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An SOD rich melon extract Extramel® prevents aortic lipids and liver steatosis in diet-induced model of atherosclerosis

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Abstract *Background and aims:* Oxidative stress has been involved in the early steps of atherosclerosis and previous studies on hypercholesterolemic hamsters have shown that non-enzymatic antioxidant could prevent fatty streak formation. Therefore, we investigated whether a melon juice extract (Extramel®) rich in superoxide dismutase (SOD) would prevent the development of early atherosclerosis.
Methods and results: The effects of Extramel® on plasma cholesterol, aortic fatty streak formation, hepatic steatosis, superoxide anion tissue production and NAD(P)H oxidase expression were studied in hamsters fed with an atherogenic diet (HF), receiving by gavage either water or Extramel® at 0.7, 2.8 or 5.6 mg/d. After 12 weeks of oral administration, Extramel® lowered plasma cholesterol and non-HDL cholesterol and induced blood and liver SOD activities. It also strongly reduced the area of aortic fatty streak by 49–85%, cardiac (45%) and liver (67%) production of superoxide anion and liver $p22^{phox}$ subunit of NAD(P)H oxidase expression by 66%, and attenuated the development of hepatic steatosis.
Conclusion: These findings support the view that chronic consumption of melon juice extract rich in SOD has potential beneficial effects with respect to the development of atherosclerosis and liver steatosis, emphasizing its use as potential dietary therapy.

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Introduction

Dietary factors are thought to play a key role in the regulation of the oxidant status. An imbalance between nutrients, and in particular those involved in antioxidant status, could explain the onset of an enhanced production of free radicals. A diet low in antioxidants contributes to the occurrence of an oxidative stress [1]. The physiological reactive oxygen species (ROS) production is regulated by enzymic defense systems and by dietary antioxidant molecules that keep transition metals in an inactive state for giving ROS. Because several studies suggest that polymorphic variations in endogenous antioxidants are linked to increased risk for atherosclerosis [2], ROS-induced depletion of antioxidants is a key factor for the initiation of atherosclerosis which is thought to be closely dependent upon an imbalance between ROS generation and natural cell antioxidant capacity in favor of the former [3]. The importance of dietary antioxidants in human health is clear and some epidemiological studies showed the potential health benefits of the consumption of antioxidant rich fruits and vegetables [4]. From a theoretical point of view, antioxidant enzymes could exhibit a major advantage over dietary antioxidants: they catalyse the detoxification of their substrates [5] whereas the latter are consumed by reacting with ROS. New approaches to antioxidant therapy appeared recently. Nelson et al. [5] reported that ingestion of an extract of five medicinal plants *Protandim* by healthy humans increased the circulating superoxide dismutase (SOD) and catalase activities and reduced the levels of thiobarbituric acid reactive-substances (TBARS), a marker of lipid peroxidation that correlates with oxidative stress and atherosclerosis [6]. Glisodin®, a melon juice concentrate coated with gliadin containing high levels of SOD and other antioxidant enzymes, has been developed and its antioxidant and anti-inflammatory properties have been demonstrated [7]. Given for 4 weeks to mice, it exhibited properties similar to *Protandim* [8] leading up to a fourfold increase of circulating and hepatic tissue antioxidant enzyme activities. In healthy volunteers, oral Glisodin® was associated with less lipid peroxidation [9]. Very recently, these authors demonstrated that a pre-treatment with Glisodin® may be a therapeutic option to reduce oxidative cell injury affiliated with aortic cross-clamping in pigs [10].

Here, we tested the hypothesis whether feeding this melon juice concentrate coated with palm oil (Extramel®) to hamsters on a high-fat diet for a 12-week period would prevent the development of the pathology.

Methods

Extramel® is a freeze-dried melon juice concentrate of a specific variety of melon (not GMO) containing high levels of SOD. It is coated with palm oil and is called Extramel® microgranules that contain 14 IU SOD/mg powder, manufactured by Bionov company (France).

Sixty weanling male golden Syrian hamsters (Janvier, Le Genest-St-Isle, France) weighing ≈ 65 g were randomly divided into five groups of 12 animals. They were housed at 23 ± 1 °C, subjected to a 12-h light:dark cycle and handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines [11].

