JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Apple Polyphenols and Fibers Attenuate Atherosclerosis in Apolipoprotein E-Deficient Mice

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Atherosclerosis, which is closely linked to nutritional habits, is a major cause of mortality in Western countries. Most of the previous investigations carried out on health effects of apples have been focused on their capacity to lower lipid concentration as well as on their antioxidant effects. The aim of the present study was to investigate the antiatherosclerotic effects of apple polyphenols and fibers. A crude apple polyphenol extract and low-viscosity apple fibers isolated from cider apples were administered separately or in association with the diet of apo E-deficient mice. After 4 months of supplementation, lipemia and oxidative stress biomarkers were measured and atherosclerotic lesions assessed by histomorphometry. Total plasmatic cholesterol and triacylgycerol levels were not affected by supplementation, and hepatic cholesterol level was lower in the group supplemented with both fibers and polyphenols. Uric acid concentrations and antioxidant capacity (FRAP) in plasma were reduced in all groups supplemented with polyphenols or fibers. The mean lesion area was reduced by 17, 38, and 38%, respectively, for the polyphenol, fiber, and polyphenol + fiber groups. Apple constituents supplied at nutritional doses therefore limit the development of atherosclerotic lesions in the aorta of apo E-deficient mice. On the basis of the results, we hypothesize that apple fibers and polyphenols may play a role in preventing atherosclerosis disease by decreasing uric acid plasma level.

KEYWORDS: Apple; polyphenols; fibers; apo E-deficient mice; atherosclerosis; uric acid

INTRODUCTION

A high consumption of fruits and vegetables has commonly been associated with a reduction of the risk of cardiovascular diseases in epidemiological studies (*1*). The protective role of fruits and vegetables is also supported by human intervention studies. The regular consumption of plant foodstuff, for several weeks or months, was shown to reduce cholesterolemia, oxidative stress, homocysteinemia, endothelial dysfunction, and blood pressure (*2–6*). In Western countries, apples account for an important part of the fruit intake and constitute an important dietary source of polyphenols, particularly flavonoids. It has been estimated that apples could provide approximately 22% of the *per capita* consumption of polyphenols in the United States (*7*). Apples, like many fruits, are also well recognized as sources of dietary fiber. Apple consumption has been associated with a reduced risk of cardiovascular diseases in the Women's Health Study since women ingesting apples had a 13−22% decrease in cardiovascular disease risk (*8*). In a Finnish study examining the link between flavonoid intake and coronary mortality, intake of flavonols with apple and tea as main contributors was inversely correlated with coronary mortality in women (*9*). Most investigations were focused on the lipid lowering effects and antioxidant properties of apples (*10–13*). These effects were mainly attributed to their content in fibers and polyphenols. Detailed studies on lipid-lowering effects have been conducted in animal models using whole apples or specific apple constituents. Aprikian et al. showed that a lyophilized apple-based diet improved lipid status and peroxidative parameters in obese Zucker rats (*10*). Gonzalez et al. also showed that apple pectins decrease cholesterol levels in liver and serum and also increase its release in feces (*14*). In spite of these cholesterol-lowering and antioxidative effects, the effects of apple or apple constituents on the development of atherosclerosis have never been examined. We investigated here the antiatherosclerotic effects of apple polyphenols and fibers in apolipoprotein E-deficient (apoE-KO) mice. The lack of apolipoprotein E results in an impaired clearance of lipoprotein and the spontaneous development of atherosclerotic lesions, when the mice are on a standard chow diet. Twenty week old mice exhibit atherosclerotic plaques resembling those observed in humans. This animal model has

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10.1021/jf800419s CCC: $40.75
Published on Web 06/18/2008

Table 1. Detailed Composition of Apple Polyphenol and Fiber Extracts

composition (% of dry matter)	apple fiber extract	apple polyphenol extract
moisture	9.4	5.3
lipids	0.2	1.0
proteins	1.6	3.0
carbohydrates	83.9	42.9
fibers	68.8	
simple sugars	15.1	42.9
minerals	3.9	3.0
polyphenols		44.8
procyanidins		27.0
flavonols		8.6
dihydrochalcones		5.9
hydroxycinnamic acids		2.1
catechins		1.2

