JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Commercial Extract of Fruits and Vegetables, Oxxynea, Acts as a Powerful Antiatherosclerotic Supplement in an Animal Model by Reducing Cholesterolemia, Oxidative Stress, and NADPH Oxidase Expression

THIBAULT SUTRA,[†,‡] KELLY DÉCORDÉ,[†] JÉROME RISS,[†] CONSTANT DALLAS,[§] JEAN-PAUL CRISTOL,[‡] AND JEAN-MAX ROUANET*,[†]

EA 3762 Nutrition & Aliments, Place E. Bataillon, Université Montpellier 2, 34095 Montpellier, France, EA 2993 Nutrition Humaine & Athérogénèse, Université Montpellier 1, CHU La Peyronie, 34925 Montpellier, France, and Nutraceutic Business Consulting (NB Consulting), 24 rue René Caillie, 34500 Béziers, France

The effects of fruit and vegetable extract (Oxxynea) on plasma cholesterol, early atherosclerosis, cardiac production of superoxide anion, and NAD(P)H oxidase expression were studied in an animal model of atherosclerosis. Thirty six hamsters were divided into two groups of 18 and fed an atherogenic diet for 12 weeks. They received by gavage either water or Oxxynea in water at a human dose equivalent of 10 fruits and vegetables per day. Oxxynea lowered plasma cholesterol and non-HDL cholesterol, but not HDL-cholesterol, and increased plasma antioxidant capacity. It also strongly reduced the area of aortic fatty streak deposition by 77%, cardiac production of superoxide anion by 45%, and $p22^{phox}$ subunit of NAD(P)H oxidase expression by 59%. These findings support the view that chronic consumption of antioxidants supplied by fruits and vegetables has potential beneficial effects with respect to the development of atherosclerosis. The underlying mechanism is related mainly to inhibiting pro-oxidant factors and improving the serum lipid profile.

KEYWORDS: Atherosclerosis; hamsters; fruits and vegetables; antioxidant compounds; NADPH oxidase

INTRODUCTION

Mortality from cardiovascular disease is the leading cause of death in the industrialized world. Diet is believed to play a major role in the development of this disease, and much research is being focused on identifying ways to prevent it through changes in dietary habits. Oxidation of low-density lipoproteins (LDL) is traditionally accepted as initiating processes leading to the development of atherosclerosis. The earliest events in the development of the pathology are endothelial dysfunction and oxidative stress in the vascular cell wall, activation of inflammatory cells, and migration of vascular smooth muscle cells to the intima with the modification of the extracellular matrix, leading to the artery remodeling (*1*). Development of atherosclerosis is thought to be closely dependent upon increased oxidative stress, that is, an imbalance between reactive oxygen species (ROS) generation (chiefly superoxide anions, hydrogen peroxide, hydroxyl radicals) and natural cell antioxidant capacity in favor of the former (*2*). ROS can regulate many signaling pathways, such as infiltration of monocytes in intima and vascular smooth muscle cell proliferation. The cause of oxidative stress observed in atherosclerosis awaits clarification. Recent findings have suggested that the major source of ROS in the vascular wall, and also in vascular smooth muscle cells, is the NAD(P)H oxidase system. This is a membrane-associated enzyme, composed of five subunits, catalyzing the one-electron reduction of oxygen, using NADH or NADPH as the electron donor. NAD(P)H oxidase generates significant amounts of superoxide radicals, and an association between enzymatic activity and clinical risk factors in atherosclerosis has been shown (*3*). Moreover, expression of membrane subunits of NAD(P)H oxidase, such as $p22^{phox}$, Nox 1, and Nox 4, is modulated in atherosclerotic arteries (*4*) and in vascular injury (*5*) by various cytokines like interferon (IFN)-γ and transforming growth factor (TGF)-β1. Azumi et al. (*6*) found that the severity of atherosclerotic lesion correlated with $p22^{phox}$ overexpression in coronary arteries. Excessive generation of superoxide anion by phagocyte NADPH oxidase is responsible for LDL oxidation, which is the key factor in the initation and progression of atherosclerosis (*1*, *7*). The contribution of NADPH oxidase to the pathogenesis of atherosclerosis overshoots LDL oxidation process. NAD(P)H oxidase induces the expression of adhesion molecules in endothelial cells for recruitment of monocyte-derived macrophages (*8*), leading to an amplification system (*9*) and vascular smooth muscle cells proliferation (*10*).

* Author to whom correspondence should be addressed [telephone/fax 33 (0)4 67 14 35 21; e-mail jm.rouanet@univ-montp2.fr].
[†] Université Montpellier 2.
[‡] Université Montpellier 1.
[§] NB Consulting.

