Digestive n-6 Lipid Oxidation, a Key Trigger of Vascular Dysfunction and Atherosclerosis in the Western Diet: Protective Effects of Apple Polyphenols

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Abbreviations: 4-HNE: 4-hydroxy-2-nonenal; Ach: Acetylcholine; ApoE: ApolipoproteinE; EPR: Electron Paramagnetic Resonance; HF: High Fat; NO: Nitric Oxide; oxLDL: oxidized LDL; PE: Phenolic Extract; PC: Proanthocyanidins; PUFA: polyunsaturated fatty acids; ROS: Reactive Oxygen Species; RM: Red Meat; SNP: Sodium Nitroprusside; SO: Sunflower Oil; WD: Western Diet.

Keywords: 4-Hydroxy-2-nonenal, Atherosclerosis, Endothelial function, Polyphenols, Western diet

Abstract

Scope

A main risk factor of atherosclerosis is a Western diet (WD) rich in n-6 PUFA sensitive to oxidation. Their oxidation can be initiated by heme iron of red meat leading to the formation of 4-hydroxy-2-nonenal (4-HNE), a cytotoxic aldehyde. An increased 4-HNE production is implicated in endothelial dysfunction and atherosclerosis. By contrast, a diet rich in proanthocyanidins reduces oxidative stress and arterial diseases. This study evaluates the effects of a WD on vascular integrity in $ApoE^{-/-}$ mice and the protective capacity of apple extract and puree rich in antioxidant proanthocyanidins.

Methods and results

$ApoE^{-/-}$ mice were fed during 12 weeks with a WD with or without n-6 PUFA. Moreover, two WD + n-6 PUFA groups were supplemented with apple puree or phenolic extract. We report an increase in digestive 4-HNE production associated with a rise in plasmatic 4-HNE and oxLDL concentrations. Oxidizable n-6 PUFA consumption was associated with a worsened endothelial dysfunction and atherosclerosis. Interestingly, supplementations with apple polyphenol extract or puree prevented these impairments while reducing oxidative stress.

Conclusion

n-6 lipid oxidation during digestion may be a key factor of vascular impairments. Nevertheless, an antioxidant strategy could limit 4-HNE formation during digestion and thus durably protect vascular function.

INTRODUCTION

Atherosclerosis, a leading cause of mortality, is a multifactorial and degenerative disease characterized by the progressive accumulation of lipids and inflammatory cells on the wall of large and medium arteries. Such changes result in blood flow impairments, chronic inflammation and oxidative stress in the vascular wall. [1,2] Lipid accumulation within the arterial wall is preceded by an altered vascular endothelial function, which is explained by a complex interplay between reduced nitric oxide (NO) bioavailability, oxidative stress and inflammation. [2,3] One of the major risk factors of atherosclerosis development is the Western diet rich in n-6 polyunsaturated fatty acids (n-6 PUFA) which are highly sensitive to oxidation. In the digestive tract, food is exposed to postprandial oxidative stress and therefore the gastric tract has been proposed as a major site for lipid oxidation. [4–6] Indeed, in the Western diet, heme iron in the form of myoglobin from red meat can initiate lipid oxidation leading to the formation of 4-hydroxy-2-nonenal (4-HNE). 4-HNE is a specific genotoxic and cytotoxic α,β-unsaturated hydroxyalkenal generated from n-6 PUFA and found in some oxidative stress-related and inflammatory diseases. [7] Keller et al. (2015) [8] have shown that marked 4-HNE administered to rats by intragastric gavage was largely absorbed by the intestinal barrier since it was found in urine, liver, or kidney. [8] This highly reactive aldehyde binds covalently (via Michael addition or Schiff base formation) with free amino or thiol groups of cysteine, lysine and histidine. [9] This adduction results in an impairment of the protein conformation and function inducing subsequent cellular dysfunctions and tissular damages. As a matter of fact, the covalent binding of 4-HNE to ApoB100, which yields oxidized LDL (oxLDL), is recognized as a key step in the development of an endothelial dysfunction and the subsequent atheromatous plaque formation. [10,11]

In the last decades, epidemiological, clinical and experimental studies have demonstrated that a healthy diet plays a central role in the prevention of atherosclerosis. As a matter of fact, inverse associations are repeatedly outlined between coronary artery disease and stroke and the consumption of fruit and vegetables. [12,13] In recent controlled trials with cocoa and other flavonoid-rich fruit and vegetables, the vascular function was improved along with the plasma NO status and inflammation-related markers. [14,15] As a matter of fact, proanthocyanidins (PCs) appear as potential mediators in reducing blood pressure and improving endothelium-mediated dilation whereas their plasma metabolites were found to reduce monocyte adhesion to endothelial cells through modulation of gene expression and TNF-α. [16,17,18,19] PCs are the second polyphenol class in the French diet with apple being the major contributor. [20]

Monomeric and oligomeric PCs represent 80% of the apple phenolic pool followed by hydroxycinnamic acids (15%), flavonols and dihydrochalcones. After ingestion, native forms of polyphenols are bioaccessible in the stomach after their release from the plant matrix. [21] Antioxidant apple polyphenols were found to inhibit lipid oxidation in *in vitro* gastric digestion. [22] Through their catechol core, they can reduce the pro-oxidant hypervalent iron form of myoglobin ($MbFe^{IV}=O$) to metmyoglobin ($MbFe^{III}$) as well as chelate free iron preventing lipid oxidation initiation. [23] Antioxidant effects of apple polyphenols in the gastrointestinal tract could limit 4-HNE formation and its subsequent absorption. Limiting 4-HNE absorption can reduce LDL oxidation and thus protect the vascular function.

The aims of this study were to evaluate 1) the effects of a chronic Western diet and the subsequent lipid oxidation occurring in the digestive tract on both the vascular function and atherosclerosis development in $ApoE^{-/-}$ mice, and 2) the consequences of a primary prevention strategy by a supplementation in polyphenols rich in PCs.

MATERIALS AND METHODS

Animal model and study design

All investigations are conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH, National Academies Press US, 8th edition, 2011) as well as European Parliament Directive 2010/63/EU (experimentation n°: 84.004. Eight weeks old male C57Bl6J mice (n = 100) knockout for apolipoproteinE ($ApoE^{-/-}$) (Charles River, France / Breeding in HP2 laboratory, Grenoble) were used for this study. Mice were housed five by cage under controlled conditions of temperature (21 ± 1 °C), hygrometry (60 ± 10%) and lightening (12:12 hours light-dark cycle), with access to water and food *ad libitum*. After two weeks of acclimatization, male mice were randomly assigned to five groups (n = 20 per group): a first group with a normocholesterolemic standard diet (SD group) (SAFE A04), a Western diet group with High Fat with red meat (WD group), a second Western diet group complemented with sunflower oil (WD+SO group) and two other groups with a WD+SO diet supplemented with apple polyphenols in the form of apple puree (WD+SO+PUREE group) or the corresponding phenolic extract (WD+SO+PE group). SD

group was only conducted in order to confirm the effects of our Western Diet in a model of ApoE $^{-/-}$ mice. Consequently, differences observed between SD and WD groups are reported in supplemental data.

Diet protocols were sustained during 12 weeks and food intake was quantified as a mean per cage (n = 3 cages with 6/7 mice per cage) each three days during the whole protocol. During this period, weight gain was measured every week and dietary intake was evaluated three times per week. Faeces were harvested at the start, middle and end of the protocol. During the last week, blood pressure was evaluated by tail cuff method in 12 animals per group and atheromatous plaque size by echography on the 8 other animals of the group. At the end of the 12 weeks-diet period, an overnight fasting was performed and mice were euthanized by sodium pentobarbital injection (120 mg/kg I.P.). Blood was collected for biochemical analyses. Hearts were excised with aortic arch and fixed in O.C.T then frozen in liquid nitrogen and stored at -80 °C for histological analysis. Finally, thoracic aorta was removed in order to perform *ex-vivo* analysis of vascular function (n = 8/grp), quantification of atheromatous plaque development (n = 8 for aortic staining with Oil-Red O) or biochemical assays (n = 4 per group)

Dietary protocol

Regarding the diet protocol, the first group was fed with a High Fat and red meat diet (HF 230, Safe, France) (Western diet, WD). Composition in % kcal: proteins 16.1, carbohydrates 24.2, lipids 59.7 for a total of 12.1 kcal per day. Composition in % of diet weight: High Fat 79.5, red meat 19.9, cellulose 0.64, n-6 PUFA 10.5, cholesterol 0.076, Vitamin E 0.014 and heme iron 0.0013. The second group was fed with a WD diet complemented with sunflower oil (SO, Rustica from Leclerc, lot A21815) as a source of oxidizable n-6 PUFA (WD+SO group). Composition in % kcal: proteins 12.3, carbohydrates 18.5, lipids 69.2 for a total of 13.0 kcal per day. Composition in % of diet weight: High Fat 68.6, sunflower oil 13.7, red meat 17.1, cellulose 0.55, n-6 PUFA 28.3, cholesterol 0.066, Vitamin E 0.021 and heme iron 0.0011. The 2 last groups were fed with a WD+SO diet supplemented with apple (*Reinette de Flandre,* Ambricourt, France) polyphenols in the form of apple puree (whole apple matrix except seeds) for the WD+SO+PUREE group or the corresponding phenolic extract for the WD+SO+PE group. Composition for WD+SO+PUREE group in % kcal: proteins 12.1, carbohydrates 19.4, lipids 68.5 for a total of 11.7 kcal per day; in % of diet weight: High Fat 60.6, sunflower oil

12.1, red meat 15.1, Puree 12.1 (polyphenols 0.060), n-6 PUFA 28.3, cholesterol 0.058, Vitamin E 0.019 and heme iron 0.0010. Composition for WD+SO+PE group in % kcal: proteins 12.1, carbohydrates 19.6, lipids 68.3 for a total of 12.4 kcal per day; in % of diet weight: High Fat 67.0, sunflower oil 13.4, red meat 16.7, extract 2.3 (polyphenols 0.061), cellulose 0.54, n-6 PUFA 28.3, cholesterol 0.064, Vitamin E 0.021 and heme iron 0.0011.

