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Mangifera indica L. extract (Vimang®) and its main polyphenol mangiferin prevent mitochondrial oxidative stress in atherosclerosis-prone hypercholesterolemic mouse

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ABSTRACT

Atherosclerosis is linked to a number of oxidative events ranging from low-density lipoprotein (LDL) oxidation to the increased production of intracellular reactive oxygen species (ROS). We have recently demonstrated that liver mitochondria isolated from the atherosclerosis-prone hypercholesterolemic LDL receptor knockout ($LDLr^{-/-}$) mice have lower content of NADP(H)-linked substrates than the controls and, as consequence, higher sensitivity to oxidative stress and mitochondrial membrane permeability transition (MPT). In the present work, we show that oral supplementation with the antioxidants *Mangifera indica* L. extract (Vimang®) or its main polyphenol mangiferin shifted the sensitivity of $LDLr^{-/-}$ liver mitochondria to MPT to control levels. These *in vivo* treatments with Vimang® and mangiferin also significantly reduced ROS generation by both isolated $LDLr^{-/-}$ liver mitochondria and spleen lymphocytes. In addition, these antioxidant treatments prevented mitochondrial NAD(P)H-linked substrates depletion and NADPH spontaneous oxidation. In summary, Vimang® and mangiferin spared the endogenous reducing equivalents (NADPH) in $LDLr^{-/-}$ mice mitochondria correcting their lower antioxidant capacity and restoring the organelle redox homeostasis. The effective bioavailability of these compounds makes them suitable antioxidants with potential use in atherosclerosis susceptible conditions.

1. Introduction

Elevation of plasma concentrations of low-density lipoprotein (LDL) is a key event in the development of atherosclerosis [1–3], a leading cause of death in the western world. A well documented and consistent hypothesis for atherogenesis [4] postulates that this disorder is triggered by oxidative modifications of LDL [5,6] when exposed to reactive oxygen species (ROS) from vascular wall cells [7–9].

Recent results from our group demonstrated that the atherosclerosis-prone hypercholesterolemic LDL receptor knockout ($LDLr^{-/-}$) mice present a cellular oxidative stress related to mitochondrial dysfunction [10]. Compared to the wild type, $LDLr^{-/-}$ mitochondria have a lower capacity to sustain their nicotinamine adenine dinucleotide (NADPH) pool in the reduced state [10,11], which compromises the source of reducing equivalents to reconstitute the mitochondrial enzymatic antioxidant system [12]. As a consequence, these $LDLr^{-/-}$ mitochondria accumulate larger amounts of reactive oxygen species and present higher susceptibility to develop Ca^{2+}-induced membrane permeability transition (MPT) [10], a condition associated with cell death [12]. Experiments *in vivo* provided direct evidence of higher cholesterol *de novo* synthesis, which consumes NADPH reducing equivalents in hypercholesterolemic mice tissues [10,11]. We then tested the hypothesis that inhibition of cholesterol synthesis could correct the decreased mitochondrial antioxidant defences in $LDLr^{-/-}$ mice by sparing mitochondrial NADPH. However, when therapeutic doses of statins (HMGCoA reductase inhibitors) were used in this animal model, the susceptibility to MPT further increased due to a direct action of the compounds on the mitochondrial membrane [13]. Another way to counteract the mitochondrial oxidative stress in these mice would be through treatment with suitable antioxidants.

Recently, a standard aqueous stem bark extract from selected species of *Mangifera indica* L. (Anacardiaceae) under the brand

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name of Vimang® was shown to exert *in vivo* and *in vitro* antioxidant activities [14–17]. Chemical analysis of this extract identified a variety of polyphenols, phenolic esters, flavan-3-ols and a xanthone (mangiferin), with the latter being the predominant component of this extract [18] and the main responsible for its antioxidant performance [19–21]. Vimang® treatment was shown to protect against serum oxidative stress in elderly humans [17], and mice treated with this extract were protected against 12-*O*-tetradecanoylphorbol-13-acetate induced lipid peroxidation in serum, in hepatic mitochondria and microsomes, and in brain homogenate supernatants [14]. Thus, these results suggest that it could be used to target the mitochondrial oxidative stress in LDLr$^{-/-}$ mice.

Therefore, the aim of the present study was to evaluate the efficacy of *in vivo* treatment of LDLr$^{-/-}$ mice with the antioxidants Vimang® and mangiferin in controlling their liver mitochondria redox unbalance.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), ADP, cyclosporin A, EGTA, succinate, rotenone, safranine, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), arsenazo III and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were purchased from Sigma–Aldrich (St. Louis, MO). All other reagents were commercially products of the highest purity grade available.

2.2. Preparation of Mangifera indica L. extract (Vimang®) and isolation of mangiferin

M. indica L. was collected from a cultivated field located in the region of Pinar del Río, Cuba. Voucher specimens of the plant (Code 41722) were deposited at the Herbarium of Academy of Sciences, guarded by the Institute of Ecology and Systematic from Ministry of Science, Technology and Environment, Havana, Cuba. Stem bark extract of *M. indica* was prepared by decoction with water for 1 h and then it was concentrated by evaporation and spray-dried to obtain a fine homogeneous brown powder with a particle size of 30–60 μm [18]. The chemical composition of the extract has been reported elsewhere [18] and has enabled the isolation and identification of phenolic acids (gallic acid, 3,4-dihydroxy benzoic acid, benzoic acid), phenolic esters (gallic acid methyl ester, gallic acid propyl ester, benzoic acid propyl ester), flavan-3-ols (catechin and epicatechin) and the xanthone mangiferin, which is the major component of Vimang® (16%).

Mangiferin, a C-glucosylxanthone (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside), was supplied by the Laboratory of Analytical Chemistry, Centre of Pharmaceutical Chemistry (Cuba) and had been isolated from *M. indica* stem bark standardized extract by extraction with methanol, and a yellow powder was obtained with 95% of purity determined by HPLC [18].