Table 2. Formulation of the Experimental Diets[a]

ingredients (%)	control	AF	AP	AF+AP
wheat starch	62.95	46.25	62.20	45.50
casein	20	20	20	20
corn oil	7	7	7	7
cellulose	5	5	5	5
mineral mix AIN-93G	3.5	3.5	3.5	3.5
vitamin mix AIN-93G	1	1	1	1
L-cystine	0.3	0.3	0.3	0.3
choline bitartrate	0.25	0.25	0.25	0.25
tert-butylhydroquinone	0.0014	0.0014	0.0014	0.0014
apple fiber extract		16.70		16.70
apple polyphenol extract			0.75	0.75

[a] For details concerning apple polyphenol and fiber extracts see **Table 1**. AF, apple fibers; AP, apple polyphenols; AF+AP, apple fibers + apple polyhenols.

been used extensively to study the relationship between lipid metabolism, inflammation, and atherogenesis as well as to study the influence of diet or drugs on the development of lesions (*15*). In this work, apple polyphenols (AP) and apple fibers (AF) have been administered separately (AP and AF groups) or in association (AP+AF group) with apo E-deficient mice for 4 months to evaluate their effects on the development of the lesion, lipid, and oxidative stress parameters.

MATERIALS AND METHODS

Animals and Diets. *Products.* Polyphenols and fiber fractions were obtained from cider apples (Val de Vire Bioactives, Condé sur Vire, France). The composition of these extracts is detailed in **Table 1**. Polyphenol content in the polyphenol extract (Pomactiv HFV) was determined by HPLC before (all polyphenols except procyanidins) and after (procyanidins) phloroglucinolysis (*16, 17*). The fiber fraction (Pomelite) contains fibers (fraction insoluble in alcohol). The fiber content was determined as the sum of constituting sugars estimated as alditol acetate by GC (*18*). The sugar composition of the fibers was as follows: galacturonic acid (66%), rhamnose (2%), arabinose (15%), galactose (5%), xylose (2%), fucose (1%), mannose (2%), and glucose (8%). Fiber chain length varied between 250 and 1000 sugar units.

Mouse Feeding Study. Pairs of homozygous apo E-deficient mice were provided by Jackson Laboratories (Charles River Laboratories, L'Arbresle, France). The males used for the present study were obtained through interbreeding these homozygous mice in the animal experimental unit of the INRA Research Centre. Animals were housed in groups of four in wire-bottomed cages in a temperature-controlled room (22 ± 0.8 °C) with a 12 h light−dark cycle and a relative humidity of 55 ± 10%. Mice were maintained and handled according to the recommendations of the INRA Ethics Committee, decree no.87-848. Mean body weight at the start of the experiment was 22.1 ± 0.3 g. All mice were fed a powdered purified AIN-93G diet in which all carbohydrates were supplied as wheat starch. At 9 weeks of age, mice were divided into four groups (16 mice per group). One group received a control diet, and the three other groups a diet supplemented with either apple polyphenols extracts (AP), apple fibers (AF), or a mix of the apple fibers and polyphenols extracts (AF+AP). These experimental diets were isoenergetic, and their detailed compositions are given in **Table 2**. Food and distilled water were provided *ad libitum* during the following 16 weeks. At the end of the experiment, mice were sacrificed under pentobarbital anesthesia. Blood was collected from the abdominal aorta into heparinized tubes. Plasma was prepared by centrifugation at 12,000*g* for 2 min, and samples were stored at −20 °C. The organs were washed with heparinized physiological saline by direct injection in the left ventricle of the heart. The heart (with aorta) and liver were weighed and immediately frozen in liquid N_2 and stored at −80 °C until analysis. The whole cecum and the cecum contents were weighed individually for each mouse.

Plasma Lipids, Total Antioxidant Capacity, and Uric Acid. Plasma total cholesterol and triacylglycerol (TAG) were measured using enzymatic assays (Biomérieux,Marcy-l'Etoile, France). The ferric reducing ability of plasma (FRAP) was determined in plasma using the Benzie and Strain method (*19*), which measures the reduction of ferric ions to ferrous ions. The colorimetric measurement was performed at 593 nm, and the reaction was monitored for up to 4 min on 25 μL samples. Uric acid was determined in plasma using a commercial kit (ref: 61923 AU PAP 150, Biomérieux, Marcy-l'Etoile, France) and a Kone automated apparatus (Progress Plus, Konelab, Thermo-Electron SA). Optical density was measured at 510 nm using 4 μL plasma samples and quality controls (Calimat, ref 62 373 and Unitrol, ref 62 321, Biomérieux, Marcy-l'Etoile, France).