Blood pressure measurements

Before the first week and during the last week of the dietary protocol, systolic, diastolic and mean blood pressures were assessed in conscious mice by tail-cuff method using CODA tail cuff system (Kent Scientific, Torrington, CT, USA). To reduce stress influence, mice underwent 3 periods of habituation to blood pressure measurements during the week preceding final recording. Mice were warmed for 30 min before measurements so that their skin temperature reached 33-34 °C during measurements. All measurements were made by the same experimenter between 9 AM and 12 AM. Obtained results were the mean of at least 10 valid measurements out of the 20 performed in a recording session.

Ultrasound imaging (echography) of atheromatous plaque

Echographic assessment was performed using a Vevo 3100™ imaging system (Fujifilm VisualSonics, Toronto, Canada) equipped with the MX550D™ probe (22-55 MHz). Images were analyzed post-processing using the VevoLab™ software (Fujifilm VisualSonics, Toronto, Canada). Echographic assessment as well as image analysis were performed in a blind manner. Mice anesthesia was induced with 4% isoflurane (IsoFlo™, Zoetis, Parsippany, USA) in medical air mix (22% O_2, 78% N_2) (Air Liquide, Paris, France), ventilated at 1 L/min, and then maintained with 1.5% isoflurane after effective sleep. Animals were placed in a supine position on a heating pad and their body temperature was monitored throughout the exam, as well as their breathing rate and electrocardiogram. Cross-sectional images of ascending aorta were obtained from a modified right parasternal view. Aortic plaque surface areas were determined from 3 measurements at the same spot. All measurements were performed between two inspiratory peaks.

Isolated aortic rings and vasoreactivity

Under anesthesia (120 mg/kg I.P.), thoracic aortas were quickly removed and placed in cold Krebs-Henseleit bicarbonate buffer (composition in mM: NaCl 118, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11). After removal of adherent perivascular adipose tissues, aortas were cut into 2 mm long rings. Then, aortic rings were set onto stainless steel connected to an isometric force transducer, linked to an amplifier and a computerized acquisition system, to record changes in isometric force (EMKA technologies, EMKA Paris, France). They were suspended in organ bathes containing Krebs-Henseleit buffer at 37 °C continuously bubbled with gas mixture (95% O_2 – 5% CO_2). Resting tension was adjusted to 0.8 g and aortic rings were allowed to stabilize during 60 min. During this period, Krebs-Henseleit buffer was changed two times and rings were re-stretched to maintain a tension of 0.8 g. First, smooth muscle cells and endothelial integrity were assessed by a single dose of phenylephrine (PE, 10^{-6} M) followed by a single vasorelaxing dose of acetylcholine (ACh, 10^{-5} M). Then, each vessel ring was pre-constricted with phenylephrine (10^{-6} M). After pre-constriction reached a plateau, endothelium-dependent and independent relaxations were examined by challenging aortic rings with cumulative concentrations of ACh (10^{-9} to 10^{-5} M) or sodium nitroprusside (SNP, 10^{-9} M to 10^{-5} M) respectively. Vasodilatation was expressed as a percentage of maximum contractile response to PE. Maximal relaxation (Emax) and sensitivity (EC50) to each drug were used to characterize vasorelaxation.

Blood analysis

Blood analyses were performed on plasma obtained by centrifuging blood at 300 *g* for 10 min at 4 °C and plasma was stored at -80 °C for biochemical analyses. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) concentrations were determined by the CHOD-PAP method (Biolabo SAS, France). Triglycerides (TG) were assessed by the GPO method (Biolabo SAS, France). Finally, malondialdehyde-modified oxLDL were assessed by an ELISA kit (Cloud-Clone Corp., USA).

Biochemical assays

Measurement of reactive oxygen species (ROS) by electron paramagnetic resonance (EPR)

ROS production was measured by EPR in aortic homogenates as previously described. [24] Briefly, 50 µL of homogenates were incubated with 5 µL of Krebs-Hepes buffer (pH 7.4) containing 1 mM of CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) and 25 µM deferroxamine methane sulfonate. This solution was placed inside the e-scan spectrometer (Bruker, Ettlingen, Germany) for data acquisition at 37 °C. The acquisition EPR parameters were: microwave frequency = 9.652 GHz; modulation frequency = 86 kHz, modulation amplitude = 0.01 G; center field = 3495.9 G, sweep width = 300 G; microwave power = 1.0 mW; number of scans = 10 and receiver gain = 2.10^{-1}. Adducts generated by the probe reaction with free radicals were acquired and the spectra sequentially recorded for about 5 min in order to calculate the ROS production rate. The ROS production was normalized to the protein content of each sample and then expressed in µmol $min^{-1}mg^{-1}$.

Nitrites in aorta

Nitrites from descending aorta, used as an index of total NO production, were quantified by using the high-sensitivity nitrite assay kit (Measure-iT™ High-Sensitivity Nitrite Assay Kit; Invitrogen™) and was performed according to the manufacturer's instructions. Measurements were performed at the end of 12 weeks of protocol diet. NO production was expressed relative to protein content of each sample.

Quantification of faeces and plasma 4-HNE

Lipid oxidation during digestion was evaluated by the quantification of 4-HNE in faeces and plasma. Faeces were collected 24 hours after diet change at the start, middle and end of the 12 weeks protocol. Faeces (200 mg) were crushed with mortar and pestle in liquid nitrogen. The resulting powder (ca. 30 mg) was vortexed once with 500 µL of acetonitrile containing 50 ng of 4-HNE-D3 (internal standard) during 5 min. After centrifugation (16 000 *g*, 5 min, room temperature), the supernatant was reacted with 100 µL of a solution of 2,4-dinitrophenylhydrazine (75 mg of 2,4-DNPH added with 0.6 mL of formic acid and 10 mL of acetonitrile) during 1 hour at room temperature. For plasma, 70 µL were vortexed with 200 µL of acetonitrile and 20 µL of 4-HNE-D3 (10 ng/mL in ACN) during 5 min. After centrifugation as above, the supernatant is dried under nitrogen and reacted with 50 µL of a solution of 2,4-DNPH (25 mg of 2,4-DNPH added with 0.2 mL of formic acid and 10 mL of acetonitrile) during 1 hour at room temperature. Separation and quantification of derivatized 4-HNE were

performed by using LC/MS/APCI (EVOQ Elite, Bruker Daltonics, Bremen, Germany) with an ACQUITY HSS T3 column (50 mm x 2.1 mm; 1.7 µm, Waters, Saint-Quentin-en-Yvelines, France) at 40 °C. For chromatographic separation, the mobile phase was constituted by a binary solvent system with water/formic acid (99.95/0.05, v/v, solvent A) and acetonitrile (solvent B) at the flow rate of 0.4 mL/min. The volume injected was 4 µL. The elution gradient was as follows: 0-1 min isocratic 50% B; 1-9 min, linear 50-98% B; 9-10 min, isocratic 98% B; 10-10.1 min, linear 98-50% B and 10.1-11 min, isocratic 50% B. Mass spectrometry conditions were as follows: ionization by APCI in negative mode, spray current 20 µA; cone temperature 300 °C; heated probe temperature 300 °C; cone gas flow 20; probe gas flow 40; nebulizer gas flow 50. Spectra were recorded in the MRM mode. Derivatized 4-HNE-D3 was quantified by following the transition from ion at *m/z* 338 to ion at *m/z* 167 and derivatized 4-HNE with transition from *m/z* 335 to *m/z* 167. The retention time of both compounds was 3.5 min. For quantification, 5 point-calibrations were injected at constant concentration for 4-HNE-D3 (0.28 µM) and between 1.02 µM and 0.064 µM for 4-HNE. The adducts with DNPH were stable over 6 h with a variation lower than 5%.