2.3. Animals and antioxidant treatments

The animals used in this study were the homozygous LDLr-null mice (LDLr$^{-/-}$) (Jackson Labs, Bar Harbor, ME) and control wild type C57BL6 mice as previously described [10]. The experiments were in accordance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animals Welfare (1992). Male and female mice, 4–6-month-old and weighing 20–26 g, were used in this study. The mice had free access to standard laboratory rodent chow diet (Nuvital CR1, Paraná, Brazil), water *ad libitum* and were housed at 22 ± 2 °C on a 12 h light–dark cycle. Animals were divided into four groups of five animals each: control wild type (WT), untreated LDLr$^{-/-}$, mangiferin-treated LDLr$^{-/-}$ and Vimang®-treated LDLr$^{-/-}$. Aqueous solutions of Vimang® or mangiferin were orally administered by a feeding needle at the doses equivalent to 250 and 40 mg/kg body weight per day, respectively, during 7 days. The control wild type and the untreated LDLr$^{-/-}$ mice were administered with distilled water. At the day 8, overnight fasted animals were killed by cervical dislocation and livers were rapidly extracted. Blood was obtained by cardiac puncture. Plasma total cholesterol levels were determined by an enzymatic-colorimetric assay (Boehringer Mannheim) according to the manufacturer's instructions.

2.4. Isolation of rat liver mitochondria and standard incubation procedure

Mitochondria were isolated by conventional differential centrifugation from the liver of adult animals fasted overnight [22]. The livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was resuspended in 250 mM sucrose to a final protein concentration of 80–100 mg/ml, measured by the method of Biuret with BSA as protein standard [23].

The experiments were carried out in standard medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, 10 mM HEPES buffer (pH 7.2), 2 μM rotenone and 5 mM succinate as a FAD-linked respiratory substrate. Other additions are indicated in the figure legends.

2.5. Mitochondrial respiration

Oxygen consumption in mitochondrial suspensions was polarographically determined with a Clark-type electrode (Yellow Springs Instruments Co.) in a 1.3-ml glass chamber equipped with a magnetic stirrer at 28 °C. Liver mitochondria (2 mg/ml) were added to standard respiration medium. Respiration rates are given in nmol O_2/mg protein/min. The experiment was calibrated using the oxygen content of air saturated medium of 425 ng atoms/ml at 28 °C [24].

2.6. Measurement of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

The mitochondrial membrane potential was estimated as fluorescence changes of safranin [25], recorded in a model Hitachi F-4010 spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 5 nm. Mitochondria (0.5 mg/ml) were incubated in 2 ml of standard medium supplemented with 5 μM safranin. After 30 s, 5 mM succinate was added. After 3 min, when $\Delta\Psi$ was stable, 30 μM Ca^{2+} was added to the medium and the periods of time until $\Delta\Psi$ collapse were estimated. Relative changes in membrane potential were expressed in arbitrary fluorescence units.

2.7. Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension measured at 540 nm in a Hitachi U-3000 spectrophotometer equipped with magnetic stirring and temperature control (28 °C). Mitochondria were incubated in the standard incubation medium at 0.5 mg of mitochondrial protein/ml.

2.8. Determination of NAD(P) redox state

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed in a Hitachi F-4010 spectrofluorimeter operating at 366 nm excitation and 450 nm emission [26]. All incubations were conducted in the presence of cyclosporin A, an inhibitor of MPT, in order to avoid fluorescence changes secondary to mitochondrial swelling, release of matrix NAD(P)H, or inhibition of NAD(P)H transhydrogenase activity due to increased membrane proton leakage.

2.9. Spleen mononuclear cell (SMC) isolation

Intact cells were isolated from spleen homogenate by the Ficoll-Hypaque technique [27]. Briefly, three spleens were homogenized in 13 ml of PBS buffer, laid onto Ficoll-Hypaque solution, and centrifuged at 400 × *g* for 25 min at temperature. The interface cell layer was recovered with a large bore Pasteur pipette and washed twice in PBS buffer, and the cells were counted in a Newbauer hemocytometer, using trypan blue exclusion to determine cell viability.

2.10. Reactive oxygen species production

Production of reactive oxygen species by liver mitochondria was followed by measuring the conversion of Amplex Red (Molecular Probes, Eugene, OR) in the presence of extramitochondrial horseradish peroxidase, to highly fluorescent resorufin, by H_2O_2 [28]. Mitochondria (1 mg/ml) were incubated in standard incubation medium supplemented with 10 μM Amplex Red and 1 U/ml horseradish peroxidase at 28 °C with continuous stirring. Resorufin fluorescence was measured in a Hitachi F-4010 spectrofluorimeter at 563 nm for excitation and 587 nm for emission. Intracellular ROS generation (SMC 6×10^6 cells/ml) was assessed using 1 μM dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes Inc., Eugene, Oregon, USA) according to Garcia-Ruiz et al. [29], at 488 nm for excitation and 525 nm for emission, with a slit width of 3 nm.

2.11. Lipid peroxidation assay

Lipid peroxidation was estimated from thiobarbituric reactive substances (TBARS) generation and was expressed as malonyldialdehyde concentration [30]. The mitochondrial suspension (400 μl, 1 mg/ml final concentration) was incubated with 400 μl of 1% thiobarbituric acid (prepared in 50 mM NaOH), 190 μl of 10% H_3PO_4, and 10 μl BHT 10 mM for 20 min at 90 °C. The thiobarbituric acid complexes were extracted with 2 ml *n*-butanol and absorbance of the extract was measured at 535 nm. Malonyldialdehyde concentration was estimated from $\varepsilon = 1.56 \times 10^5$/M cm.

2.12. Determination of Ca^{2+} movements

Variations in free Ca^{2+} concentration were followed by measuring the changes in the absorbance spectrum of the metallochromic indicator arsenazo III [31], using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm at 28 °C. The calibrations were performed by the sequential addition of known concentrations of EGTA.

2.13. Data analysis

Data from the experiments were analyzed by one-way ANOVA followed by Tukey's post-hoc test performed by Origin 7.5 software (OringinLab Corp., Northampton, MA). When one parameter was compared between two groups, Student's *t*-test was used. Data are presented as mean ± S.D. of at least five experiments conducted with different preparations.

3. Results

3.1. Plasma cholesterol levels in LDLr$^{-/-}$ hypercholesterolemic mice are not affected by Vimang® and mangiferin treatment

Table 1 shows that total cholesterol levels in LDLr$^{-/-}$ mice are elevated by five-fold when compared to control wild type mice. No statistically significant alterations in plasma cholesterol levels were observed after treatment of control and LDLr$^{-/-}$ mice with either Vimang® (250 mg/kg) or mangiferin (40 mg/kg).