10.1021/jf070029n CCC: $37.00
Published on Web 04/20/2007

The importance of antioxidants in human health has become increasingly clear, and some epidemiological studies showed the potential health benefits of dietary antioxidants (*11*). Fruits and vegetables consumption is inversely related to cancer and coronary heart disease mortality, and this appears not to be due exclusively to high levels of vitamins and fibers (*12*, *13*). Several studies have shown that flavonoids also contribute to the overall antioxidant capacity of fruits and vegetables and also to the beneficial effects, a view supported by recent research demonstrating that dietary flavonoids protect against vascular diseases and reduce the risk of myocardial infaction (*13*). There is growing interest in flavonoids and phenolic compounds because they are potent antioxidants and inhibit low-density lipoprotein (LDL) oxidation in vitro (*14*), properties that are associated with their ability to scavenge free radicals and chelate metals. An increased consumption of phenolics has been correlated with a reduced risk of cardiovascular diseases and certain types of cancers (*15*, *16*). Moreover, polyphenols have been shown to directly interact with NAD(P)H oxidase, inhibiting most of the ROS production in the vessel wall (*17*). Cumulatively or synergistically, these dietary antioxidants provide bioactive mechanisms to reduce oxidative stress.

With the exception of recent research by Adams et al. (*18*) with a transgenic mice model, few studies have investigated the effect of plant material on atherosclerosis and oxidative stress in rodents (*19*, *20*); moreover, these studies were only focused on the effect of vegetables. Golden Syrian hamsters fed a fat-rich diet develop dyslipidemia and atherosclerotic plaques, similar in many respects to human atheroma (*21*−*23*). Hamsters were selected for this study because of their responsiveness to plasma cholesterol lowering and anti-atherogenic interventions (*24*). Moreover, hamster has a plasma lipoprotein distribution similar to that of humans and LDL as the major plasma cholesterol carrier. To induce an oxidative stress, their high cholesterol and high fat diet was rendered deficient in vitamin C and E and in selenium. This study was designed to trigger the arterial wall response to such a stress (fatty streak formation and aortic atherosclerosis emergence) and then to look at the modulation of this effect by a commercial fruit and vegetable extract, Oxxynea. In addition, for the first time, modulation of oxidative stress parameters including cardiac production of superoxide anions and NAD(P)H oxidase expression was measured in this model.

MATERIALS AND METHODS

Fruits and Vegetable Extract. According to the manufacturer (NB Consulting, Béziers, France), the powdered Oxxynea extract was obtained from 22 varieties of antioxidant-rich fruits and vegetables including apple, asparagus, bilberry, apricot, black currant, broccoli, carrot, cherry, cucumber, garlic, grapefruit, green cabbage, olive, onion, orange, papaya, pineapple, red and white grapes, strawberry, tea, tomato, and wheat germ. Oxxynea contains high level of catechins, that is, sum of procyanidin dimers B1, B2, B3, and B4 (1.14 g/100 g) and monomeric catechins (catechin, 0.55 g/100 g; epicatechin, 3.08 g/100 g; epichatechin-3-*O*-gallate, 4.10 g/100 g; epigallocatechin, 4.17 g/100 g; epigallocatechin-3-*O*-gallate, 21.33 g/100 g). Other phenolic compounds such as gallic acid and anthocyanins were detected in lower amounts (0.15 and 0.6 g/100 g, respectively). The extract also contained low levels of lycopene (28 mg/100 g) and vitamin C (4.92 mg/100 g).

Oxygen Radical Absorbance Capacity (ORAC) Value. The ORAC-fluorescein assay was based on the method of Ou et al. (*25*) that was subsequently modified by Davalos et al. (*26*). Briefly, the reaction was performed in 75 mM phosphate buffer (pH 7.4), and the final assay mixture (200 μL) contained fluorescein (120 μL, 70 nM final concentration) as oxidizable substrate, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, 60 μL, 12 mM final concentration) as oxygen radical generator, and antioxidant (20 μL, either trolox [1−8 μM, final concentration] or Oxxynea [at different concentrations]). The reaction was performed at 37 °C, and fluorescence was recorded every minute for 80 min. ORAC values was expressed as trolox equivalents by using the standard curve calculated for each experiment. The final ORAC value for Oxxynea was 6100 μmol of trolox equiv/g.

Animals. Male golden Syrian hamsters (Janvier, Le Genest-St-Isle, France) weighing 85−95 g were randomly divided into groups with approximately equal mean group body weights. The animals were housed in plastic cages in a temperature-controlled room (23 ± 1 °C) subjected to a 12 h light:dark cycle (lights on at 0700 h) with free access to both food and water. Hamsters were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines (*27*).

Diets and Feeding Procedures. Two experiments were carried out concomitantly. Experiment 1 was used to determine at what point hamsters on the atherogenic diet exhibited oxidative stress and hypercholesterolemia. In such a way, four groups of six hamsters each received either a standard or an atherogenic diet for 15, 30, 45, and 84 days. The standard diet consisted of 200 g/kg casein and 3 g/kg L-methionine, 447 g/kg corn starch, 175 g/kg sucrose, 50 g/kg cellulose, 80 g/kg vegetable oil (corn oil/sunflower oil, 1/1), mineral mix (35 g/kg), and vitamin mix (10 mg/kg). The atherogenic diet has been previously described (*22*) and consisted of 200 g/kg casein and 3 g/kg L-methionine, 393 g/kg corn starch, 154 g/kg sucrose, 50 g/kg cellulose, 150 g/kg lard, 5 g/kg cholesterol, mineral mix (35 g/kg), and vitamin mix (10 mg/kg). Vitamin and mineral mixes were formulated according to AIN-93 guidelines (*28*) and supplied by Scientific Animal Food & Engineering (SAFE, Augy, France). The atherogenic diet did not contain selenium, vitamin C, and vitamin E.