OIL red coloration

Atheromatous plaque size was assessed by measuring lipid aggregation in thoraco-abdominal aortas that were fixed in paraformaldehyde (4%) during 24 hours at 4 °C. Then, aortas were rinsed three times with Krebs-Henseleit buffer and incubated overnight in an Oil-Red-O solution in isopropanol/water (60/40, v/v, 0.33% of Oil-Red-O, Sigma-Aldrich, Saint-Louis, USA) at room temperature. Atheromatous plaque size was assessed by capturing images directly from a color camera (Motic Moticam 2300, Motic China group Co.) attached to a binocular loupe. Images were displayed on a RGB monitor by using Motic Image 2.0 (Motic China group Co.). Analysis was carried out using Image J® software (Image J, NIH, USA). Oil red staining was expressed as the percentage of aortic area.

Histological sections and colorations

The upper part of the heart (with aortic root) was cut using a cryostat (Leica CM1950; Leica systems, France) at -20 °C. The distal portion of the aortic sinus was recognized by the appearance of the three leaflets of the valves. Sections of 8 µm thickness were performed. For

each aortic root, we quantified atheromatous plaque size and necrotic core size (acellular area) with Oil-Red-O and Hematoxylin and Eosin staining (Sigma-Aldrich, France) and fibrosis (Red Sirius, Labomoderne, Paris, France) from five sections separated from each other by 80 µm. Photography was performed using a digital camera (Baumer VCXU31C, Baumer SAS France), mounted with a macro lens VS Technology 0513 (VS Technology Corporation, Japan) and Baumer GAPI acquisition software (Baumer SAS France). Quantitative analyses were performed blindly using Image J® software to assess the stained area in mm² and express it relative to the diameter of aorta in mm (atheromatous plaque size and necrotic core size) or in percentage of atheroma (fibrosis).

Statistical analysis

Data were expressed as mean ± SEM. Normality was evaluated using D'Agostino and Pearson test when n≥8. Otherwise, Shapiro-Wilk test was performed. When applicable, comparison of multiple experimental conditions were performed using analysis of variance (ANOVA) or repeated measures ANOVA followed by a Tukey post hoc test. A value of $p<0.05$ was considered statistically significant. Statistics were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, USA).

RESULTS

Diet Characterization

ApoE$^{-/-}$ mice, classically used to evaluate atherosclerosis development, were distributed to four groups: a group with a high fat and red meat diet (WD), a second group with a WD diet complemented with sunflower oil as a source of oxidizable n-6 PUFA (WD+SO group) and two other groups with a WD+SO diet supplemented with apple polyphenols in the presence of the apple matrix (Puree) or as a phenolic extract, respectively WD+SO+PUREE and WD+SO+PE groups. In parallel, a negative control group of ApoE$^{-/-}$ mice fed with standard diet was performed (SD, n=20, SAFE A04) to evaluate the deleterious effects of WD (Supplementary data Figure 2).

As previously reported, the composition of the sunflower oil used was: 9.8% of saturated fatty acids (6.3% C16; 3.3% C18; 0.2% C20), 27.8% of monounsaturated fatty acids (0.1%

C16:1; 27.0% C18:1n-9; 0.6% C18:1n-7; 0.1% C20:1) and 62.4% of polyunsaturated fatty acids (62.4% C18:2; 0.01% C18:3). [25] The α-tocopherol content was 665 ppm.

Identification and quantification of apple polyphenols were performed by UPLC/DAD/ESI-MS and thioacidolysis as previously reported. [25] Forty-six compounds distributed in four phenolic classes were identified: proanthocyanidins, hydroxycinnamic acids, dihydrochalcones and flavonols. The selected variety (*Reinette de Flandre*) is markedly rich in polyphenols (4.3 g/kg) compared to classically consumed dessert apples (1 g/ kg). [26] Polyphenols were even more concentrated in puree after thermal treatment of *Reinette de Flandre* reaching 5 g/kg. Monomeric and oligomeric PCs represented 5-7% and 67-68%, respectively, of the phenolic pool followed by the hydroxycinnamic acid, 5-caffeoylquinic acid (21-22%). Quercetin derivatives and dihydrochalcones were only minor contributors. [21] In our model of apple supplementation, no major difference was observed for polyphenol contents and composition between Puree and PE. Apples used to prepare the diet also contained, 3.15 g of glucose, 5.85 g of fructose, 1.09 g of saccharose, 0.04 g of citric acid, 1.61 g of malic acid, 17.7 mg of dehydroascorbic acid and 5.9 mg of ascorbic acid per 100 g of fresh fruit.

Lipid oxidation during digestion and blood oxidative state

In our model of Western Diets (high fat associated with red meat), body weight increased all along the protocol to the same extent in the 4 groups (Figures 1A and 1B). In line with these results, we observed no difference regarding adiposity index defined as (epididymal fat + inguinal fat + perinephric fat)/mouse weight (Figure 1C), blood HDL (Figure 1D), LDL (Figure 1E), HDL-C/LDL-C (Figure 1F), total cholesterol (Figure 1G) and triglycerides (Figure 1H) between groups. These results suggest that there is no difference between groups regarding lipid absorption. In addition, no difference between groups in term of fasting glycemia as well as glucose and insulin tolerance (supplemental data) was observed. To evaluate the impact of a Western diet enriched in n-6 PUFA on lipid oxidation during digestion and the ability of Puree and PE to modulate this phenomenon, we next measured the level of 4-HNE in faeces. Before the beginning of the diet (Week 0), only low contents of 4-HNE were reported at the detection limit of LC/MS/APCI (Figure 2A). After 6 (Figure 2B) and 12 (Figure 2C) weeks of diet a stronger increase in 4-HNE was observed in the group supplemented with SO (WD+SO) compared to the WD group. It is worth noting that 4-HNE was neither detected in freshly reconstituted diets nor after 48 hours at room temperature (data

not shown). This suggests that lipid oxidation can occur during *in vivo* gastrointestinal digestion. The higher levels of 4-HNE in WD+SO compared to WD can be linked to the diet contents in n-6 PUFA, which are respectively of 28.3% and 10.5% for the WD+SO and WD diets. Interestingly, the increase observed by the addition of n-6 PUFA was largely prevented by both Puree and PE supplementations (Figure 2BC). Next, to evaluate whether lipid oxidation products were also observed in systemic circulation, we measured, after 12 weeks of diet, plasmatic concentrations of 4-HNE also known to be reliable biomarkers of oxidative stress. [9] As reported in Figures 2D and E, plasmatic level of 4-HNE was higher in the WD+SO group compared to WD by 87%. In line with the results obtained on 4-HNE in faeces, this phenomenon was blunted by both apple Puree and PE supplementations by 32% and 51%, respectively (Figure 2D). Finally, we measured oxLDL, another biomarker of oxidative stress, which is also known as a strong biomarker of cardiovascular risk. [10] The same pattern was observed since oxLDL level was significantly increased by 14% in the WD+SO group compared to WD (Figure 2E) while the supplementation by both Puree and PE markedly attenuated this phenomenon (-22% and -29%, respectively) (Figure 2E).

4-HNE and cardiovascular risk factors

High levels of 4-HNE and oxLDL are associated in the literature with an increased cardiovascular risk. [10] Then, we evaluated how our Western diet model enriched in n-6 PUFA, supplemented or not with apple polyphenols, affected some key elements of cardiovascular risk such as arterial blood pressure, endothelial function and finally atheromatous plaque development. We first evaluated arterial blood pressure by the tail-cuff method. When the Western diet was enriched with n-6 PUFA (WD+SO), both systolic and mean arterial pressures were increased compared to WD (Figure 3AC). However, no change in arterial diastolic pressure was observed between WD and WD+SO groups (Figure 3B). When the WD+SO diet was supplemented with either Puree or PE, systolic, diastolic and mean arterial blood pressures were significantly lower than in WD+SO group (Figure 3ABC).

Another key element of increased cardiovascular risk factor, which can contribute to the regulation of blood arterial pressure is endothelial function. Thus, we next evaluated the impact of our experimental conditions on vascular endothelial function on isolated aortic rings. In the aorta of animals fed for 12 weeks with a WD enriched in n-6 PUFA, maximal

vascular relaxation in response to ACh was reduced by 16% compared to WD (Figure 3D left and central panel). Considering that no difference was observed between these 2 groups regarding the response to SNP (Figure 3D right panel), these results strongly support that a Western diet, enriched in n-6 PUFA, is associated with a vascular endothelium impairment without smooth muscle cell alteration. In line with our previous results, endothelial dysfunction was prevented when the animals were supplemented with both apple Puree and polyphenol extract (Figure 3D left and central panel). Indeed, the maximal response to ACh was increased by 41% in WD+SO+PUREE and 40% in WD+SO+PE groups compared to WD+SO. An interesting point is that contrary to the WD+SO group which presented an impairment of vasodilation to ACh in 75% of the tested arteries (with an impairment threshold of < 70% of precontraction), no impaired aorta was reported in both supplemented groups. No impact on the dose-response to SNP was observed.