3.2. Vimang® and mangiferin treatment of LDLr$^{-/-}$ mice correct their mitochondrial capacity to retain accumulated Ca^{2+} and sustain membrane potential

Damage to the inner mitochondrial membrane can be assessed by the classic swelling technique, which monitors the net influx of the osmotic support associated with a non-specific increase in

Table 1
Plasma cholesterol levels in LDLr$^{-/-}$ hypercholesterolemic mice treated with Vimang® or mangiferin

Groups	Cholesterol (mg/dl) Baseline	Cholesterol (mg/dl) One week treatment
Untreated LDLr$^{-/-}$ mice	315 ± 14	318 ± 34
Vimang® (250 mg/kg)	310 ± 16	313 ± 14^{ns}
Mangiferin (40 mg/kg)	307 ± 14	302 ± 11^{ns}
Control (wild mice)	66 ± 2	64.1 ± 3[a]

Results are expressed as mean ± S.D. ($n = 5$).
^{ns}, not significant ($p > 0.05$) *vs.* untreated LDLr$^{-/-}$ mice.
[a] Statistical difference at $p < 0.05$.

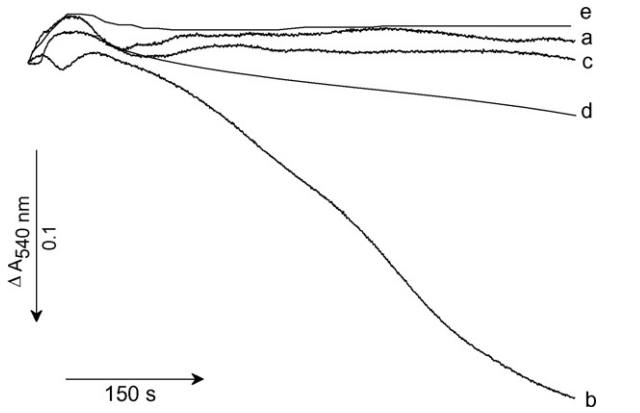

Fig. 1. *In vivo* Vimang® and mangiferin supplementations inhibited Ca^{2+}-induced liver mitochondrial swelling in LDL receptor knockout mice. Mouse liver mitochondria (0.5 mg/ml) were incubated at 28 °C under continuous stirring in standard medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, 10 mM HEPES buffer (pH 7.2), 30 μM Ca^{2+}, 2 μM rotenone and 5 mM succinate as FAD-linked respiratory substrate. Reaction medium contains: mitochondria isolated from control wild type mice (WT) (*line a*), from untreated LDLr$^{-/-}$ mice (*line b*), from Vimang®-treated LDLr$^{-/-}$ mice (*line c*), from mangiferin-treated LDLr$^{-/-}$ mice (*line d*). *Line e* represents experimental conditions of *line b* plus 1 μM CsA. The downward deflection indicates mitochondrial swelling. Results are representative of five experiments conducted with independent mitochondrial preparations. The absorbance at 480 s was: 0.742 ± 0.037 (*line a*), 0.564 ± 0.028 (*line b*), 0.733 ± 0.041 (*line c*) and 0.701 ± 0.017 (*line d*). Differences statistically significant ($p < 0.05$) were found between (*line a*) and (*line b*), between (*line c*) and (*line b*) and between (*line d*) and (*line b*). *Line c* does not differ from *line a*.

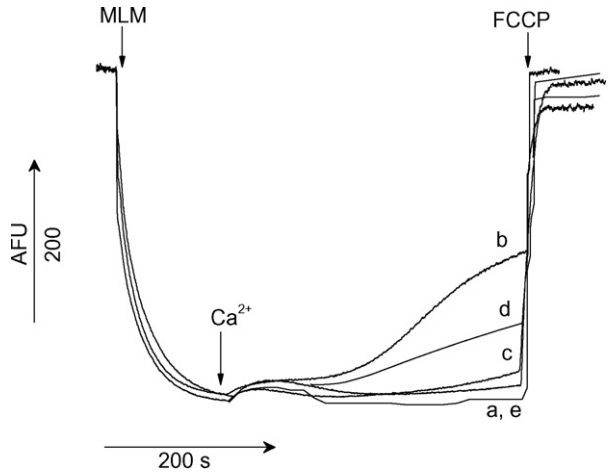

Fig. 2. Vimang® and mangiferin inhibited Ca^{2+}-induced mitochondrial transmembrane electrical potential ($\Delta\Psi$) disruption in $LDLr^{-/-}$ mice. $\Delta\Psi$ was estimated as described in Section 2 and expressed as arbitrary fluorescence units (AFU). Experimental conditions are described in the legend of Fig. 1, except that safranine (10 μM) was added to the reaction medium. Reaction medium also contains: liver mitochondria from WT mice (*line a*), from untreated $LDLr^{-/-}$ mice (*line b*), from Vimang®-treated $LDLr^{-/-}$ mice (*line c*), from mangiferin-treated $LDLr^{-/-}$ mice (*line d*). *Line e* represents experimental conditions of *line b* plus 1 μM CsA. Mice liver mitochondria (MLM 0.5 mg/ml), Ca^{2+} (30 μM) and the uncoupler FCCP (1 μM) were added as indicated by the arrows. Results are representative of five experiments conducted with independent mitochondrial preparations. The fluorescence units at 480 s were: 70.51 ± 5.92 (*line a*), 227.30 ± 10.26 (*line b*), 85.69 ± 3.70 (*line c*) and 142.90 ± 12.09 (*line d*). Differences statistically significant were found between (*line a*) and (*line b*), between (*line c*) and (*line b*) and between (*line d*) and (*line b*), at $p < 0.05$ level.

membrane permeability. It was previously shown that liver mitochondria from $LDLr^{-/-}$ mice have an increased susceptibility to Ca^{2+} dependent permeability transition [10]. Fig. 1 confirms the above mentioned results as revealed by the large decrease in turbidity of