In experiment 2, two groups of 18 hamsters were fed the atherogenic diet for 84 days. The hamsters of each group were fed daily by gavage either tap water (group 1; control) or a solution of Oxxynea in water (group 2; experimental). The volume of the solutions force-fed was adjusted daily to the weight of the hamsters and was established by extrapolating 500 mL/d water drinking for a 70 kg human. This represents a volume of 7.14 mL/(kg of body weight·d). Based upon the ORAC value of fruits and vegetables starting material and issuing Oxxynea, and according to a recommended consumption of 10 servings of fruits and vegetables/d for a human, that is, ∼800 g/d, hamsters from the experimental group received 21.4 mg of Oxxynea/(kg body weight·d) dissolved in water.

Analytical Procedures. At the end of each experimental period, hamsters were deprived of food for 18 h and were anesthetized with an IP injection of pentobarbital (60 mg/mL at a dosage of 60 mg/kg body weight). In experiment 1, only plasma cholesterol, cardiac superoxide anion production, and NADPH oxidase expression were measured as described below. In experiment 2, blood was drawn by cardiac puncture with heparin moistened syringes, and plasma was prepared by centrifugation at 2000*g* for 10 min at 4 °C, then stored at −80 °C prior to analysis. Plasma concentrations of total cholesterol (TC) and HDL cholesterol (HDL-C) were measured by an enzymatic technique (Konelab, Thermo Electron Corp., Vantaa, Finland). Plasma nonHDL-C was calculated from the difference between TC and HDL-C.

The antioxidant capacity of plasma was measured as trolox equivalent, that is, a quantitative value for general antioxidant levels in biological samples (*29*, *30*), which was assayed in plasma with a quantitative colorimetric technique according to the kit supplier's instructions (Kit NX2332; Randox, Mauguio, France). The assay is based on the incubation of a peroxidase and H_2O_2 with 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) to produce the radical cation $ABTS^{\circ+}$. This has a relatively stable blue−green color, which is measured at 600 nm. Antioxidants (albumin, uric acid, ascorbic acid, α-tocopherol, glutathione, beta-carotene, etc.) in the sample suppressed $ABTS^{\circ+}$ color production to a degree proportional to their concentration.

Aortic Tissue Processing. Following blood collection and liver removal, the intact aorta from 12 hamsters was first perfused with phosphate buffered saline containing 1 mmol/L $CaCl_2$ and 15 mmol/L glucose for 5 min, then with 0.1 mmol/L sodium cacodylate buffer pH 7.4 containing 2.5 mmol/L $CaCl_2$, 2.5% paraformaldehyde, and 1.5%

glutaraldehyde for the fixation of the vasculature. The aorta was carefully dissected between sigmoid valves and 3−4 cm after the aortic arch and thoroughly cleaned of loose adventitial tissue; the aortic arch was cut free, open longitudinally along the outside of the arch, pin corked, immersed in fresh fixative solution, and stored at 4 °C until staining. The aortic arches were then first rinsed for 48 h in 0.1 mol/L sodium cacodylate buffer pH 7.4 containing 30 mmol/L $CaCl_2$ and 250 mmol/L sucrose. The arches were then rinsed in distilled water, stained for 40 s in Harris hematoxylin, rinsed in distilled water, and then quickly in 70% isopropyl alcohol; finally, they were stained in Oil red O for 30 min according to Nunnari et al. (*31*), rinsed in 70% isopropyl alcohol, and back to distilled water. Each aortic arch was then directly displayed on a glass slide, endothelium side up, covered with Aquamount mounting medium and cover slips, and observed en face by light microscopy. All segments were photographed using a video digitizer. A computerized image analysis system (ImageJ, Scion Corp., Frederick, MD) attached to a compound light microscope was used to measure the total Oil Red O stained area of each aortic arch. The area covered by foam cells (aortic fatty streak lesion area or AFSA) was expressed as a percentage of the total area surveyed.

Determination of Superoxide Anion Production. Superoxide anion production was evaluated in hamsters that were not used for AFSA measurement ($n = 6$ per group). Briefly, the left ventricle (150 mg) (*41*) was placed in Krebs buffer containing 250 μM of lucigenin, and the intensity of luminescence was recorded on a luminometer (Perkin-Elmer Wallac, Victor, Turku, Finland). Results were expressed as count/mg of protein.

Immunoblotting. Proteins were extracted as previously described (*32*) from the frozen left ventricles of six hamsters previously used for measurement of superoxide anion production. Samples were homogenized using an ultra turrax T25 basic (Irka-Werke) in an ice cold extraction buffer containing 120 mM NaCl, 25 mM KCl, 2 mM $CaCl_2$, 15 mM Tris−Cl pH 7.5, 0.5% Triton X-100, 1 mM PMSF, 0.1 mM DTT, 10 M leupeptin, and 1 M pepstatin. Protein concentrations in sample were determined by Bio-Rad Dc protein assay using BSA as a standard. Proteins (50 μg) were separated with 12% SDS-PAGE and then transferred to a nitrocellulose membrane (45 min, 100 V). Membranes were incubated for 2 h with primary antibody against $p22^{phox}$ (1/200, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer. After six washes (6 °C, 5 min) in TBS/Tween under gentle agitation, blots were incubated for 45 min with horseradish peroxidase-labeled antibody (1/5000). After further washes, blots were treated with enhanced chemiluminescence detection reagents (ECL, Amersham), and areas (mm^2) were measured using the BIO-Profil 1D software (Fisher Bioblock).