Hepatic Lipids. Liver samples were homogenized in KCl (9 g/L) with a Polytron homogenizer (Kinermatica GmbH, Lucerne, Switzerland), and lipids were chloroform−methanol (2:1, v/v) extracted under overnight agitation according to the method described previously (*20*). The chloroform phase was recovered after centrifugation and evaporated under dry air. TAG from the lipid residue were saponified with 0.5 mol/L KOH-ethanol at 70 °C for 30 min followed by the addition of 0.15 mol/L $MgSO_4$ to neutralize the mixture. After centrifugation (2000*g*; 5 min), the concentrations of glycerol in the supernatant fractions were determined. The cholesterol in the lipid residue was dissolved with isopropanol. Total cholesterol and TAG levels were determined by enzymic assay (Biomérieux, Marcy-l'Etoile, France). Absorbance at 492 nm was measured in a spectrophotometer (Uvikon 941 plus series; Kontron instruments, St Quentin en Yvelines, France).

Atherosclerotic Lesions. Atherosclerotic lesions were assessed by measuring lipid deposition in the aortic sinus (*21*). The peripheral fat of the upper aorta was removed and the thoracic and abdominal aortas discarded. The heart with the aortic arch was dissected under a stereo microscope and frozen in liquid N_2 in a cutting embedding medium for serial cryo-sectioning covering 400 μm of the aorta root. The heart was cut with a microtome (HM 560, Walldorf, Germany) at −20 °C. Sections (10 μm thick) were collected at every 100 μm throughout the aortic sinus (300 μm of the distal portion) and analyzed. The distal portion of the aortic sinus at the junction of the aorta and the heart was recognized by the three valve cusps. Microtome sections were evaluated for fatty streak lesions after staining with Oil red O and counterstaining with hematoxylin. Each section was evaluated for Oil red O staining area by capturing images directly from a color camera (Sony XC-71P CCD RGB, Kenmore, WA, USA) attached to an Olympus light microscope (Reichert-Jung Polyvar, Vienna, Austria). Images were displayed on a RGB monitor by using Visilog software (Noesis, Crolles, France). Image analysis was carried out using the ImageJ free software (http://rsb.info.nih.gov/ij/). In order to reduce errors induced by sectioning angle, results were expressed as the percentage of the cross-sectional vessel area stained with Oil red O.

Statistical Analysis. Results are expressed as mean values with their SEM. Data were analyzed by one-way ANOVA coupled with the Student−Newman−Keuls multiple comparison test (Statistica, Statsoft, USA) except for the analysis of the lesions areas for which a two-way ANOVA (mice groups and aortic sections) (REGWG test; Statview; SAS Institute Inc., Cary, NC, USA) was used. Differences were considered significant when the p value was ≤ 0.05.

Table 3. Body, liver and Caecum Weight in Male Homozygous Apolipoprotein E-Deficient Mice after 4 Months on Diets Supplemented with Apple Polyphenol or Fiber[a]

	control	AF	AP	AF+AP
body weight (g)	31.94 ± 0.77[a]	31.13 ± 0.63[a]	30.79 ± 0.63[a]	29.81 ± 0.66[a]
liver (g/100 g b.w.)	4.28 ± 0.15[a]	4.51 ± 0.24[a]	4.80 ± 0.12[a]	4.79 ± 0.16[a]
whole cecum (g)	0.18 ± 0.01[a]	0.75 ± 0.05[b]	0.22 ± 0.01[a]	0.82 ± 0.03[b]
cecum wall (g)	0.13 ± 0.06[a]	0.25 ± 0.01[b]	0.09 ± 0.01[a]	0.24 ± 0.01[b]

[a] Values are the means ± SEM ($n = 16$). Values not sharing the same superscript letter differ significantly ($p < 0.05$). AF, apple fibers; AP, apple polyphenols; AF+AP, apple fibers + apple polyhenols.