Since, endothelial dysfunction is a key trigger in the pathogenesis of atherosclerosis [10] we next assessed the consequences of endothelial dysfunction on the development of atherosclerosis. In our study, all experiments have been performed on $ApoE^{-/-}$ mice, which are prompt to develop subsequent atherosclerotic lesions at aortic bifurcations in the aortic arch, in the descending aorta and in other large arteries. [27]. The enrichment of WD with n-6 PUFA increased atheromatous plaque size in aortic arch section as measured by echography (Figure 4A). However, no difference in atherosclerosis was observed by Oil-Red-O coloration in descending thoraco-abdominal aorta or by histological staining of aortic root in this WD+SO group when compared to the WD group (Figure 4BC). To evaluate plaque stability, percentage of fibrosis and necrotic core area size in atheromatous plaque were assessed. No difference between any groups was observed regarding fibrosis (Figure 4D). However, we observed that the necrotic core area increased in mice by 52% compared to the WD group when the Western diet was enriched in n-6 PUFA (Figure 4E). In line with the protective effect of both apple Puree and PE on endothelial dysfunction, reduced atheromatous plaque developments were reported by echography in aortic cross (respectively by -20% and -20%) and by Oil-Red O histological staining in aortic root (respectively by -73% and -37%) as well as by Oil-Red O staining in thoraco-abdominal aorta (respectively -51% and -45% when compared to WD+SO) (Figure 4ABC). Another interesting result was that both apple Puree and PE were able to prevent the increase of the necrotic core area size by 55% and 42%, respectively (Figure 4E). This last result indicates that plaque stability, which is affected negatively in mice fed with WD+SO, was preserved with polyphenol supplementation.

ROS production and NO pathway in endothelial function

In the vascular wall, the sensitive equilibrium between NO bioavailability and ROS production contributes mainly to the regulation of the endothelial function. Moreover, increased ROS production and alteration in NO bioactivity are known to be the key triggers of endothelial dysfunction and atherosclerosis. [28] Thus, we measured the impact of our experimental conditions on NO and ROS production in the aortic wall as well as eNOS expression and phosphorylation on its main activation site serine^{1177} (eNOS^{Ser1177}). No difference was observed between WD and WD+SO groups regarding total eNOS expression and phosphorylation on Ser^{1177} (Suppl Figure 2). However, with both supplementations, eNOS level tended to be lower when compared to the WD+SO group (p=0.06 and 0.08) and they both seem to increase eNOS activation state through its phosphorylation on serine^{1177} (Suppl Figure 2). To evaluate the impact of our experimental conditions on NO bioavailability, we next measured the level of nitrites in aortic tissues. We report an increased nitrite level in WD+SO group compared to WD mice (Figure 5A). Neither Puree (WD+SO+PUREE) nor polyphenols supplementations (WD+SO+PE) were able to impact nitrite levels compared to WD+SO group. Finally, since in pro-oxidative conditions, ROS can react with NO to generate nitro-oxidative stress, which can also contribute to reduce NO bioavailability, we also measured ROS production by EPR. We report that WD+SO led to increased ROS production compared to WD (Figure 5B). By contrast, apple Puree significantly reduced ROS production by 30% whereas polyphenol extract only tended to reduce it (-26 %, p=0.08) when compared to the WD+SO diet. No statistical difference between PUREE and PE groups could be further evidenced. Altogether, these results point out that despite the beneficial effect of apple polyphenol supplementations on endothelial function, their impact on the NO pathway appears limited. Nonetheless, the modulation of aortic ROS production may contribute to explain some changes in NO bioavailability in our groups.

DISCUSSION

The aim of this study was to give an insight into the mechanism involved in vascular dysfunction induced by the consumption of a Western diet enriched with n-6 PUFA. ApoE $^{-/-}$ mouse is the most widely used animal model to evaluate the effects of a diet on atherosclerosis development. ApoE$^{-/-}$ mice display an increased peroxidation of lipoproteins, an elevated level of endothelial cell adhesion molecules, an increased proliferation and migration of VSMCs as well as a decreased anti-atherogenic function. [29] Moreover, the anti-inflammatory properties of APOE, combined with its impact on lipoprotein metabolism, explain why ApoE knockout mice display a very strong development of atherosclerosis compared to other mouse models. [30–32] However, this model has imperfections and other models such as LDLr $^{-/-}$ have been considered. Nonetheless, our diets have a low cholesterol content (<0.01% of diet weight) and it has been reported that long diets with higher cholesterol contents are needed to induce significant lesion development in LDLr $^{-/-}$ mice. [33,34] Consequently, the ApoE$^{-/-}$ model appeared as the most appropriate to study the link between lipid oxidation, endothelial function and atherosclerosis development with our diets.

The main results of our work with ApoE $^{-/-}$ mice showed that oxidative stress generated in the gastrointestinal tract (4-HNE) is correlated with an increased cardiovascular risk (endothelial dysfunction and atherosclerosis) and that preventing lipid oxidation could avoid these impairments.

Lipid oxidation products can be generated *in vivo* and the gastric tract has been proposed as the major site for diet-induced lipid oxidation. [35] This was confirmed in our study since we observed that enrichment of a Western diet with n-6 PUFA strongly increases the formation of 4-HNE during digestion, as shown by the higher recovery of 4-HNE in faeces from WD+SO group. This result is in line with previous studies reporting that a Western meal based on sunflower oil and beef led to the formation of TBARS, lipid-derived conjugated dienes and 4-HNE during gastric digestion in mini-pig. [36,37]

In order to prevent the deleterious effects of 4-HNE (oxidative stress, cell proliferation, or cell death), [38] we considered using an antioxidant strategy. Since, we previously reported that an apple phenolic extract and an apple matrix (Puree) were able to reduce lipid oxidation and 4-HNE formation in an *in vitro* model of digestion, [25] we tried to use both of them *in vivo*. By adding either the whole apple matrix (WD+SO+PUREE) or the corresponding phenolic extract (PE) to the diet, we managed to prevent lipid oxidation during digestion. We can

hypothesize that in our model, a reduction of the pro-oxidant hypervalent heme iron form ($MbFe^{IV}=O$) into metmyoglobin ($MbFe^{III}$) by chlorogenic acid as well as by monomeric and oligomeric PCs may have occurred. This reduction inhibits the initiation phase of the lipid oxidation process and has been previously reported as a major antioxidant effect of PCs and hydroxycinnamic acids in *in vitro* digestion. [5] A remarkable result is that both WD+SO+PUREE and WD+SO+PE managed to prevent digestive lipid oxidation with the same extent. This result could be explained by an increased polyphenol bioaccessibility in WD+SO+PUREE group when compared to raw fruit that we have reported in a previous work. [25] The relative similarity in polyphenol bioaccessibilities for both apple matrix and phenolic extract may explain the very similar results obtained throughout this study for both supplementations.

Next, we hypothesized that lipid oxidation products generated during digestion may have an effect on vascular health (endothelial dysfunction, atherosclerosis) after their absorption. Indeed, we report here that higher lipid oxidation in the digestive tract (4-HNE) is associated with a higher plasmatic concentration of 4-HNE. These data are in accordance with results from Keller et al. who observed 4-HNE in urine and various organs (liver, kidney, brain, heart) after its ingestion. [8] It further indicates that even if the majority of 4-HNE could be excreted in faeces, a part of it is absorbed and can be implicated in vascular impairments. Moreover, it has been shown that eating a fatty diet can alter the intestinal barrier physiological state by increasing its permeability [39] which could lead to an increased absorption of 4-HNE.

In our work, fasting plasma concentration of 4-HNE after chronic WD+SO diet was 2.7 µM (0.43 mg/L). Such concentration of 4-HNE is known to trigger a variety of biological responses such as inflammation and cell proliferation. [9] Moreover, this plasmatic level of 4-HNE is also known to induce oxidative stress in the vascular system. It is important to note that 4-HNE values observed in our work were assessed after a prolonged fasting state and that we can expect higher values in the postprandial state. Nevertheless, the increased oxidative stress was confirmed here since the group with the higher 4-HNE concentration displays an increase in ROS production and plasmatic malondialdehyde-modified oxLDL. Yet, an increase in oxLDL production has been largely reported in the literature as a key step in the development of vascular impairments such as endothelial dysfunction or atherosclerosis, which are generally described as the consequence of oxidative stress. [40,41] Moreover, it is considered that high NO levels can account for nitro-oxidative stress in obese mice. [42] The

high levels of nitrites observed in the WD+SO group may be the results of the reaction between NO and superoxide to form peroxynitrite, a potent cytotoxic reactive species. Such a nitro-oxidative stress is largely reported as a key factor of vascular endothelial impairments and atherosclerosis. [43] Consequently, it was not surprising to observe the worst endothelial function and the largest atherosclerosis development in the group with the highest lipid oxidation.