Statistical Analyses. Data are shown as the means ± SEM. Data were subjected to logarithmic transformation where necessary to achieve homogeneity of variances. Statistical analysis of the data was carried out using the Stat View IV software (Abacus Concepts, Berkeley, CA) by one-way ANOVA followed by Fisher's protected least significant difference test. Differences were considered significant at $P < 0.05$.

RESULTS

Evolution of Cholesterol Concentration, Superoxide Production, and NADPH Oxidase Expression during Early Development of Atherosclerosis (Experiment 1). Plasma cholesterol significantly increased in the hamsters fed the atherogenic diet just from the first 15 days as compared to the controls animals. No alteration in cholesterol was observed in the control hamsters fed the standard diet, whereas in hamsters fed the atherogenic diet, plasma cholesterol level significantly increased from day 30 (7.76 ± 0.46 mmol/L) to day 45 (10.18 ± 1.47 mmol/L) and leveled to 84 days (**Figure 1a**).

The time course of cardiac superoxide production was also established. Whereas the cardiac superoxide level was constant during 84 days in the control hamsters, we noted an increase of superoxide production in atherogenic hamsters compared at 15, 30, 45, and 84 days by 18.5% (not significant, NS), 21.5% (NS), 21.8% ($p = 0.0548$), and 94.1% ($p < 0.0001$), respectively, such differences being only significant at 84 days (**Figure 1b**). In addition, cardiac superoxide levels increased by 20.5% (NS) from day 15 to day 45 and were highest at 84 days (**Figure 1b**) in hamsters fed atherogenic diet. In agreement with the cardiac superoxide production at 84 days of atherogenic diet, the measure of cardiac NADPH oxidase expression by western blot showed that cholesterol diet triggered a significant expression of $p22^{phox}$ (**Figure 3**) by 146% ($p = 0.001$).

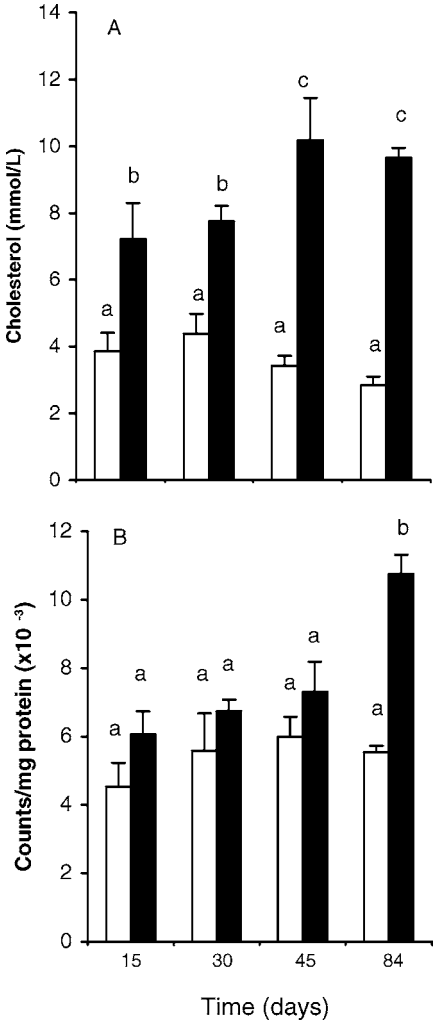

Figure 1. Time course experiment of plasma cholesterol concentration (**A**) and superoxide anion production (**B**) in hamsters fed a standard diet (white bars) and in hamsters fed an atherogenic diet (black bars) during experiment 1. Values are expressed as mean ± SEM of triplicate wells ($n = 6$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

Oxxynea Improves Blood Lipid Profile. Nutritional parameters are shown in **Table 1**. No significant difference appeared in food intake and final body weight between the two groups. Plasma lipids are summarized in **Table 2**. Oxxynea significantly reduced plasma total cholesterol by 11.7% ($p < 0.0001$) and non-HDL cholesterol by 14% ($p = 0.0066$), but not HDL-cholesterol, as compared to the control group. Consequently, the atherogenic index calculated as total cholesterol/HDL-cholesterol was lowered by 8.3% ($p = 0.0139$) in hamsters receiving Oxxynea.