Table 4. Plasma Lipid and Uric Acid Concentrations and Total Antioxidant Capacity in Male Homozygous Apolipoprotein E-Deficient Mice after 4 Months on Diets Supplemented with Apple Polyphenol or Fiber[a]

	control	AF	AP	AF+AP
plasma cholesterol (mmol/L)	9.2 ± 0.7[a]	8.1 ± 0.9[a]	10.0 ± 0.7[a]	10.5 ± 0.7[a]
plasma triacylglycerol (mmol/L)	1.0 ± 0.2[a]	0.8 ± 0.1[a]	1.2 ± 0.1[a]	1.2 ± 0.1[a]
hepatic cholesterol (mg/g)	3.1 ± 0.2[a]	2.7 ± 0.2[a]	2.6 ± 0.1[a]	2.4 ± 0.1[b]
hepatic triacylglycerol (mg/g)	42.4 ± 7.6[a]	35.0 ± 4.6[a]	44.0 ± 6.1[a]	41.7 ± 8.2[a]
FRAP (μmol Fe^{2+}/mL)	413.8 ± 28.3[a]	299.7 ± 9.4[b]	398.7 ± 24.2[a,c]	366.1 ± 12.8[b,c]
uric acid (mg/L)	23.9 ± 3.1[a]	8.7 ± 1.6[b]	13.9 ± 1.6[b]	12.4 ± 1.0[b]

[a] Values are the means ± SEM ($n = 16$). Values not sharing the same superscript letter differ significantly ($p < 0.05$). AF, apple fibers; AP, apple polyphenols; AF+AP, apple fibers + apple polyhenols.

RESULTS

Body and Organ Weight. No difference in body and liver weight was observed between groups after feeding the diets supplemented with apple polyphenol extracts or apple fibers (**Table 3**). An increase in cecal weight, both whole cecum and cecum wall, was observed in the mice fed the diet supplemented with apple fibers (AF and AP+AF groups).

Lipids, Antioxidant Capacity, and Uric Acid in Plasma and Liver. Plasma total cholesterol and triacylglycerol showed similar levels in all groups (**Table 4**). Hepatic triacylglycerol levels were not modified, but hepatic cholesterol levels were significantly decreased in the AP+AF group. Uric acid, the major endogenous antioxidant in the plasma, was estimated. All of the diets supplemented with apple constituents induced a strong decrease of the uric acid level in plasma (**Table 4**). AF was the most efficient with a 63% reduction with respect to control mice. The AP and AP+AF groups showed a similar reduction of uric acid level (42 and 48%). A similar trend was observed for the plasma total antioxidant capacity (FRAP assay), which was significantly reduced in the AF and AF+AP groups by, respectively, 27 and 11% with respect to the control group (**Table 4**).

Atherosclerotic Lesions in the Aortic Sinus. Mice fed the diets supplemented with either polyphenols, fibers, or both showed a significant reduction of the mean lesion area with respect to the control group. The mean lesion area observed with the AF, AP, and AF+AP groups were, respectively, 38.3, 16.5, and 38.6% lower compared to that of the control group (**Figure 1**).

DISCUSSION

The aim of this study was to explore the antiatherogenic effect of different bioactive constituents of apple (polyphenols and fibers) in an animal model of atherosclerosis. A crude apple polyphenol extract containing all apple polyphenols in proportions similar to those found in the fruit (*17*) and an apple fiber extract was administered in the diet of apo E-deficient mice at a level close to the nutritional intake in humans. The supplementation level applied to the present experiment corresponds to an equivalent intake in humans of 1.6 g of polyphenols and 50 g of fiber per day, when expressed on the basis of diet content (for a human food intake estimate of 500 g dry weight). These values are close to the average total polyphenol dietary intake commonly estimated to 1 g/day (*22*) or to the dietary recommendations for fiber intake (30 g/day). The catechin intake level in the present experiment corresponds to an intake of 36 mg in humans, a value also close to the actual intake measured in various populations (11 to 121 mg/day) (*22*).

None of the supplemented diets modified the mouse body and liver weight. However, apple fibers induced an increase in the cecum wall weight of mice most likely due to the stimulation of the fermentation in the large intestine and the production of short-chain fatty acids (SFCA) known for their trophic effect on the cecum (*23*). All of the supplemented diets significantly reduced the development of the atherosclerotic plaques in the aortic sinus when compared to the control diet (**Figure 1**). Apple fibers showed a higher inhibitory effect than apple polyphenols. Association of apple fibers and polyphenols did not show a stronger effect than apple fibers alone. This inhibition of the development of the atherosclerotic lesions by apple fibers might be explained by the SCFA produced in the colon, which may inhibit smooth muscle cell proliferation and modulate the expression of various genes involved in oxidative stress and atherosclerosis processes (*24, 25*). Altogether, these results are consistent with previous publications showing a reduction of the progression of the atherosclerotic lesions upon consumption of tea extract (*26*), pomegranate juice (*27*), and grape extracts (*28*) all rich in polyphenols, or of inulin (*29*).