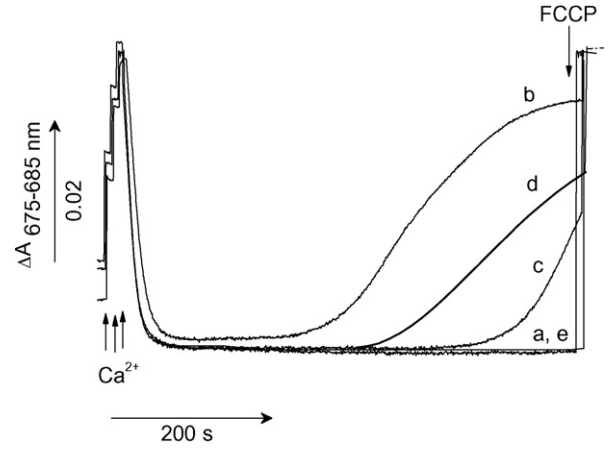

Fig. 3. Vimang® and mangiferin treatment prevented Ca^{2+} efflux from $LDLr^{-/-}$ mice mitochondria. Ca^{2+} movements were estimated as described in Section 2, using the specific Ca^{2+} indicator arsenazo III (20 μM). Experimental conditions are described in the legend of Fig. 1. Reaction medium contains: liver mitochondria from WT mice (*line a*), from untreated $LDLr^{-/-}$ mice (*line b*), from Vimang®-treated $LDLr^{-/-}$ mice (*line c*), from mangiferin-treated $LDLr^{-/-}$ mice (*line d*). *Line e* represents experimental conditions of *line b* plus 1 μM CsA. Mice liver mitochondria (MLM 0.5 mg/ml), Ca^{2+} (three additions of 10 μM each) and the uncoupler FCCP (1 μM) were added as indicated by the arrows. Results are representative of five experiments conducted with independent mitochondrial preparations. The values of Δabsorbance at 480 s were: 0.0141 ± 0.0004 (*line a*), 0.0478 ± 0.0002 (*line b*), 0.0205 ± 0.0005 (*line c*) and 0.0339 ± 0.0007 (*line d*). Differences statistically significant were found between (*line a*) and (*line b*), between (*line c*) and (*line b*) and between (*line d*) and (*line b*), at $p < 0.05$ level.

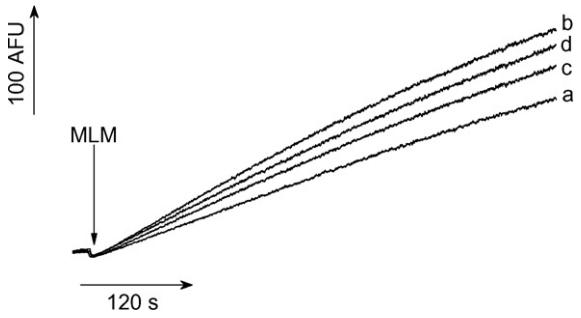

Fig. 4. Vimang® and mangiferin inhibited H_2O_2 production by the LDL receptor knockout mice mitochondria, monitored by oxidation of Amplex Red to the fluorescent compound resorufin. Results are expressed as arbitrary fluorescence units (AFU). MLM (1 mg/ml) from WT (*line a*), untreated $LDLr^{-/-}$ mice (*line b*), Vimang®-treated $LDLr^{-/-}$ mice (*line c*) and mangiferin-treated $LDLr^{-/-}$ mice (*line d*) were added to a standard reaction medium containing 10 μM Amplex Red and 1 U/ml HRP in the presence of 100 μM EGTA. MLM were added as indicated by the arrow. Results are representative of five experiments conducted with independent mitochondrial preparations. Maximum rates (slopes) of H_2O_2 production (AFU/mg/min) were as follows: 15.59 ± 1.97 (*line a*), 22.45 ± 1.31 (*line b*), 18.70 ± 1.05 (*line c*) and 20.17 ± 0.76 (*line d*). Differences statistically significant were found between (*line a*) and (*line b*), between (*line b*) and (*line c*) and between (*line b*) and (*line d*) at $p < 0.05$ level.

the mitochondrial suspension followed at 540 nm in $LDLr^{-/-}$ *versus* control WT (*line b vs. line a*). This swelling was associated with $\Delta\Psi$ depolarization (Fig. 2, *line b vs. line a*) and a faster mitochondrial Ca^{2+} release (Fig. 3, *line b vs. line a*).

Vimang® treatment almost completely inhibited the swelling process (Fig. 1, *line c*) similarly to the protection elicited by the classic MPT inhibitor cyclosporin A (*line e*). It also significantly protected against mitochondrial $\Delta\Psi$ dissipation and calcium efflux (*lines c* in Figs. 2 and 3, respectively). Similar protection was elicited by mangiferin treatment (see *lines d* in Figs. 1–3), although to a lesser extend when compared to the Vimang®-treated mice mitochondria.

3.3. *Vimang® and mangiferin treatments decrease ROS generation and lipid peroxidation in $LDLr^{-/-}$ hypercholesterolemic mice liver mitochondria and spleen lymphocytes*

We have shown that MPT is favored by oxidative damage to membrane proteins [12]. To evaluate the influence of Vimang® and mangiferin treatment on the $LDLr^{-/-}$ mice mitochondrial ROS production, we measured the H_2O_2 released in the medium by using the Amplex Red assay, in the absence of Ca^{2+} (100 μM EGTA). Fig. 4 shows that mitochondria from Vimang®-treated $LDLr^{-/-}$ mice released significantly less hydrogen peroxide than mitochondria from untreated $LDLr^{-/-}$ mice (*line c vs. line b*). Mangiferin treatment also diminished ROS production by mitochondria from $LDLr^{-/-}$ mice (*line d*). This suggests an important contribution of this compound to the overall antioxidant effect of the extract. Accordingly, the lipid peroxidation assay showed a potent inhibition of TBARS formation in mitochondria from $LDLr^{-/-}$ mice treated with Vimang® or mangiferin ($p < 0.01$) (Fig. 5), which is in line with the inhibition of ROS production.

To ascertain whether the effects of Vimang® extract or mangiferin were restricted to liver mitochondria, ROS generation was also measured in whole mononuclear cells isolated from mice spleen. Fig. 6 shows that, as in liver mitochondria, $LDLr^{-/-}$ mice lymphocytes (*line b*) produce more ROS than cells from control wild type mice (*line a*). Vimang® treatment of $LDLr^{-/-}$ mice reduced ROS production by 50% (*line c*). Mangiferin only slightly reduced ROS production (*line d*) by $LDLr^{-/-}$ mice lymphocytes.