They were fed with a high-fat diet (HF) for 12 weeks and received daily by gavage either tap water (HF) or aqueous solution of Extramel® at 0.7 (E1), 2.8 (E2) or 5.6 (E3) mg/d, corresponding to 10, 40 and 80 IU SOD/d, respectively. The HF diet consisted of 200 g/kg casein and 3 g/kg L-methionine, 393 g/kg corn starch, 154 g/kg sucrose, 50 g/kg cellulose, 150 g/kg lard, 5 g/kg cholesterol, mineral mix (35 g/kg) and vitamin mix (10 mg/kg). For reference, a group was fed with a standard diet (STD) which consisted of 200 g/kg casein and 3 g/kg L-methionine, 447 g/kg corn starch, 175 g/kg sucrose, 50 g/kg cellulose, 80 g/kg vegetable oil, mineral mix (35 g/kg) and vitamin mix (10 mg/kg). Vitamin and mineral mixes were formulated according to the AIN-93 guidelines [12]; in the HF diet, mixes did not contain selenium, vitamin C and vitamin E. The volume of solutions force-fed was adjusted daily to the weight of hamsters, i.e. 7.14 mL/(kg body wt d).

After 12 weeks, the hamsters were deprived of food overnight and blood samples were collected. Plasma and erythrocytes were prepared by centrifugation at 2000 *g* for 10 min. The liver was excised, weighed and sectioned for analyses and stored at −80 °C. Concomitantly, samples were removed for histological analysis. Liver was homogenized in ice cold 0.1 mol/L potassium phosphate buffer (pH 7.4) and the homogenate was spun at 13,000 *g* for 15 min at 4 °C. Glutathione peroxidase (GSHPx) and SOD activities were assayed on the supernatant on an automat Pentra 400 (HORIBA ABX, Montpellier, France) using commercial kits (Ransod kit no. SD 125 and Ransel no. RS505, Randox Laboratories LTD, Crumlin, UK, respectively). SOD activity in erythrocytes was measured using the Ransod Kit no. SD 125, after washing the cells in saline, followed by lysis in cold redistilled water.

Plasma lipid levels were measured by routine enzymatic methods (KonePro, Konelab, Epoo, Finland). Liver lipids were extracted according to Folch et al. [13]. Liver samples were fixed in 10% neutral buffered formaldehyde for pathologic analysis. Formalin-fixed livers were paraffin embedded and then serial sections (3 μm) were prepared. The deparaffinized sections were stained with hematoxylin and eosin.

The thoracic aorta was removed and samples (30 mg) were placed in vials containing 4 mL of methanol and 10 mL of chloroform and treated according to Rudel et al. [14] then total cholesterol concentration was determined enzymatically (no. CH 200, Randox Laboratories LTD, Crumlin, UK).

Following blood collection and liver removal, the intact aorta was first perfused with phosphate buffered saline containing 1 mmol/L $CaCl_2$ and 15 mmol/L glucose for 5 min, then with 0.1 mmol/L sodium cacodylate buffer, pH 7.4, containing 2.5 mmol/L $CaCl_2$, 2.5% paraformaldehyde and 1.5% glutaraldehyde for the fixation of the vasculature. The aorta was carefully dissected and processed as previously described [15], lipids being stained in Oil Red O. An image acquisition and analysis system (ImageJ, Scion Corporation, Frederick, MD) incorporated in an Olympus microscope was used to capture and analyze the total Oil Red O stained area of each aortic arch. The area covered by foam cells (aortic fatty streak lesion area

or AFSA) was expressed as a percentage of the total area surveyed.

Cardiac and hepatic superoxide anion ($O_2^{\cdot-}$) production was evaluated by lucigenin (10 μM)-enhanced chemiluminescence intensity [16] measured on a luminometer (Perkin Elmer Wallac, Victor, Turku, Finland). Results were expressed as count/mg of protein.

Proteins were extracted as previously described [16] from liver samples, then separated with 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Normalization to β-actin (anti-actin, 1:5000, Chemicon International, Temecula, CA) carried out to verify the uniformity of protein load and transfer efficiency across the tested tissues.

Data are shown as the mean ± SEM. Statistical analysis of the data was carried out using the StatView IV software (Abacus Concepts, Berkeley, CA) by one-way ANOVA followed by Fisher's protected least significant difference test. Differences were considered significant at $P < 0.05$.