We previously pointed out the deleterious effects of digestive lipid oxidation on vascular integrity and the fact that both WD+SO+PUREE and WD+SO+PE diets were able to prevent lipid oxidation. We therefore hypothesized that inhibition of lipid oxidation by polyphenol consumption may prevent vascular impairments. This hypothesis was confirmed regarding the decrease of ROS production and vascular impairments (reduced endothelial impairments and atherosclerosis development). Surprisingly, vascular function was not only restored but even positively enhanced in both supplemented groups. Few assumptions can be performed to explain the beneficial effect of our diets on vascular function. In a first place, our protocol does not allow us to exclude a cell-mediated effect of polyphenols included in the extract and puree since these supplementation were not tested on a group with a standard diet. Indeed, a direct effect of phase-II and colonic metabolites from proanthocyanidins on vascular function could not be excluded according to our results and the literature. [19,44,45]. Nonetheless, it has been previously reported that 4-HNE was responsible for an impairment of Akt phosphorylation, which is a main regulator of vascular function especially through eNOS phosphorylation on Ser1177. [46] Despite eNOS phosphorylation on its activation site seems to be increased in both WD+SO+PUREE and WD+SO+PE groups, the extent of this increase was not satisfactory and certainly did not appear to be a key element to explain our results on endothelial function. This idea is reinforced by the lack of effect of both PUREE and PE on nitrites level when compared to the WD+SO group. Nevertheless, we have to consider that even if nitrite levels are unchanged, a decrease of oxidative stress can result in a reduction of NO reaction with O_2^- to form $ONOO^-$. Thus, considering that both PUREE and PE treatment reduced or tended to reduce ROS production in the vascular wall, we can hypothesize that the bioavailability of NO may be increased. This hypothesis is supported by the impact of our experimental conditions on endothelial vasodilatation to ACh. Indeed, on aortic tissue endothelium-dependent vasodilatation is almost exclusively dependent of the eNOS-NO pathway, since the pre-incubation of L-NAME almost completely abolishes it in aortas of

ApoE$^{-/-}$ mice. [47–49] Further studies, evaluating eNOS coupling, NO bioavailability and the level of nitro-oxidative stress will be needed to confirm this point.

These results are in accordance with numerous studies reporting the interest of a chronic uptake of apple polyphenols to reduce risk factors (arterial pressure, blood lipid profile, inflammation, oxidative stress) and cardiovascular diseases (anti-angiogenic, anti-atherogenic and anti-hypertensive) in rodents or humans. [45,50–53] However, there is no study as this time that are able to link these effects with a potential antioxidant effect during digestion.

CONCLUSION

In conclusion, a Western diet rich in n-6 PUFA appears to exacerbate lipid oxidation during digestion leading to the formation of 4-HNE among other lipid oxidation products. The absorption of these products is associated with plasmatic oxidative stress, endothelial dysfunction and development of atherosclerosis in the ApoE$^{-/-}$ mice model. The involvement of digestive lipid oxidation in these phenomena was confirmed since the inhibition of lipid oxidation and, consequently, 4-HNE synthesis in the gastrointestinal tract, by a supplementation in apple polyphenols under two different matrix forms, remarkably prevents those impairments. Consequently, the consumption of proanthocyanidin-rich fruits such as apple or pear should be encouraged to attenuate the deleterious effect of the Western diet.

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Disclosures

Authors declare that they have no conflict of interest. Claire Dufour and Gregory Meyer designed the study protocols and secured funding.

Author Contributions

G.B.; C.P.; A.H.; M.D.; C.G.; S.R.; A-L.C. B.G. G.M. performed experiments. C.A. developed ApoE$^{-/-}$ model and histological protocols. G.B., G.M., C.D. designed the scope, analyzed the data and wrote the manuscript. C.R., C.A. and B.G. revised the manuscript. All authors read and approved the final manuscript.

REFERENCES

[1] J.-J. Chiu, S. Chien, *Physiol. Rev.* **2011**, *91*, 327–387.

[2] R. Ross, *Am. Heart J.* **1999**, *138*, S419–S420.

[3] G. K. Hansson, A.-K. L. Robertson, C. Söderberg-Nauclér, *Annu. Rev. Pathol. Mech. Dis.* **2006**, *1*, 297–329.

[4] J. Kanner, *Mol. Nutr. Food Res.* **2007**, *51*, 1094–1101.

[5] B. Lorrain, O. Dangles, M. Loonis, M. Armand, C. Dufour, *J. Agric. Food Chem.* **2012**, *60*, 9074–9081.

[6] E. Vulcain, P. Goupy, C. Caris-Veyrat, O. Dangles, *Free Radic. Res.* **2005**, *39*, 547–563.

[7] S. J. Chapple, X. Cheng, G. E. Mann, *Redox Biol.* **2013**, *1*, 319–331.

[8] J. Keller, M. Baradat, I. Jouanin, L. Debrauwer, F. Guéraud, *Redox Biol.* **2015**, *4*, 136–148.

[9] A. Nègre-Salvayre, S. Garoby-Salom, A. Swiader, M. Rouahi, M. Pucelle, R. Salvayre, *Free Radic. Biol. Med.* **2017**, *111*, 127–139.

[10] S. Gargiulo, G. Testa, P. Gamba, E. Staurenghi, G. Poli, G. Leonarduzzi, *Free Radic. Biol. Med.* **2017**, *111*, 140–150.

[11] I. Staprans, X.-M. Pan, J. H. Rapp, K. R. Feingold, *Mol. Nutr. Food Res.* **2005**, *49*, 1075–1082.

[12] D. Aune, E. Giovannucci, P. Boffetta, L. T. Fadnes, N. Keum, T. Norat, D. C. Greenwood, E. Riboli, L. J. Vatten, S. Tonstad, *Int. J. Epidemiol.* **2017**, *46*, 1029–1056.

[13] J. Zhan, Y.-J. Liu, L.-B. Cai, F.-R. Xu, T. Xie, Q.-Q. He, *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 1650–1663.

[14] L. Hooper, C. Kay, A. Abdelhamid, P. A. Kroon, J. S. Cohn, E. B. Rimm, A. Cassidy, *Am. J. Clin. Nutr.* **2012**, *95*, 740–751.

[15] A. L. Macready, T. W. George, M. F. Chong, D. S. Alimbetov, Y. Jin, A. Vidal, J. P. E. Spencer, O. B. Kennedy, K. M. Tuohy, A.-M. Minihane, M. H. Gordon, J. A. Lovegrove, FLAVURS Study Group, *Am. J. Clin. Nutr.* **2014**, *99*, 479–489.

[16] C. C. Lee, J. H. Kim, J. S. Kim, Y. S. Oh, S. M. Han, J. H. Y. Park, K. W. Lee, C. Y. Lee, *Int. J. Mol. Sci.* **2017**, *18*, 1363.

[17] S. Claude, C. Boby, A. Rodriguez-Mateos, J. P. E. Spencer, N. Gérard, C. Morand, D. Milenkovic, *Mol. Nutr. Food Res.* **2014**, *58*, 1016–1027.

[18] C. D. Kay, L. Hooper, P. A. Kroon, E. B. Rimm, A. Cassidy, *Mol. Nutr. Food Res.* **2012**, *56*, 1605–1616.

[19] R. Sansone, A. Rodriguez-Mateos, J. Heuel, D. Falk, D. Schuler, R. Wagstaff, G. G. C. Kuhnle, J. P. E. Spencer, H. Schroeter, M. W. Merx, M. Kelm, C. Heiss, for the Flaviola Consortium, European Union 7th Framework Program, *Br. J. Nutr.* **2015**, *114*, 1246–1255.

[20] J. Pérez-Jiménez, L. Fezeu, M. Touvier, N. Arnault, C. Manach, S. Hercberg, P. Galan, A. Scalbert, *Am. J. Clin. Nutr.* **2011**, *93*, 1220–1228.

[21] C. Dufour, M. Loonis, M. Delosière, C. Buffière, N. Hafnaoui, V. Santé-Lhoutellier, D. Rémond, *Food Chem.* **2018**, *240*, 314–322.

[22] G. Boléa, C. Ginies, M.-J. Vallier, C. Dufour, *Food Funct.* **2019**, *10*, 3942–3954.

[23] B. Lorrain, O. Dangles, C. Genot, C. Dufour, *J. Agric. Food Chem.* **2010**, *58*, 676–683.

[24] S. Battault, F. Singh, S. Gayrard, J. Zoll, C. Reboul, G. Meyer, *Hypertens. Res.* **2016**, *39*, 70–78.