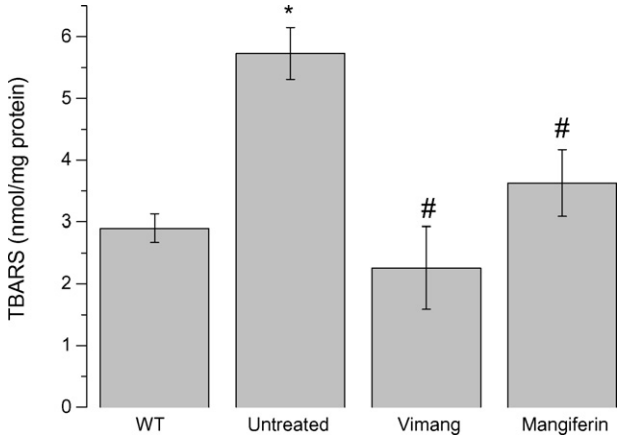

Fig. 5. Vimang® and mangiferin supplementation prevented mitochondrial membrane lipid peroxidation in LDLr$^{-/-}$ mice. Experimental conditions are described in the legend of Fig. 4. Mice liver mitochondria (1 mg/ml) were incubated 5 min in standard medium containing 2 μM rotenone, 5 mM succinate and 100 μM EGTA. thiobarbituric reactive substances (TBARS) expressed as malonyldialdehyde concentration was determined as described under Section 2. Values represent average of five independent experiments ±S.D. *$p < 0.01$ when comparing WT with untreated LDLr$^{-/-}$ mice, #$p < 0.01$ when comparing untreated *vs.* Vimang® or mangiferin-treated LDLr$^{-/-}$ mice.

3.4. Influence of Vimang® and mangiferin treatment on the LDLr$^{-/-}$ hypercholesterolemic mice mitochondrial NADPH redox status

It was shown that LDLr$^{-/-}$ mouse mitochondria were incapable to sustain a reduced state of matrix NADPH, the main mitochondrial source of antioxidant defence against ROS, likely due to high rates of lipogenesis [10]. Thus, we evaluated the influence of Vimang® treatment on spontaneous NADPH oxidation. Pyridine nucleotide oxidation was followed fluorometrically under experimental conditions similar to those used in the ROS assays. A time-dependent decrease in fluorescence of untreated LDLr$^{-/-}$ mice (Fig. 7, *line b*) but not in control WT mice mitochondria (*line a*) was observed.

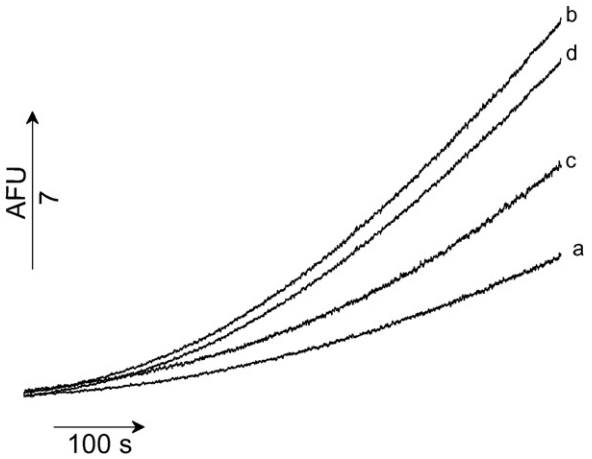

Fig. 6. Generation of reactive oxygen species by whole spleen mononuclear cells (SMC). Cells from WT (*line a*), untreated LDLr$^{-/-}$ mice (*line b*), Vimang®-treated LDLr$^{-/-}$ mice (*line c*), and mangiferin-treated LDLr$^{-/-}$ mice (*line d*), were added to standard reaction medium containing 1 μM H_2DCFDA. Maximum rates (slopes) of DCF production (nmol/min/2.4 × 10^6cells) are as follows: 0.12 ± 0.01 (*line a*), 0.19 ± 0.02 (*line c*), 0.25 ± 0.01 (*line d*), 0.27 ± 0.01 (*line b*). Differences statistically significant were found between (*line a*) and (*line b*), and between (*line b*) and (*line c*) at $p < 0.05$ level. Results are representative of five experiments conducted with independent mitochondrial preparations.

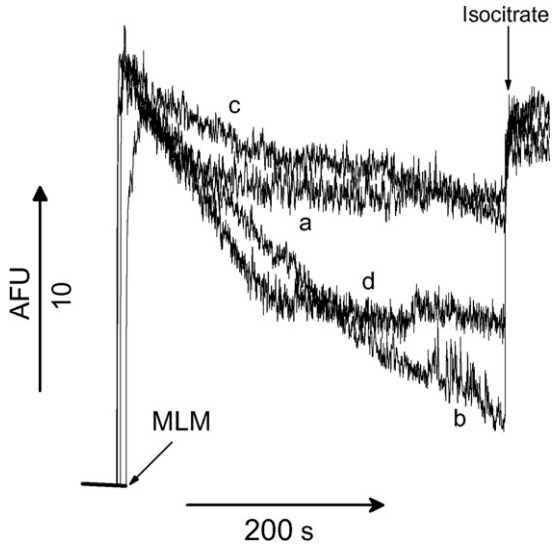

Fig. 7. Spontaneous oxidation of mitochondrial pyridine nucleotides was partially inhibited in LDLr$^{-/-}$ mice treated with Vimang® or mangiferin. MLM (1 mg/ml) from WT (*line a*), untreated LDLr$^{-/-}$ mice (*line b*), Vimang®-treated LDLr$^{-/-}$ mice (*line c*), and mangiferin-treated LDLr$^{-/-}$ mice (*line d*) were added to standard reaction medium in the presence 100 μM EGTA and 5 μM rotenone. Isocitrate (1 mM) was added as indicated. Results are representative of five experiments conducted with independent mitochondrial preparations. The fluorescence units at 420 s (before isocitrate addition) were: 79.42 ± 2.59 (*line a*), 67.76 ± 1.52 (*line b*), 77.79 ± 1.72 (*line c*) and 72.82 ± 2.42 (*line d*). Differences statistically significant were found between (*line a*) and (*line b*), between (*line b*) and (*line c*) and between (*line d*) and (*line b*), at $p < 0.05$ level. *Line c* does not differ from *line a*.