Oxxynea Improves Antioxidant Status and Decreased $O_2^{\cdot -}$ by Preventing NADPH Oxidase Expression. In experiment 2, Oxxynea significantly increased by 10% the plasma antioxidant capacity induced by the atherogenic diet ($p = 0.0244$) (**Table 2**). As shown in **Figures 2** and **3**, superoxide

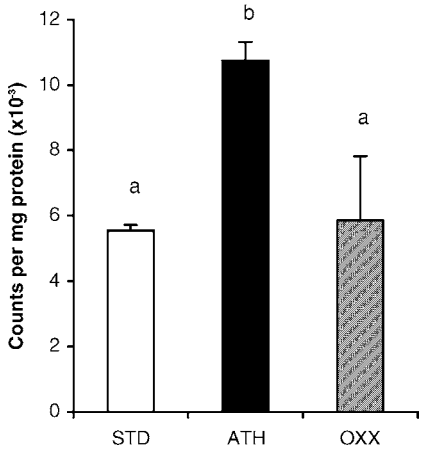

Figure 2. Cardiac superoxide anion production in hamsters fed a standard or an atherogenic diet with (OXX) or without (ATH) Oxxynea. Values are expressed as mean ± SEM of triplicate wells ($n = 6$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

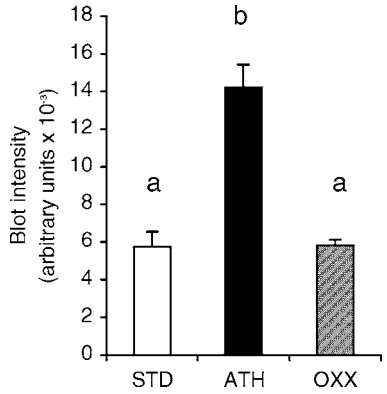

Figure 3. Expression of the cardiac p22^{phox} subunit of NAD(P)H oxidase in hamsters fed a standard diet (STD) or an atherogenic diet with (OXX) or without (ATH) Oxxynea during 84 days. The densitometric measurement shows arbitrary area units. Values are expressed as mean ± SEM ($n = 6$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

Table 1. Effects of Ingestion of a Fruit and Vegetable Extract (Oxxynea) on Body Weight and Food Intake of Hamsters Fed an Atherogenic Diet[a] (Experiment 2)

group	atherogenic diet	atherogenic diet + Oxxynea
initial body weight, g	91.3 ± 2.1[a]	86.7 ± 5.8[a]
final body weight, g	130.9 ± 9.7[a]	129.6 ± 1.8[a]
food intake, g/d	3.47 ± 0.90[a]	3.49 ± 0.60[a]

[a] Values are means ± SEM, $n = 18$. Data were analyzed by one-way ANOVA followed by the least significant difference test. For each dietary treatment, means in a column with different letters differ, $P < 0.05$.

anion production (**Figure 2**) and expression of p22^{phox} (**Figure 3**) decreased by 45.5% ($p < 0.0001$) and 59.1% ($p = 0.001$), respectively, in hamsters receiving Oxxynea.

Oxxynea Powerfully Inhibits Lipid Deposition in Aortic Arch. Average aortic fatty streak accumulation (AFSA), measured as the percentage of Oil Red O staining relative to the total area surveyed (**Figure 4**), was not detected in hamsters fed the standard diet (experiment 1). In addition, AFSA was significantly reduced by 77% ($p = 0.001$) in the hamsters receiving Oxxynea as compared to control animals on the atherogenic diet (experiment 2).

Table 2. Effects of Ingestion of a Fruit and Vegetable Extract (Oxxynea) on Plasma Lipid Concentrations and on Plasma Antioxidant Capacity (PAC) in Hamsters Fed an Atherogenic Diet[a] (Experiment 2)

group	atherogenic diet	atherogenic diet + Oxxynea
TC[b] (mmol/L)	9.54 ± 0.20[a]	8.42 ± 0.16[b]
HDLC[c] (mmol/L)	6.01 ± 0.27[a]	5.90 ± 0.16[a]
non-HDLC (mmol/L)	3.12 ± 0.15[a]	2.68 ± 0.17[b]
atherogenic index[d]	1.56 ± 0.04[a]	1.43 ± 0.03[b]
PAC (mmol/L)	1.29 ± 0.06[a]	1.42 ± 0.10[b]

[a] Values are means ± SEM, $n = 18$. Data were analyzed by one-way ANOVA followed by the least significant difference test. For each dietary treatment, means in a column with different letters differ, $P < 0.05$. [b] TC : total cholesterol. [c] HDLC : high-density lipoprotein cholesterol. [d] Total cholesterol/HDL-cholesterol.

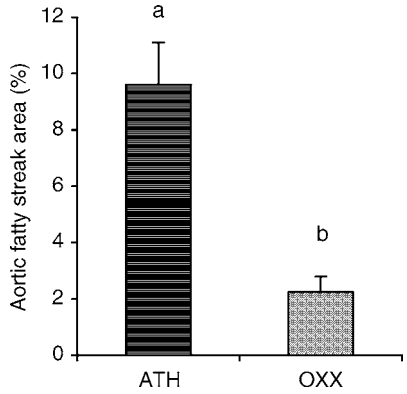

Figure 4. Effects of ingestion of water (ATH) or a fruit and vegetable extract Oxxynea (OXX), on aortic fatty streak area in hamsters fed an atherogenic diet for 84 days (experiment 2). Values are expressed as mean ± SEM ($n = 12$). Bars with different index letters differ ($P < 0.05$).