[25] G. Boléa, C. Ginies, M.-J. Vallier, C. Dufour, *Food Funct.* **2019**, *10*, 3942–3954.

[26] Guyot, S., Le Bourvellec, C., Marnet, N., and Drilleau, J.F., *Procyanidins Are the Most Abundant Polyphenols in Dessert Apples at Maturity.*, **2002**.

[27] D. E. Venegas-Pino, N. Banko, M. I. Khan, Y. Shi, G. H. Werstuck, *J. Vis. Exp.* **2013**, 50933.

[28] V. Victor, *Curr. Pharm. Des.* **2009**, *15*, 2986–2987.

[29] S. Oppi, T. F. Lüscher, S. Stein, *Front. Cardiovasc. Med.* **2019**, *6*, 46.

[30] A. S. Plump, J. D. Smith, T. Hayek, K. Aalto-Setälä, A. Walsh, J. G. Verstuyft, E. M. Rubin, J. L. Breslow, *Cell* **1992**, *71*, 343–353.

[31] Y. Nakashima, A. S. Plump, E. W. Raines, J. L. Breslow, R. Ross, *Arterioscler. Thromb. J. Vasc. Biol.* **1994**, *14*, 133–140.

[32] J. D. Smith, J. L. Breslow, *J. Intern. Med.* **1997**, *242*, 99–109.

[33] Y. T. Lee, H. Y. Lin, Y. W. F. Chan, K. H. C. Li, O. T. L. To, B. P. Yan, T. Liu, G. Li, W. T. Wong, W. Keung, G. Tse, *Lipids Health Dis.* **2017**, *16*, 12.

[34] G. S. Getz, C. A. Reardon, *Arterioscler. Thromb. Vasc. Biol.* **2012**, *32*, 1104–1115.

[35] J. Kanner, T. Lapidot, *Free Radic. Biol. Med.* **2001**, *31*, 1388–1395.

[36] M. Delosière, V. Santé-Lhoutellier, C. Chantelauze, D. Durand, A. Thomas, C. Joly, E. Pujos-Guillot, D. Rémond, B. Comte, C. Gladine, A. Guy, T. Durand, M. Laurentie, C. Dufour, *Food Funct.* **2016**, *7*, 3497–3504.

[37] M. Gobert, D. Rémond, M. Loonis, C. Buffière, V. Santé-Lhoutellier, C. Dufour, *Food Funct.* **2014**, *5*, 2166.

[38] S. Dalleau, M. Baradat, F. Guéraud, L. Huc, *Cell Death Differ.* **2013**, *20*, 1615–1630.

[39] S. Masumoto, A. Terao, Y. Yamamoto, T. Mukai, T. Miura, T. Shoji, *Sci. Rep.* **2016**, *6*, 31208.

[40] D. Gradinaru, C. Borsa, C. Ionescu, G. I. Prada, *Mech. Ageing Dev.* **2015**, *151*, 101–113.

[41] Y. Steffen, G. Vuillaume, K. Stolle, K. Roewer, M. Lietz, J. Schueller, S. Lebrun, T. Wallerath, *Nitric Oxide* **2012**, *27*, 176–184.

[42] A. Kleindienst, S. Battault, E. Belaidi, S. Tanguy, M. Rosselin, D. Boulghobra, G. Meyer, S. Gayrard, G. Walther, B. Geny, G. Durand, O. Cazorla, C. Reboul, *Basic Res. Cardiol.* **2016**, *111*, 40.

[43] C. R. White, T. A. Brock, L. Y. Chang, J. Crapo, P. Briscoe, D. Ku, W. A. Bradley, S. H. Gianturco, J. Gore, B. A. Freeman, *Proc. Natl. Acad. Sci.* **1994**, *91*, 1044–1048.

[44] L. Hooper, P. A. Kroon, E. B. Rimm, J. S. Cohn, I. Harvey, K. A. Le Cornu, J. J. Ryder, W. L. Hall, A. Cassidy, *Am. J. Clin. Nutr.* **2008**, *88*, 38–50.

[45] M.-H. Oak, C. Auger, E. Belcastro, S.-H. Park, H.-H. Lee, V. B. Schini-Kerth, *Free Radic. Biol. Med.* **2018**, *122*, 161–170.

[46] I. Nakashima, W. Liu, A. A. Akhand, K. Takeda, Y. Kawamoto, M. Kato, H. Suzuki, *Mol. Aspects Med.* **2003**, *24*, 231–238.

[47] M. O. R. Mian, N. Idris-Khodja, M. W. Li, A. Leibowitz, P. Paradis, Y. Rautureau, E. L. Schiffrin, *J. Pharmacol. Exp. Ther.* **2013**, *347*, 30–37.

[48] L. V. d'Uscio, L. A. Smith, Z. S. Katusic, *Stroke* **2001**, *32*, 2658–2664.

[49] C. M. Balarini, M. A. Leal, I. B. S. Gomes, T. M. C. Pereira, A. L. Gava, S. S. Meyrelles, E. C. Vasquez, *J. Transl. Med.* **2013**, *11*, 3.

[50] C. M. Andre, J. M. Greenwood, E. G. Walker, M. Rassam, M. Sullivan, D. Evers, N. B. Perry, W. A. Laing, *J. Agric. Food Chem.* **2012**, *60*, 10546–10554.

[51] D. Aune, E. Giovannucci, P. Boffetta, L. T. Fadnes, N. Keum, T. Norat, D. C. Greenwood, E. Riboli, L. J. Vatten, S. Tonstad, *Int. J. Epidemiol.* **2017**, *46*, 1029–1056.

[52] D. A. Hyson, *Adv. Nutr.* **2011**, *2*, 408–420.

[53] A. Koutsos, K. Tuohy, J. Lovegrove, *Nutrients* **2015**, *7*, 3959–3998.

Figure Legends

Figure 1. Effects of a Western diet with or without supplementation on weight gain and plasma lipoproteins. A: Mean of daily food intake during 12 weeks per mice. B: Follow up of body mass during the 12 weeks of different diets. C: Adiposity index at the end of the protocol diets. Plasma HDL (D), LDL (E), HDL-cholesterol/LDL-cholesterol (F), total cholesterol (G) and triglyceride (H), were assessed at the end of the dietary protocols. Values are expressed as means ± SEM (n=6/8 per group for LDL, HDL, HDL-c/LDL-c, total cholesterol and triglyceride). One way ANOVA (Tukey's multiple comparisons test).

Figure 2. 4-Hydroxy-2-nonenal (4-HNE) formation during gastrointestinal digestion and lipid oxidation products in plasma. 4-HNE concentration in faeces before (A), at 6 weeks (B) and 12 weeks (C) of diet measured by LC/MS/APCI. Plasma 4-HNE (D) and oxLDL (E) at the end of the 12 weeks. Values are expressed as means ± SEM (plasma 4-HNE n=5/7 per grp, oxLDL n=6/7 per grp). *P<0.05 *vs.* WD. #P<0.05 *vs.* WD+SO. §P<0.05 *vs.* WD. $P<0.05 *vs.* WD+SO+PUREE. One way ANOVA (Tukey's multiple comparisons test).

Figure 3. Effects of Western diets and protection by apple polyphenol supplementation on vascular function. Systolic (A), diastolic (B) and mean (C) blood pressure measured at the end of dietary protocols by tail cuff method. D: Dose-dependent response to cumulative doses of acetylcholine (ACh) and sodium nitroprusside (SNP) on aortic rings. *Left:* Dose-dependent response to cumulative doses of ACh. *Middle:* Maximal relaxation of aortic rings to ACh. *Right*: Dose-dependent response to cumulative doses of sodium nitroprusside (SNP). Data are expressed in percent of relaxation relative to maximal contraction obtained with 1 µM of phenylephrine. Values are expressed as means ± SEM (blood pressure n=9/11 per group, vasoreactivity n=6/8 per group). *P<0.05 *vs.* WD group. #P<0.05 *vs.* WD+SO group. §P<0.05 *vs.* WD. $P<0.05 *vs.* WD+SO+PUREE. £P<0.05 *vs.* Week 0. Dose-response were analyzed with a repeated measures ANOVA followed with a Tukey multiple comparisons test. Blood pressures and maximal relaxation were analyzed using a one way ANOVA (Tukey's multiple comparisons test.)

Figure 4. Development and characterization of atheromatous plaque size induced by Western diets and protection by apple polyphenol supplementation. (A) Evaluation of aortic cross atheromatous plaque size performed by echography. (B) Atheromatous plaque size in descending thoraco-abdominal aorta assessed by Oil-Red-O coloration. (C) Atheromatous plaque size in aortic root relative to aortic diameter. (D) Percent of fibrosis in atheromatous plaque of aortic valve and (E) Necrotic core size in atheromatous plaque of aortic root relative to aortic diameter assessed on histological sections. All measurements were performed at the end of dietary protocol. Values are expressed as means ± SEM (echography n=8 per group, histological sections n=5 animals per group). One way ANOVA (Tukey's multiple comparisons test.) *P<0.05 *vs.* WD group. #P<0.05 *vs.* WD+SO group. §P<0.05 *vs.* WD. $P<0.05 *vs.* WD+SO+PUREE.