This fluorescence decrease reflects NADPH oxidation in a manner reversed by isocitrate. Mitochondria from Vimang®-treated LDLr$^{-/-}$ mice sustained most of their endogenous NADPH in the reduced state up to 300 s (*line c*). At the end of the experiment (600 s), Vimang®-treated LDLr$^{-/-}$ mitochondria conserved more than 90% of the pyridine nucleotides in the reduced state as estimated by the addition of isocitrate. In liver mitochondria, isocitrate quickly restores the fluorescence of the LDLr$^{-/-}$ mitochondria to the level of controls. Mangiferin treatment (*line d*) also decreased

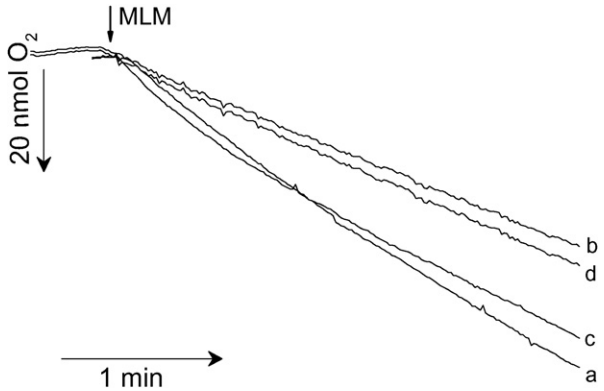

Fig. 8. Vimang® and mangiferin supplementation increased the rate of oxygen consumption sustained by endogenous substrates of mitochondria isolated from LDLr$^{-/-}$ mice. MLM (2.0 mg/ml) from WT mice (*line a*), untreated LDLr$^{-/-}$ mice (*line b*), Vimang®-treated LDLr$^{-/-}$ mice (*line c*), and mangiferin-treated LDLr$^{-/-}$ mice (*line d*) were added to standard reaction medium in the presence of 100 μM EGTA and 1 mM ADP. The rates of O_2 consumption (nmol O_2 /mg protein/min) were as follows: 6.25 ± 1.07 (*line a*), 2.79 ± 0.62 (*line b*), 6.25 ± 1.06 nmol (*line c*), and 3.70 ± 0.67 (*line d*). *Line b* is significant different from *lines a* and *c* at $p < 0.05$. *Line c* does not differ from *line a*. Results are representative of five experiments conducted with independent mitochondrial preparations.

the spontaneous NADPH oxidation in treated LDLr$^{-/-}$ mitochondria although to a lesser extend than Vimang® treatment.

The above results led to the hypothesis that the increase in the antioxidant capacity of hypercholesterolemic LDLr$^{-/-}$ mice mitochondria promoted by the antioxidant supplementation could spare the endogenous NAD(P)H pool. In order to ascertain this proposition we measured the rates of respiration supported by endogenous NAD(P)H-linked substrates in liver mitochondria isolated from control and LDLr$^{-/-}$ mice. Fig. 8 shows that mitochondria from untreated LDLr$^{-/-}$ hypercholesterolemic mice consume O_2 at lower rates (*line b*) than that from WT mice (*line a*). On the other hand, *in vivo* treatment of LDLr$^{-/-}$ mice with the antioxidant Vimang® restored the mitochondrial respiration rate to the level of the control (*line c*). No significant differences in O_2 consumption rates were observed when control and LDLr$^{-/-}$ mitochondria were assayed in the presence of 1 mM isocitrate (results not shown), showing that the activities of the respective dehydrogenases were similar and that the limiting factor in the LDLr$^{-/-}$ mitochondria respiration was, indeed, the endogenous content of NAD(P)H-linked substrates. Mangiferin treatment (*line d*) only slightly increased mitochondrial O_2 consumption rate in LDLr$^{-/-}$ mice.

4. Discussion

There is a large body of evidences linking atherosclerosis with a number of oxidative events ranging from LDL oxidation to the production of intracellular ROS (for review see refs. [4,32]). Mitochondria can produce large amounts of ROS and play an important role in the life and death of a cell. Thus, mitochondrial dysfunction and oxidative damage contribute to a number of diseases, including atherosclerosis (for review see refs. [33,34]). Previous experiments provided evidence that higher cholesterol *de novo* synthesis in the LDLr$^{-/-}$ mice liver decreases mitochondrial NADPH reducing equivalents [10,11], thus increasing the mitochondrial susceptibility to oxidative stress and MPT [10,12]. Although inhibition of cholesterol synthesis by statins treatment seemed to be the ideal strategy to correct the lower mitochondria antioxidant defence in the LDLr$^{-/-}$ mice, we showed that statins worsened the condition of MPT in their liver and skeletal muscle mitochondria [13]. Therefore, we turned our attention to an alternative mitochondrial targeted antioxidant strategy. The present study shows that the administration of either *M. indica* L. extract (Vimang®) or partially purified mangiferin fraction *in vivo* to the LDLr$^{-/-}$ mice reduced their mitochondrial oxidative stress and protected against the organelle susceptibility to permeability transition.

The poor performance of most antioxidant strategies used in clinical trials to reduce atherosclerosis [34] provides a real challenge to targeting at oxidative stress associated with this disease. Pharmacological intervention relying on non-selective antioxidants often appears to be ineffective. A distinction between one electron (1e) and two electron (2e) oxidants is of particular importance since most reported trials used species that are solely effective against 1e-oxidation reactions [32]. We recently demonstrated that mitochondrial accumulation of H_2O_2, but not $O_2^{\bullet -}$, occurs in the LDLr$^{-/-}$ mice [11]. This finding highlights the importance of this 2e-oxidant for atherosclerosis susceptibility. Reactions involving H_2O_2 are not inhibited by classic antioxidants such as vitamin E [32]. As a consequence, excess H_2O_2 production would not be altered by most antioxidant interventions that have been published so far. *In vitro*, Vimang® showed a powerful scavenger activity for both 1e- (hydroxyl radicals) and 2e- (hypochlorous acid) oxidants and, in addition, acted as an iron chelator *in vitro* ([15,16,35], reviewed in ref. [36]) and *in vivo* [37].

