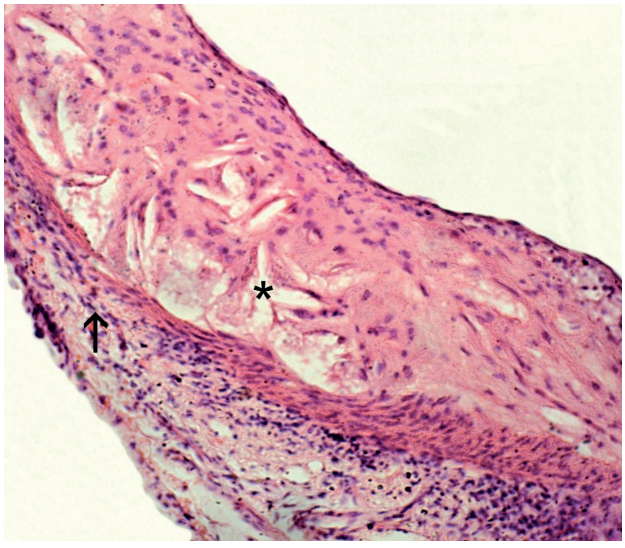

Fig. 2. Histological image corresponding to the aortic wall of a 20-24 week old mouse. It shows cholesterol crystal deposits (asterisk) included in fibrocellular tissue that expand the vascular intima compressing the muscle layer (arrow). H & E, 10 ×.

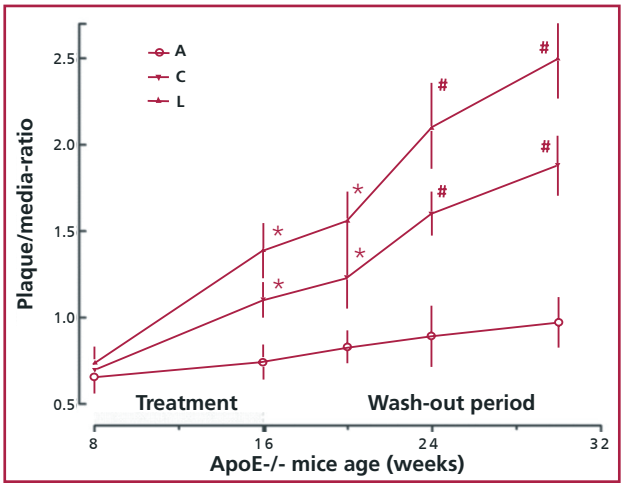

Fig. 4. Effect of cola consumption on plaque/media ratio in ApoE-/- mice during the study period.
Ordinate: Plaque/media ratio. Abscissa: age (weeks).
* $p < 0.05$, # $p < 0.01$ compared with the same age W group.

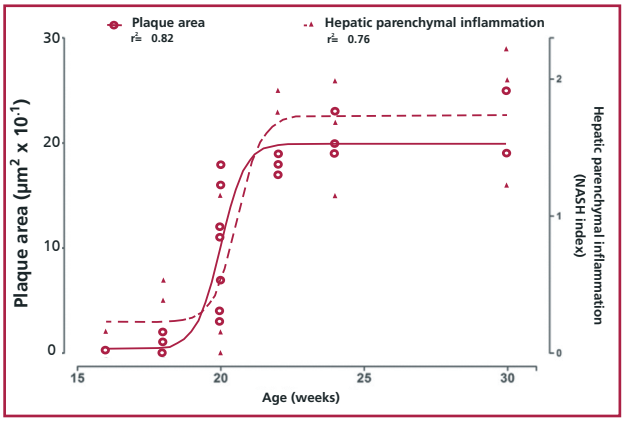

Fig. 5. Plaque area and hepatic parenchymal inflammation in ApoE-/- mice after cola beverage discontinuation.
Ordinate: plaque area (µm2 × 103). Abscissa: age (weeks).

consumption (10). However, interpretation of these results could have been challenged considering that the determinations made at a single time point after end of treatment and recovery period might have been insufficient to observe damage reversal. The results found in the present study allow us to rule out this alternative explanation and to confirm the rapid growth of atherosclerotic lesions as a function of time after cessation of cola beverage consumption. The rapid growth of atherosclerotic lesions observed in treated animals (i.e., who consumed cola beverages at an early age) exceeded the growth associated to the natural evolution of atherosclerosis observed in animals that never consumed cola beverages. In recent studies, it has been reported that safrole -2', 3' - oxide (SFO), the main metabolite of safrole (sassafras essential oil and a minor component of nutmeg essential oil), is found in cola beverages and could aggravate atherosclerotic lesions in ApoE-/- mice (17).