Figure 5. Dietary effects on vascular NO and ROS production. (A) Quantification of nitrite in aorta. (B) Total ROS production measured by electron paramagnetic resonance in aortic tissue

716 homogenate. Values are expressed as means ± SEM (nitrite and ROS production n=4/grp).
717 *P<0.05 *vs.* WD group. #P<0.05 *vs.* WD+SO group. §P<0.05 *vs.* WD. $P<0.05 *vs.*
718 WD+SO+PUREE. ROS and Nitrite productions were analyzed using a one way ANOVA
719 (Tukey's multiple comparisons test.)

720

721

722

FIGURE 1

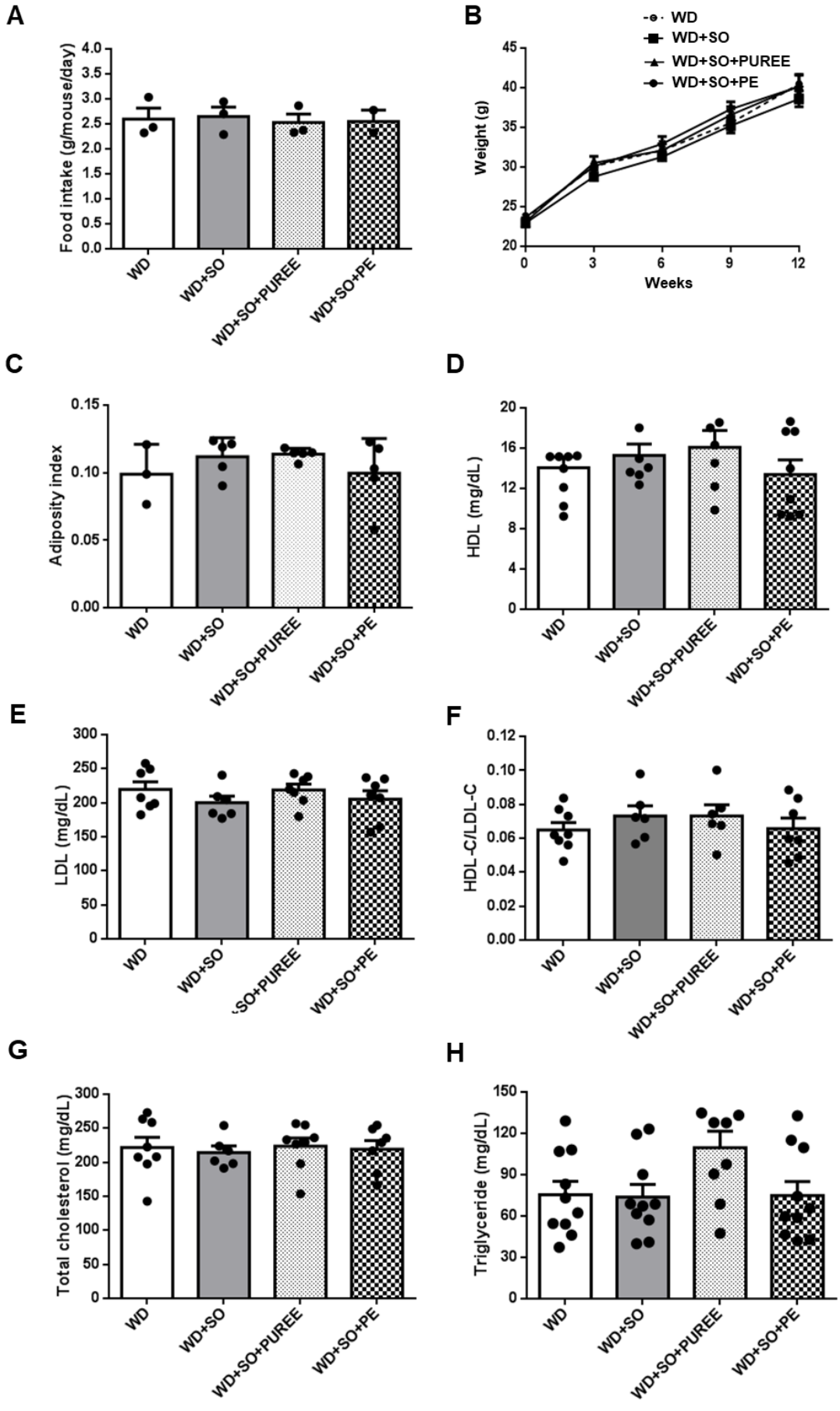
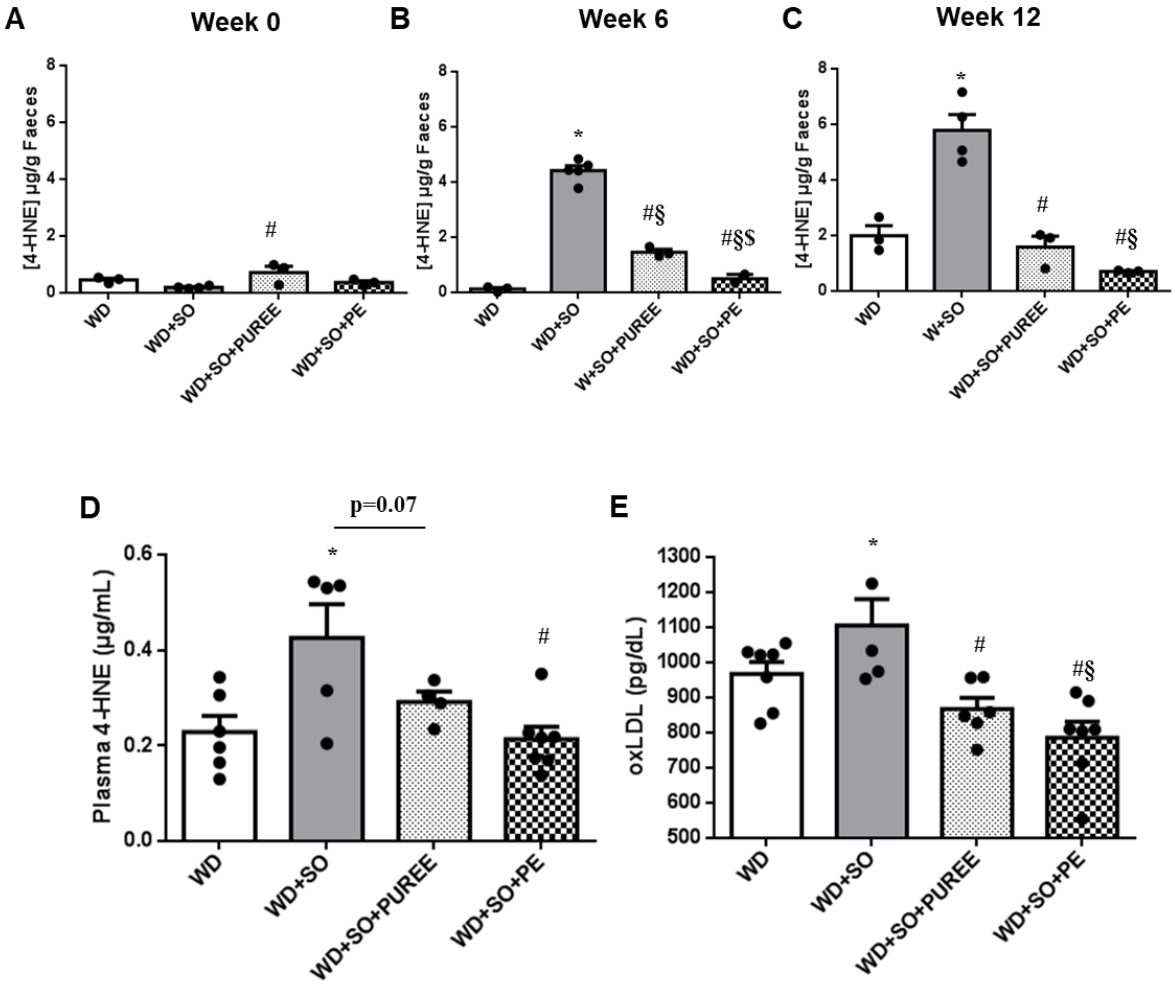

A
Food intake (g/mouse/day)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
B
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
Weight (g)
45
40
35
30
25
20
0
3
6
9
12
Weeks
C
Adiposity index
0.15
0.10
0.05
0.00
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
D
HDL (mg/dL)
20
16
12
8
4
0
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
E
LDL (mg/dL)
300
250
200
150
100
50
0
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
F
HDL-C/LDL-C
0.12
0.10
0.08
0.06
0.04
0.02
0.00
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
G
Total cholesterol (mg/dL)
300
250
200
150
100
50
0
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
H
Triglyceride (mg/dL)
150
120
90
60
30
0
WD
WD+SO
WD+SO+PUREE
WD+SO+PE