Apo E-deficient mice accumulate chylomicron and VLDL in blood as a result of a defect in their clearance by the liver (*15*), resulting in a plasma cholesterol level about five times higher (9.2 ± 0.7 mmol/L, **Table 4**) than that in C57BL/6J mice (1.8 mmol/L; in Mouse Phenome Database; Jackson Laboratory, Bar Harbor, ME, USA; http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=strains/details&strainid=7). None of the supplemented diets used (either apple polyphenols or fibers or both) modified the cholesterol and triacylglycerol levels in plasma. In the liver, only the diet associating apple fibers and apple polyphenols led to a reduction of the cholesterol level. This last result is consistent with a previous rat study from our laboratory in which the addition of both apple pectin and polyphenol-rich lyophilized apple to the diet was shown to lower

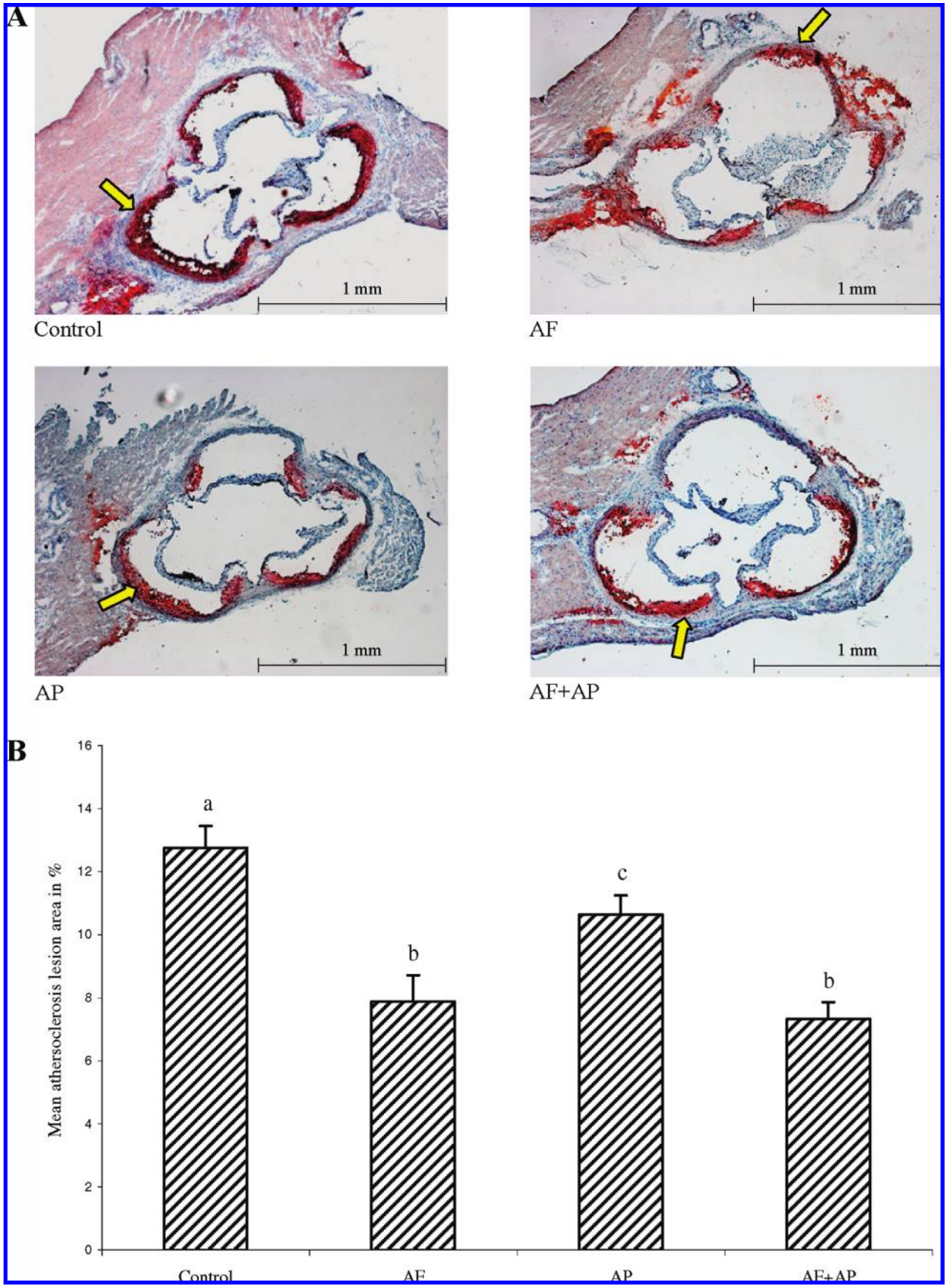

Figure 1. (**A**) Sections of aortic sinus stained by Oil red O from apo E-deficient mice fed a control, AF, AP, and AF+AP diet for 16 weeks. Yellow arrows indicate lipid deposition in aortic sinus. (**B**) Mean atherosclerotic lesion area. Values are represented by the mean ± SEM for 16 mice per group. Mean values not sharing a letter were significantly different ($p < 0.05$). AF, apple fibers; AP, apple polyphenols; AF+AP, apple fibers + apple polyhenols.

hepatic cholesterol levels (*30*). The absence of effects of apple polyphenols or of procyandins similar to those present in apple on liver lipids has previously been reported in the rat (*30–32*). A similar reduction of atherosclerotic lesions with tea catechins,

also independent of any effect on cholesterol has previously been reported in the same apo E-deficient mouse model (*26*).

The plasmatic antioxidant capacity or markers of oxidative stress are often measured to assess the effects of antioxidants such as polyphenols. Uric acid is the main contributor to the plasma antioxidant capacity (*19*), and an increase of its concentration in plasma has repeatedly but not systematically been reported after the consumption of polyphenols or polyphenol-rich foods (*33*). The consumption by human subjects of 5 apples was also shown to increase the plasma antioxidant capacity and plasma urate, but these effects were attributed to the fructose contained in the fruit (*34*). In the present study, apple fibers and polyphenols were found to significantly decrease the concentration of uric acid and FRAP levels in the plasma. A similar reduction of serum uric acid levels has been observed in rats fed pectin-enriched diets (*35*).