The Vimang® redox-active compounds are probably their polyphenols. For this class of compounds, beneficial effects on cardiovascular disease have been described, for instance, in humans treated with black tea [38]. In addition, limited but positive data suggest that green tea may inhibit atherogenesis in animal models [39]. The polyphenol resveratrol found in red wine was shown to decrease experimental atherosclerosis [40,41]. However, several phenolic lipid-soluble antioxidants such as probucol and analogs have provided conflicting results regarding the atherosclerotic lesion progression in animal model studies (reviewed in refs. [32,42]).

Previous studies have already shown the antioxidant capacity of Vimang® and mangiferin *in vitro* and *in vivo*. In ischemia-reperfusion models, Vimang® antioxidant protection was verified in rat liver and gerbil brain [43,44]. In addition, protection against induced lipid peroxidation and oxidative damage by Vimang® was comparable to or higher than that of vitamins C, E, C + E and β-carotene in plasma, macrophages, liver, and brain from treated mice [14].

The amount of mangiferin given to the LDLr$^{-/-}$ mice was the same contained in the Vimang® extract dose. In most of the assays performed in the present study the protection conferred by the Vimang® extract was higher than that conferred by the xanthone mangiferin. Vimang® contains other polyphenols, including phenolic acids (gallic, 3,4-dihydroxy benzoic, and benzoic acids), phenolic esters (methyl gallate, propyl gallate, propyl benzoate) and flavan-3-ols (catechin/epicatechin). Green tea catechin derivatives have shown important antioxidant effects in treated apolipoprotein E knockout mice which presented decreased early atherosclerosis lesion development [45]. Therefore, a possible additive action between mangiferin and catechin or other polyphenols of Vimang® may explain the increased efficacy of the extract treatment in relation to the purified fraction of mangiferin. The combination of these polyphenols in the extract may have greater ability to reach the appropriate extracellular and intracellular sites of oxidant stress, especially mitochondria.

The antioxidant effect of Vimang® was not only limited to the hepatic tissue, it also reached the spleen lymphocytes, where it reduced ROS production by about 50%. This finding suggests that the antioxidant performance of Vimang® in LDLr$^{-/-}$ mice may target different organs and tissues, and probably also endothelial cells. A first metabolizing hepatic step should be considered to explain the minor effect of mangiferin on the ROS production in lymphocytes.

In summary, present results show that Vimang® and mangiferin, through their ROS scavenging abilities, spared the endogenous reducing equivalents (NADPH) in LDL receptor knockout mice mitochondria, which then could support a safe endogenous cholesterol synthesis without major disturbance of the mitochondrial glutathione-dependent detoxifying system. Since no reduction in total plasma cholesterol levels of treated mice were observed, Vimang® and mangiferin actions can be attributed solely to antioxidant mechanisms. Therefore, Vimang® and mangiferin treatments corrected the LDLr$^{-/-}$ mice mitochondrial low antioxidant capacity restoring the organelle redox homeostasis, even with an increased lipogenesis rate.

Given the recent evidences of the participation of mitochondrial oxidative stress in the atherosclerosis development [10,11,46–48], targeting antioxidants to mitochondria may be a potential new intervention strategy for cardiovascular diseases. The present data demonstrate the effective bioavailability of Vimang® and mangiferin, and therefore, justify further research to determine how their long term *in vivo* administration would influence the atherosclerosis development.