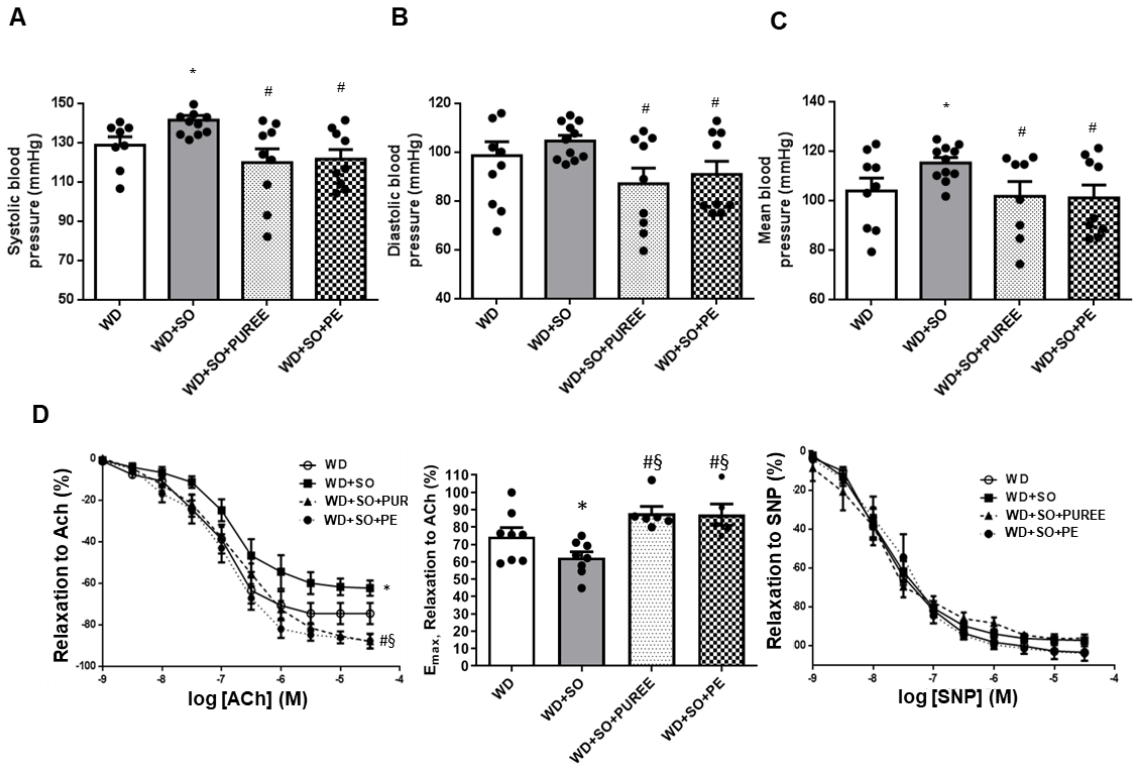
FIGURE 2



724

725

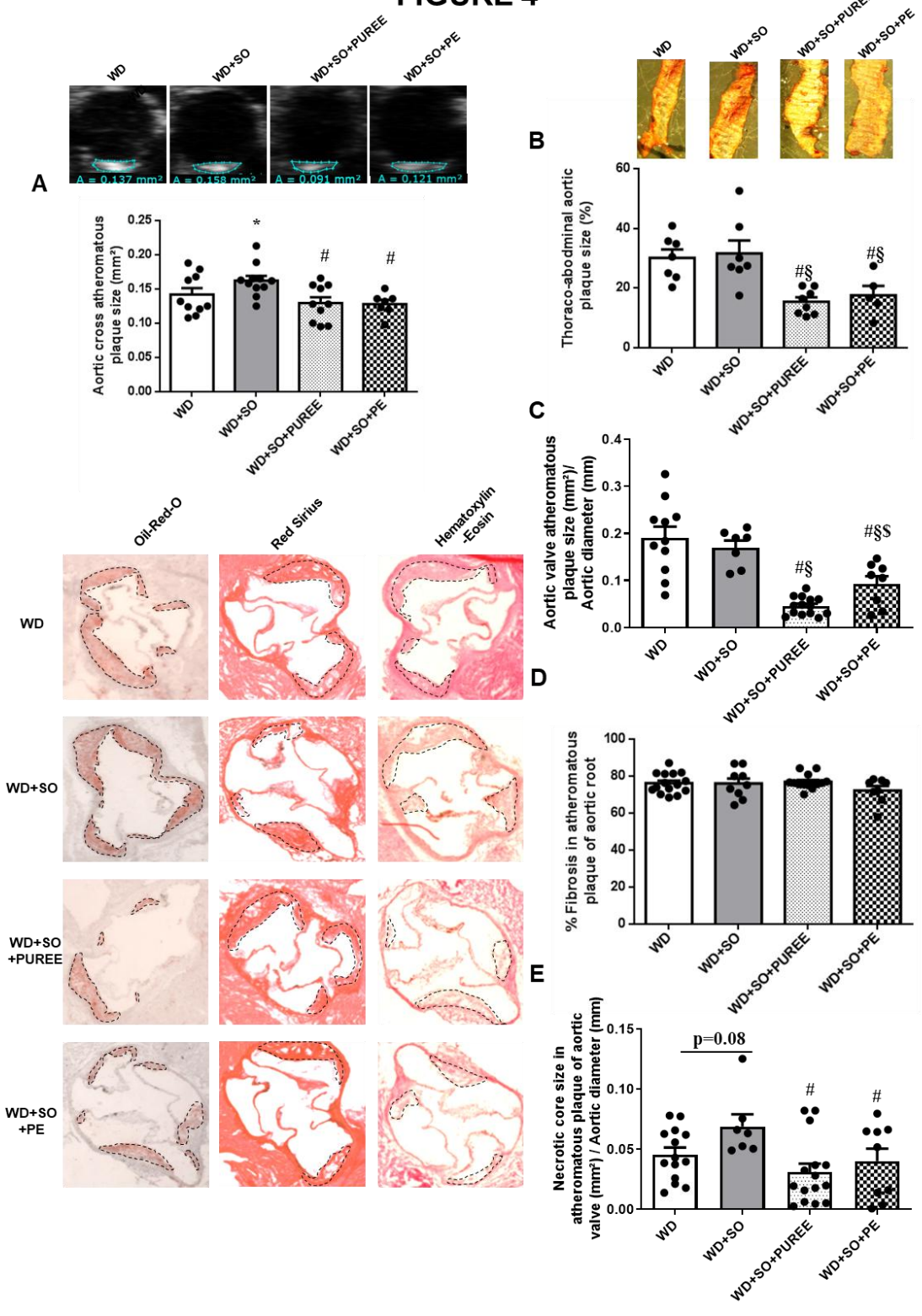
FIGURE 3



726

727

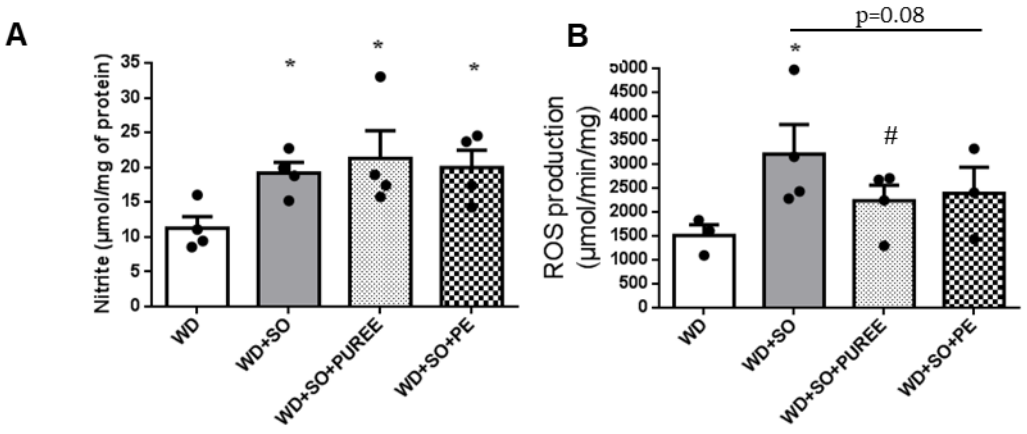
FIGURE 4



728

729

FIGURE 5


A
Nitrite (µmol/mg of protein)
35
30
25
20
15
10
5
0
*
*
*
WD
WD+SO
WD+SO+PUREE
WD+SO+PE
B
p=0.08
ROS production (µmol/min/mg)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
#
WD
WD+SO
WD+SO+PUREE
WD+SO+PE

730