Results

The HF and STD diets differ in many aspects other than fat; thus, STD group's data are included for reference only and are not included in the statistical analysis. No significant difference appeared in energy intake and body weight gain between the four groups receiving the HF diet (not shown here). Plasma lipid concentrations are shown in Table 1. On average only E2 and E3 fed hamsters displayed significantly reduced plasma total cholesterol (48%) and non-HDL cholesterol (53%) in comparison to HF-fed group. Plasma triglycerides and HDL-C and liver lipids were not modified by the treatments. Erythrocytic SOD activity was raised by increasing the dose of Extramel®, and more particularly by 13% in E3 group (Table 1). In the liver, SOD activity rose up to 140% in E3 group in comparison to HF group. The activity of GSHPx was not modified by Extramel®, except for a decrease in E2 group (Table 1). In the left cardiac ventricle, $O_2^{\cdot-}$ production (Fig. 1) was significantly decreased in E2 (26%) and E3 (45%)-fed groups whereas in E1 it did not. The hepatic $O_2^{\cdot-}$ production was only reduced by feeding E3 (67%) compared to HF controls (Fig. 2). As shown in Fig. 3, hepatic expression of $p22^{phox}$ decreased by 48% in hamsters receiving E1 or E2, and by 88% in hamsters fed with E3. Average aortic fatty streak accumulation (AFSA), measured as the percentage of Oil Red O staining relative to the total area surveyed (Table 1), was gradually decreased in hamsters given 0.7—5.6 mg/d Extramel® in comparison with controls. E1 and E2 feedings led to the less

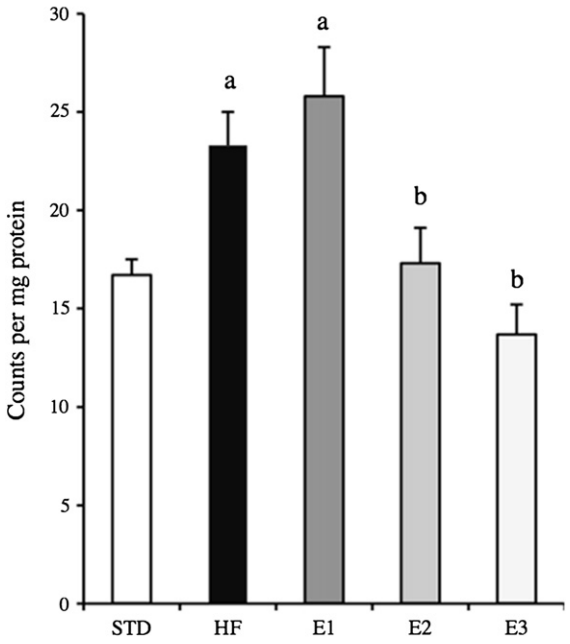

Figure 1 Cardiac superoxide anion production in hamsters fed with a standard diet (STD) or a high-fat diet (HF), or an HF diet plus Extramel® at 0.7 (E1), 2.8 (E2) or 5.6 (E3) mg/d for 12 weeks. Values are expressed as mean ± SEM ($n = 12$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

Table 1 Effect of feeding a standard diet (STD) or high fat diet (HF), or an HF diet plus Extramel® at 0.7 (E1), 2.8 (E2) or 5.6 (E3) mg/d for 12 weeks on blood and liver lipids, on antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities, and on aortic fatty streak area (AFSA) and aortic cholesterol level.