In the L group, an accelerated progression of atherosclerosis was observed in comparison with W and C groups of the same age at the different studied times. Recently, an increase in the activity of hepatic transaminases (2.8 times) together with hyperuremia (74 %) and hypercreatininemia (2.5 times) was reported after consumption of "light" cola beverage in this mouse model (10). In this context of knowledge, the findings of our study suggest the association of functional alterations in liver, kidney, and/or muscle as a mechanism involved in the acceleration of atherosclerosis in ApoE-/-mice after cessation of cola beverage consumption, and provide evidence reaffirming that ApoE-/-mice are idiosyncratically sensitive to arterial damage in response to cola beverage consumption. In this instance, to discuss the real possible mechanisms responsible for these findings would be speculative, since we have not made any determinations in this regard. In any case it should be noted that a deregulation in the complex crosstalk among mediators of inflammation, coagulation and pro-peroxidation mechanisms, to name a few, and the vascular system, would be involved in the current findings (18-20).

ApoE-/-mice that did not consume cola beverages (i.e. the W Group) showed accelerated changes in atherosclerotic plaque growth, which would be accompanied by a rapid increase in hepatic inflammation around 20 weeks of age. These results concerning the natural history of atherosclerotic lesions in ApoE-/-mice are consistent with those found by Watson et al. who reported spontaneous acceleration of atherosclerotic lesions at about 20 weeks of age in this mouse model (21). As recently reported, the chronological parallelism observed between increased aortic plaque area and hepatic inflammation in this study may reflect the peripheral expression of alterations at the genetic level. (22) Taken together, published researches using this mouse model emphasize the key involvement of the liver in the process of atherogenesis. Experimental evidence confirms the existence of liver-artery interactions, thus illustrating remote organ crosstalk in atherosclerosis. (23).

CONCLUSIONS

Cola beverage consumption, regardless of the sugar content, increased the rate of atherosclerosis progression in ApoE-/- mice, favoring the growth of aortic plaque (internal remodeling) on the thin media layer. The effects of treatment with cola beverages in 8 and 16 week old mice did not reverse even after prolonged discontinuation (30 week old mice). The data suggest that sustained consumption of cola beverages during the early stages of life can accelerate the aggravation of atherosclerotic damage in later stages of life, in a genetically favorable scenario, as is the case of atherosclerosis prone ApoE-/- mice.

RESUMEN

Las bebidas cola aceleran el crecimiento de la placa aterosclerótica en ratones ApoE-/-

Introducción

Los hábitos de alimentación poco saludables durante la infancia y la juventud se han sugerido como favorecedores de las complicaciones ateroscleróticas en edades más avanzadas. El creciente consumo de bebidas cola en las últimas décadas se ha asociado con el desarrollo de obesidad e incremento en la incidencia de aterosclerosis y enfermedades cardiovasculares. A su vez, se sabe que existe correspondencia entre el consumo de estas bebidas y etapas de la vida, el cual es mayor en los niños, los adolescentes y los adultos jóvenes.

Objetivo

Evaluar el efecto del consumo de bebidas cola sobre la aterosclerosis.

Material y métodos

Se distribuyeron ratones ApoE-/- (8 semanas de edad) en tres grupos según el consumo libre de agua (A), bebida cola azucarada (C) y bebida cola edulcorada light (L). Al cabo de 8 semanas las bebidas cola se reemplazaron por agua. Los ratones fueron sacrificados secuencialmente: antes del tra-

tamiento (8 semanas de edad) y luego de su interrupción (16, 20, 24 y 30 semanas de edad). Se extrajeron la aorta ascendente y el hígado. Se calculó la relación entre el área de la placa aórtica y el espesor de la capa media (relación placa/media). Se evaluó la inflamación del parénquima hepático según la escala de NASH.

Resultados

La relación placa/media varió según la bebida ($F_{2,54} = 3,433$, $p < 0,04$) y la edad ($F_{4,54} = 5,009$, $p < 0,03$) y fue mayor en los grupos C y L ($p < 0,05$ a las 16 y 20 semanas, $p < 0,01$ a las 24 y 30 semanas). La inflamación del parénquima hepático ($F_{2,9} = 13,29$, $p < 0,002$) y portal ($F_{2,9} = 6,30$, $p < 0,02$) aumentó cinco y dos veces, respectivamente, en función del tiempo ($p < 0,01$ y $p < 0,03$) entre las semanas 20 y 30, en contraste con la esteatosis y el daño hepatocelular, que no se modificaron. El grupo A (evolución natural de la aterosclerosis) se caracterizó por la aceleración del crecimiento del área de placa en paralelo con un rápido aumento de la inflamación hepática alrededor de la semana 20.

Conclusiones

El consumo de bebidas cola en ratones ApoE-/- entre las semanas 8 y 16 de edad aumentó la tasa de progresión de la aterosclerosis. Los datos sugieren que, en este modelo murino, el consumo sostenido de bebidas cola durante las etapas tempranas de la vida puede acelerar el agravamiento del daño aterosclerótico en etapas más tardías de la vida.

Palabras clave > Aterosclerosis - Bebidas gaseosas - Apolipoproteína E

Conflicts of interest
None declared.

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