Our results are consistent with various observations suggesting that uric acid may actually increase the risk of cardiovascular diseases (*36*). A higher serum antioxidant capacity and uric acid has been associated with increased atherosclerosis risk in a prospective cohort case-control study (*37*). A higher level of uric acid in the plasma could result from an increased xanthine oxidase activity, a known source of superoxide free radicals, resulting in an impaired vascular function as observed in hypercholesterolemic rabbits (*38*). A higher plasma antioxidant capacity, often linked to an increase of uric acid level, could therefore rather be regarded as a risk factor of atherosclerosis rather than a protective factor as commonly considered in the field of antioxidants. As stressed recently by Strazzullo et al., a high serum uric acid level may have a different meaning depending on the population (*39*). An independent association between serum uric acid level and cardiovascular disease outcomes was observed in patients with arterial hypertension or those at high cardiovascular risk but not in the general population. A high level of serum uric acid may only be detrimental in conditions of acute metabolic stress, as is likely the case in the present apo E-deficient mice.

The meaning of a relatively high uric acid level in unstressed conditions or in the general population is less clear, as well as that of variations induced by dietary antioxidants. As already stressed above, uric acid level and antioxidant capacity measured in plasma and serum are often unaffected in polyphenol intervention studies (*33*). Furthermore, the value of serum antioxidant capacity to evaluate oxidative stress status has been questioned as no correlation could be observed between serum antioxidant capacity and different oxidative stress markers measured in the tissues of rats submitted to various oxidative stress (*40*). This raises concerns about the value of such serum biomarkers, and interpretations should be made with caution when evaluating the potential health effects of dietary antioxidants.

The strong reduction of uric acid in plasma observed here after supplementation with apple fibers and polyphenols may actually indicate a reduction of oxidative stress and an improved vascular function, and explain the reduction of the size of atherosclerotic lesions. The exact mechanisms are not known. These effects could be explained by an inhibition of uric acid renal reabsorption or an inhibition of xanthine oxidase as has been shown in the rat with various polyphenols (*41, 42*). On the basis of the present results, we hypothesize that apple fibers and polyphenols may play a role in preventing atherosclerosis disease, notably by decreasing the uric acid plasma level. However, precise mechanisms implicated in these processes have to be established.

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Received for review February 11, 2008. Revised manuscript received April 2, 2008. Accepted April 28, 2008. We are grateful to Val de Vire Bioactives (Condé sur Vire, France) for their financial support.

JF800419S