DISCUSSION

This study reported the protective effect of fruit and vegetable antioxidants supplementation against diet-induced oxidative stress and atherosclerosis in hypercholesterolemic golden Syrian hamsters.

The golden Syrian hamster is a good nutritional rodent model of atherosclerosis, which could mimic the early stages of human atherosclerosis, that is, fatty streak (*33*). As previously reported, a high fat diet led to an early increase in total and non-HDL cholesterol after 15 days of diet leading to lipid deposition on aortic arch at 84 days. Interestingly, the atherogenic diet-induced hypercholesterolemia is in parallel accompanied by a tendency of superoxide anion overproduction, which reaches the significance at 45 days. In agreement with oxidative hypothesis of atherosclerosis, it could be postulated that NADPH oxidase expression and activity conspire with high non-HDL cholesterol level to induce foam cells and fatty streak.

As reported in other rodent models of atherosclerosis, such as insulin resistance (*32*) or hypertension (*34*), oxidative stress could be a key event in diet-induced atherosclerosis and cardiac remodelling. Hypercholesterolemia has been previously involved in enhanced ROS production by NADPH oxidase activity (*35*) in a model of cholesterol-fed mice. It has been further suggested that ROS overproduction could be linked to an induction of NADPH oxidase subunit in particular gp91^{phox} in neutrophils from hyperlipidemic guinea pig (*36*). Our observation of a hypercholesterolemic diet-induced NADPH oxidase expression (+146%) in cardiac tissue extends these observations. Superoxide anion and further ROS generation by monocyte-derived macrophages could oxidize LDL, being in turn responsible for

amplification loops by stimulation of phagocyte NADPH oxidase. Beyond generation of foam cells and cholesterol deposition (*37*), cholesterol-induced ROS generation could participate in left ventricle remodelling as suggested by the enhanced expression of $p22^{phox}$ in the infarcted sites.

Oxxynea, a fruit and vegetable antioxidant extract, prevented the progression of early atherosclerosis in aortic arch of cholesterol-fed hamsters (<10% foam cell coverage of aorta). In agreement with the recent report by Adams et al. (*18*) that a diet rich in green and yellow vegetables inhibits atherosclerosis in transgenic mice, we have shown that Oxxynea extract prevents fatty streak formation in aortic arch of cholesterol fed hamster. This effect could be in part due to a slight, but significant, decrease in total and non-HDL cholesterol, without affecting HDL cholesterol. The resulting improvement of atherogenic index obtained with fruit and vegetable extract extends the previous observation on the beneficial effects in lipid parameters obtained with grape polyphenols (*23*). On the other hand, nutritional antioxidants supplied from Oxxynea could act throughout the improvement of antioxidant defenses as demonstrated by significant increase in plasma antioxidant capacity. This free radical scavenging capacity evidenced in plasma is in agreement with the ORAC value observed in vitro and could account in part for protection against LDL oxidation reported for numerous polyphenols such as catechin, epicatechin, epicatechin-3-*O*-gallate, epigallocatechin, and epigallocatechin-3-*O*-gallate (*38*).

Furthermore, our findings suggest for the first time that the fruit and vegetable antioxidant extract could prevent both NAD(P)H oxidase expression and $O_2^{\circ -}$ overproduction in the heart from hypercholesterolemic hamster. Here again, NAD(P)H oxidase inhibition could be involved in prevention of LDL oxidation and further atherosclerosis steps. Beyond the vicious circles linked to LDL oxidation, the inhibition of ROS production by NAD(P)H oxidase system could also prevent other early events in cardiovascular diseases such as endothelial dysfunction or arterial remodelling. A recent study has shown that endothelium-dependent vasorelaxation is impaired in the high lipid-fed golden syrian hamster (*39*). Our current results showing that the fruit and vegetable extract inhibits the overproduction of $O_2^{\circ -}$ by NAD(P)H system strongly suggest that Oxxynea may prevent the endothelial dysfunction. Indeed, an overproduction of superoxide anion that could react with NO° to produce peroxinitrite has been involved in the hypercholesterolemia-induced impairment of vasorelaxant system (*40*). On the other hand, we have shown that an overproduction of ROS is strongly associated with cardiac remodelling, suggesting a pathogenic role of oxidative stress in its constitution (*41*). Pharmacological or nutritional intervention could prevent both NAD(PH) oxidase expression and activity and cardiac hypertrophy (*41*). Our results showing that hypercholesterolemic diet activates and that vegetable and fruit extracts inhibit NAD(P)H expression and activity in the heart reinforce the hypothesis of a nutritional modulation of ROS enzymatic producing systems.

Finally, improvement of plasma lipid profile, increase in PAC, and decrease in superoxide anion production and reduction of NAD(P)H oxidase expression ($p22^{phox}$ subunit) by Oxxynea were associated with a total prevention of aortic fatty streak lesion area. The relative contribution of each parameter such as lipid profile, plasma antioxidant defenses, and overproduction of ROS is difficult to establish. However, it is tempting to speculate on a specific role of tissular oxidative stress. Indeed, in a previous paper, it has been shown that the wine polyphenols-induced aortic fatty streak lesion area prevention was associated with lipid and plasma antioxidant capacity improvement without any effect on plasma oxidative stress markers such as MDA, AOPP, and AGEs (*21*). Taken together, these results suggest a specific role of polyphenol in vascular tissue mediated by NAD(P)H oxidase.