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References

[1] Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34–47.
[2] Stokes 3rd J, Kannel WB, Wolf PA, Cupples LA, D'Agostino RB. The relative importance of selected risk factors for various manifestations of cardiovascular disease among men and women from 35 to 64 years old: 30 years of follow-up in the Framingham Study. Circulation 1987;75:V65–73.
[3] Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 1993;92:883–93.
[4] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radic Biol Med 2000;28:1815–26.
[5] Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915–24.
[6] Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. J Clin Invest 1991;88:1785–92.
[7] Morel DW, DiCorleto PE, Chisolm GM. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. Arteriosclerosis 1984;4:357–64.
[8] Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. Arteriosclerosis 1986;6:505–10.
[9] Lamb DJ, Wilkins GM, Leake DS. The oxidative modification of low density lipoprotein by human lymphocytes. Atherosclerosis 1992;92:187–92.
[10] Oliveira HC, Cosso RG, Alberici LC, Maciel EN, Salerno AG, Dorighello GG, et al. Oxidative stress in atherosclerosis-prone mouse is due to low antioxidant capacity of mitochondria. FASEB J 2005;19:278–80.
[11] Paim BA, Velho JA, Castilho RF, Oliveira HFC, Vercesi AE. Oxidative stress in hypercholesterolemic LDL receptor knockout mice is associated with low content of mitochondrial NADP-linked substrates and is partially reversed by citrate replacement. Free Radic Biol Med 2008;44:444–51.
[12] Kowaltowski AJ, Castilho RF, Vercesi AE. Mitochondrial permeability transition and oxidative stress. FEBS Lett 2001;495:12–5.
[13] Velho JA, Okanobo H, Degasperi GR, Matsumoto MY, Alberici LC, Cosso RG, et al. Statins induce calcium-dependent mitochondrial permeability transition. Toxicology 2006;219:124–32.
[14] Sanchez GM, Re L, Giuliani A, Nunez-Selles AJ, Davison GP, Leon-Fernandez OS. Protective effects of *Mangifera indica* L. extract, mangiferin and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. Pharmacol Res 2000;42:565–73.
[15] Pardo Andreu G, Delgado R, Velho J, Inada NM, Curti C, Vercesi AE. *Mangifera indica* L. extract (Vimang) inhibits Fe^{2+}-citrate-induced lipoperoxidation in isolated rat liver mitochondria. Pharmacol Res 2005;51:427–35.
[16] Pardo Andreu G, Delgado R, Núñez-Sellés AJ, Vercesi AE. *Mangifera indica* L. extract (Vimang) inhibits 2-deoxyribose damage induced by Fe (III) plus ascorbate. Phytother Res 2006;20:120–4.
[17] Pardo-Andreu GL, Philip SJ, Riano A, Sanchez C, Viada C, Nunez-Selles AJ, et al. *Mangifera indica* L. (Vimang) protection against serum oxidative stress in elderly humans. Arch Med Res 2006;37:158–64.
[18] Nunez-Selles AJ, Velez-Castro HT, Aguero-Aguero J, Gonzalez-Gonzalez J, Naddeo F, De Simone F, et al. Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (*Mangifera indic*a L.) stem bark aqueous decoction used in Cuba as nutritional supplement. J Agric Food Chem 2002;50:762–6.
[19] Andreu GL, Delgado R, Velho JA, Curti C, Vercesi AE. Iron complexing activity of mangiferin, a naturally occurring glucosylxanthone, inhibits mitochondrial lipid peroxidation induced by Fe^{2+}–citrate. Eur J Pharmacol 2005;513:47–55.
[20] Pardo-Andreu G, Sánchez-Baldoquín C, Ávila-González R, Delgado R, Naal Z, Curti C. Fe(III) improves antioxidant and cytoprotecting activities of mangiferin. Eur J Pharmacol 2006;547:31–6.
[21] Pardo-Andreu GL, Delgado R, Núñez-Sellés AJ, Vercesi AE. Dual mechanism of mangiferin protection against iron- induced damage to 2-deoxyribose and ascorbate oxidation. Pharmacol Res 2006;53:253–60.
[22] Kaplan RS, Pedersen PL. Characterization of phosphate efflux pathways in rat liver mitochondria. Biochem J 1983;212:279–88.
[23] Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of biuret reaction. J Biol Chem 1949;177:751–66.
[24] Reynafarje B, Costa LE, Lehninger ALJ. O_2 solubility in aqueous media determined by a kinetic method. Anal Biochem 1985;145:406–18.
[25] Åkerman KEO, Wikstrom MKF. Safranine as a probe of the mitochondrial membrane potential. FEBS Lett 1976;68:191–7.
[26] Fagian MM, Pereira-da-Silva L, Martins IS, Vercesi AE. Membrane protein thiol cross-linking associated with the permeabilization of the inner mitochondrial membrane by Ca^{2+} plus prooxidants. J Biol Chem 1990;265:19955–60.
[27] Boyum A. Isolation of lymphocytes, granulocytes and macrophages. Scand J Immunol 1976;5:9–15.
[28] Votyakova TV, Reynolds IJ. DeltaPsi(m)-dependent and -independent production of reactive oxygen species by rat brain mitochondria. J Neurochem 2001;79:266–77.
[29] Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. J Biol Chem 1997;272:11369–77.
[30] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–10.
[31] Scarpa A. Measurements of cation transport with metallochromic indicators. Methods Enzymol 1979;56:301–38.
[32] Stocker R, Keaney JFJr. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004;84:1381–478.
[33] Vercesi AE, Castilho RF, Kowaltowski AJ, Oliveira HC. Mitochondrial energy metabolism and redox state in dyslipidemias. IUBMB Life 2007;59:263–8.
[34] Rodrigo R, Guichard C, Charles R. Clinical pharmacology and therapeutic use of antioxidant vitamins. Fundam Clin Pharmacol 2007;21:111–27.
[35] Pardo-Andreu GL, Sánchez-Baldoquín C, Ávila-González R, Suzuki Yamamoto ET, Revilla A, Uyemura SA, et al. Interaction of Vimang (*Mangifera indica* L. extract) with Fe(III) improves its antioxidant and cytoprotecting activity. Pharmacol Res 2006;54:389–95.
[36] Nunez-Selles AJ, Delgado-Hernández R, Garrido-Garrido G, García-Rivera D, Guevara García M, Pardo-Andreu GL. The paradox of natural products as pharmaceuticals. Pre-clinical and clinical evidences of a mango stem bark extract. Pharmacol Res 2007;55:351–8.
[37] Pardo Andreu GL, Barrios MF, Curti C, Hernández I, Merino N, Lemus Y, et al. Protective effects of *Mangifera indica* L extract (Vimang), and its major component mangiferin, on iron-induced oxidative damage to rats serum and liver. Pharmacol Res 2008;57:79–86.
[38] Duffy SJ, Keaney Jr JF, Holbrook M, Gokce N, Swerdloff PL, Frei B, et al. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. Circulation 2001;104:151–6.
[39] Frei B, Higdon JV. Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. J Nutr 2003;133:3275S–84S.
[40] Fuhrman B, Volkova N, Coleman R, Aviram M. Grape powder polyphenols attenuate atherosclerosis development in Apolipoprotein E deficient (E0) mice and reduce macrophage atherogenicity. J Nutr 2005;135:722–8.
[41] Auger C, Teissedre PL, Gerain P, Lequeux N, Bornet A, Serisier S, et al. Dietary wine phenolics catechin, quercetin, and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation. J Agric Food Chem 2005;53:2015–21.
[42] Stocker R. Dietary and pharmacological antioxidants in atherosclerosis. Curr Opin Lipidol 1999;10:589–97.
[43] Martinez Sanchez G, Candelario-Jalil E, Giuliani A, Leon OS, Sam S, Delgado R, et al. *Mangifera indica* L. extract (QF808) reduces ischaemia-induced neuronal loss and oxidative damage in the gerbil brain. Free Radic Res 2001;35:465–73.
[44] Sanchez GM, Rodriguez HMA, Giuliani A, Nunez Selles AJ, Rodriguez NP, Leon Fernandez OS, et al. Protective effect of *Mangifera indica* L. extract (Vimang) on the injury associated with hepatic ischaemia reperfusion. Phytother Res 2003;17:197–201.
[45] Chyu KY, Babbidge SM, Zhao X, Dandillaya R, Rietveld AG, Yano J, et al. Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice. Circulation 2004;109:2448–53.
[46] Ballinger S, Patterson C, Conklin CA, Hu Z, Hunter GVC, McIntyre K, et al. Mitochondrial integrity and function in atherogenesis. Circulation 2002;106:544–9.
[47] Sheu SS, Nauduri D, Anders MW. Targeting antioxidants to mitochondria: a new therapeutic direction. Biochim Biophys Acta 2006;1762:256–65.
[48] Nageswara RM, Marschall SR. Mitochondrial dysfunction in atherosclerosis. Circ Res 2007;100:460–73.