All of these results suggest that this extract acted by mechanisms operating both inside and outside a hypolipemic effect, especially an antioxidant effect. Although the constituent-(s) responsible for these effetcs remain(s) unclear, candidates such as vitamin C, vitamin E, carotenoids, selenium, and polyphenols could act synergistically or additively to prevent atherosclerosis in the hamster model. These promising results obtained in a diet-induced atherosclerosis animal model give rise to further studies in clinical fields.

LITERATURE CITED

(1) Ross, R. Atherosclerosis − an inflammatory disease. *N. Engl. J. Med.* **1999**, *340*, 115−26.

(2) Frei, B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. *Am. J. Med.* **1994**, *97*, 5S−13S, 22S−8S.

(3) Guzik, T. J.; West, N. E.; Black, E.; McDonald, D.; Ratnatunga, C.; Pillai, R.; Channon, K. M. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ. Res.* **2000**, *86*, E85−90.

(4) Lassegue, B.; Clempus, R. E. Vascular NAD(P)H oxidases : specific features, expression, and regulation. *Am. J. Physiol.: Regul., Integr. Comp. Physiol.* **2003**, *285*, R277−97.

(5) Szöcs, K.; Lassegue, B.; Sorescu, D.; Hilenki, L. L.; Valppu, L.; Couse, T. L.; Wilcox, J. N.; Quinn, M. T.; Lambeth, J. D.; Griendling, K. K. Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler., Thromb., Vasc. Biol.* **2002**, *22*, 21−7.

(6) Azumi, H.; Inoue, N.; Takeshita, S.; Rikitake, Y.; Kawashima, S.; Hayashi, Y.; Itoh, H.; Yokoyama, M. Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation* **1999**, *100*, 1494−8.

(7) Aviram, M. Modified forms of LDL and atherosclerosis. *Atherosclerosis* **1993**, *98*, 1−9.

(8) Aviram, M.; Rosenblat, M.; Etzioni, A.; Levy, R. Activation of NADPH oxidase required for macrophage-mediated oxidation of low-density lipoprotein. *Metabolism* **1996**, *45*, 1069−79.

(9) Heinloth, A.; Heermeyer, K.; Raff, U.; Wanner, C.; Galle, J. Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of human vascular endothelial cells. *J. Am. Soc. Nephrol.* **2000**, *11*, 1819−25.

(10) Zhao, G. F.; Sen, J. J.; Zhang, H.; She, M. P. Effects of oxidized low density lipoprotein on the growth of human artery smooth muscle cells. *Chin. Med. J. (Beijing, Engl. Ed.)* **2005**, *118*, 1973−8.

(11) Block, G. The data support a role for antioxidants in reducing cancer risk. *Nutr. Rev.* **1992**, *50*, 207−13.

(12) Parodi, P. W. The French paradox unmasked: the role of folate. *Med. Hypotheses* **1997**, *49*, 313−8.

(13) Hertog, M. G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med.* **1995**, *155*, 381−6.

(14) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−7.

(15) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317−33.

(16) Duthie, G.; Crozier, A. Plant-derived phenolic antioxidants. *Curr. Opin. Lipid* **2000**, *11*, 43−7.

(17) Orallo, F.; Alvarez, E.; Camina, M.; Leiro, J. M.; Gomez, E.; Fernandez, P. The possible implication of trans-Resveratrol in the cardioprotective effects of long-term moderate wine consumption. *Mol. Pharmacol.* **2002**, *61*, 294−302.

(18) Adams, M. R.; Golden, D. L.; Chen, H.; Register, T. C.; Gugger, E. T. A diet rich in green and yellow vegetables ihibits atherosclerosis in mice. *J. Nutr.* **2006**, *136*, 1886−89.
(19) Nicolle, C.; Cardinaut, M.; Aprikian, O.; Busserolle, J.; Grolier, P.; Rock, E.; Demigné, C.; Mazur, A.; Sclabert, A.; Amouroux, P.; Rémézy, C. Effect of a carrot intake on cholesterol metabolism and on anti-oxidant status in cholesterol-fed rat. *Eur. J. Nutr.* **2003**, *42*, 254−61.
(20) Nicolle, C.; Cardinaut, M.; Gueux, E.; Jaffrelo, L.; Rock, E.; Mazur, A.; Amouroux, P.; Rémézy, C. Health effect of vegetable-based diet : lettuce consumption improves cholesterol metabolism and antioxidant status in the rat. *Clin. Nutr.* **2004**, *23*, 605−14.
(21) Auger, C.; Teissèdre, P. L.; Gérain, P.; Lequeux, N.; Bornet, A.; Serisier, S.; Besançon, P.; Caporiccio, B.; Cristol, P.; Rouanet, J. M. Dietary wine phenolics catechin, quercetin and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation. *J. Agric. Food Chem.* **2005**, *53*, 2015−21.
(22) Auger, C.; Caporiccio, B.; Landrault, N.; Teissèdre, P. L.; Laurent, C.; Cros, G.; Besançon, P.; Rouanet, J. M. Red wine phenolic compounds reduce plasma lipids and apolipoprotein B, and prevent early aortic atherosclerosis in hypercholesterolemic Golden Syrian hamsters (Mesocricetus auratus). *J. Nutr.* **2002**, *132*, 1207−13.
(23) Vinson, J. A.; Teufel, K.; Wu, N. Red wine, dealcoholized red wine, and espacially grape juice, inhibit atherosclerosis in a hamster model. *Atherosclerosis* **2001**, *156*, 67−72.
(24) Kowala, M. C.; Nunnari, J. J.; Durham, S. K.; Nicolosi, R. J. Doxazosin and cholestyramine similarly decrease fatty streak formation in the aortic arch of hyperlipidemic hamsters. *Atherosclerosis* **1991**, *91*, 35−49.
(25) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−26.
(26) Davalos, A.; Gomez-Cordoves, C.; Bartolomé, B. Extending applicability of oxygen radical absorbance capacity (ORAC-Fluorescein) assay. *J. Agric. Food Chem.* **2004**, *52*, 48−54.
(27) National Research Council. *Guide for the Care and the Use of Laboratory Animals. Publication no. 85-23(rev.)*; National Institutes of Health: Bethesda, MD, 1985.
(28) Reeves, P. G.; Nielsen, F. H.; Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76 rodent diet. *J. Nutr.* **1993**, *123*, 1939−51.
(29) Rice-Evans, C.; Miller, N. J. Total antioxidant status in plasma and body fluids. *Methods Enzymol.* **1994**, *234*, 279−93.
(30) Koechlin, C.; Couillard, A.; Cristol, J. P.; Chanez, P.; Hayot, M.; Le Gallais, D.; Prefaut, C. Does systemic inflammation trigger local exercise-induced oxidative stress in COPD? *Eur. Respir. J.* **2004**, *23*, 538−44.
(31) Nunnari, J. J.; Zand, T.; Joris, I.; Majno, G. Quantification of Oil red O staining of the aorta in hypercholesterolemic rats. *Exp. Mol. Pathol.* **1989**, *51*, 1−8.
(32) Delbosc, S.; Paizanis, E.; Magous, R.; Araiz, C.; Dimo, T.; Cristol, J. P.; Cros, G.; Azay, J. Involvement of oxidative stress and NADPH oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. *Atherosclerosis* **2005**, *179*, 43−9.
(33) Nikkari, S. T.; Salokivi, T.; Jaakkola, O. The hyperlipidemic hamster as an atherosclerosis model. *Artery* **1991**, *18*, 285−90.
(34) Delbosc, S.; Cristol, J. P.; Descomps, B.; Nimran, A.; Jover, B. Simvastatin prevents angiotensin II-induced cardiac alteration and oxidative stress. *Hypertension* **2002**, *40*, 142−7.
(35) Matsumoto, T.; Miyamori, K.; Kobayashi, T.; Kamata, K. Apocynin normalizes hyperreactivity to phenylephrine in mesenteric arteries from cholesterol-fed mice by improving endothelium-derived hyperpolarizing factor response. *Free Radical Biol. Med.* **2006**, *41*, 1289−303.
(36) Maeda, K.; Yasunari, K.; Sato, E. F.; Inoue, M. Enhanced oxidative stress in neutrophils from hyperlipidemic guinea pig. *Atherosclerosis* **2005**, *181*, 87−99.
(37) Witzum, J. L.; Steinberg, D. Role of oxidized lipoprotein in atherogenesis. *J. Clin. Invest.* **1991**, *88*, 1785−92.
(38) Shafiee, M.; Carbonneau, M. A.; Urban, N.; Descomps, B.; Leger, C. L. Grape and grape seed extract capacities at protecting LDL against oxidation generated by $Cu2^{+}$, AAPH or SIN-1 and at decreasing superoxide THP-1 cell production. A comparison to other extracts or compounds. *Free Radical Res.* **2003**, *37*, 573−84.
(39) Georgescu, A.; Alexandru, M.; Constantinescu, E.; Popo, B. Effect of gap junction uncoupler heptanol on resistance arteries reactivity in experimental model of diabetes, hyperlipemia and hyperlipemia-diabetes. *Vasc. Pharmacol.* **2006**, *44*, 513−8.
(40) Wagner, A. H.; Kohler, T.; Ruckschloss, U.; Just, I.; Hecker, M. Improvement of nitric oxide-dependent vasodilatation by HMGCoA reductase inhibitor through attenuation of endothelial superoxide anion formation. *Arterioscler., Thromb., Vasc. Biol.* **2000**, *20*, 61−9.
(41) Al-Awwadi, N. A.; Araiz, C.; Bornet, A.; Delbosc, S.; Cristol, J. P.; Linck, N.; Azay, J.; Teissedre, P. L.; Cros, G. Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. *J. Agric. Food Chem.* **2005**, *53*, 151−7.

Received for review January 4, 2007. Revised manuscript received March 6, 2007. Accepted March 12, 2007.

JF070029N