	STD	HF	E1	E2	E3
Plasma					
TG (mmol/L)	0.97 ± 0.09	2.00 ± 0.53[a]	2.24 ± 0.36[a]	2.10 ± 0.61[a]	2.27 ± 0.65[a]
TC (mmol/L)	2.06 ± 0.09	9.22 ± 2.46[a]	7.25 ± 2.09[a,b]	4.62 ± 1.43[b]	4.98 ± 1.33[b]
HDL-C (mmol/L)	1.42 ± 0.64	4.97 ± 1.33[a]	4.33 ± 1.28[a]	2.56 ± 0.85[a]	2.69 ± 1.28[a]
Non-HDL-C (mmol/L)	0.64 ± 0.03	4.25 ± 1.13[a]	2.92 ± 0.8[a]	1.66 ± 0.58[b]	2.29 ± 0.05[b]
Erythrocytes					
SOD (IU/g Hb)	880 ± 21	1188 ± 19[a]	1242 ± 31[a,b]	1305 ± 28[b,c]	1342 ± 24[c]
Liver					
Lipids (mg/g)	6.60 ± 2.51	17.16 ± 2.62[a]	16.47 ± 2.44[a]	15.65 ± 4.20[a]	15.62 ± 2.18[a]
SOD (IU/mg protein)	21.9 ± 1.4	18.8 ± 1.5[a]	20.7 ± 0.7[a]	20.2 ± 0.7[a]	45.1 ± 4.6[b]
GSHPx (IU/mg protein)	2190 ± 120	2097 ± 102[a,b]	2517 ± 125[a]	1640 ± 156[b]	2401 ± 284[a]
Aorta					
AFSA (%)	0	5.8 ± 0.5[a]	2.6 ± 0.5[b]	1.9 ± 0.4[b]	1.1 ± 0.3[c]
Cholesterol (mg/g)	2.88 ± 1.2 8	8.22 ± 1.71[a]	4.23 ± 0.70[b]	3.01 ± 0.75[b]	1.22 ± 0.4[c]

Values are expressed as mean ± SEM ($n = 12$). For each dietary treatment, means in a column with different superscripts differ, $P < 0.05$. TG = triglycerides, TC = total cholesterol, HDL-C = high density lipoprotein cholesterol.

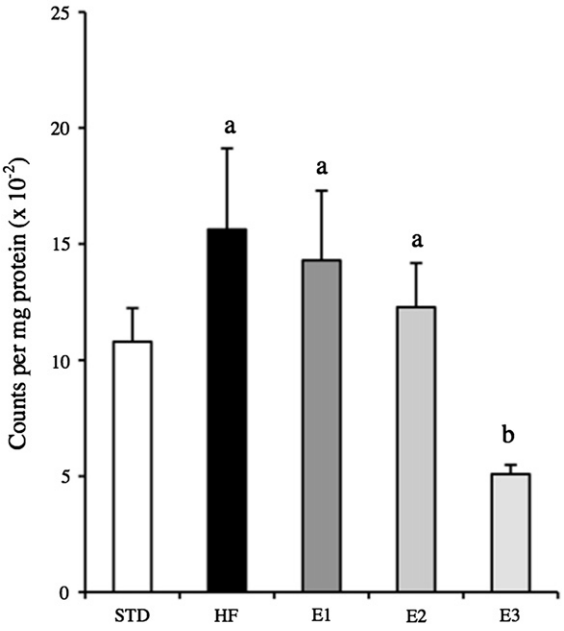

Figure 2 Liver superoxide anion production in hamsters fed with a standard diet (STD) or a high-fat diet (HF), or an HF diet plus Extramel® at 0.7 (E1), 2.8 (E2) or 5.6 (E3) mg/d for 12 weeks. Values are expressed as mean ± SEM ($n = 12$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

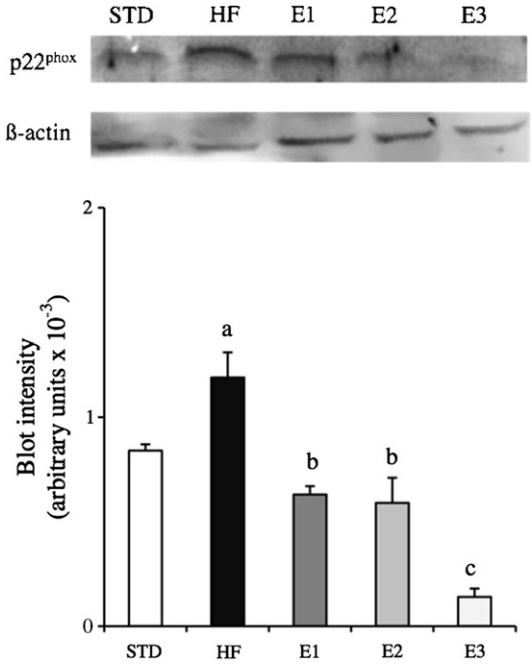

Figure 3 Expression of hepatic $p22^{phox}$ subunit of NAD(P)H oxidase in hamsters fed with a standard diet (STD) or a high-fat diet (HF), or an HF diet plus Extramel® at 0.7 (E1), 2.8 (E2) or 5.6 (E3) mg/d for 12 weeks. The densitometric measurement shows arbitrary area units. Values are expressed as mean ± SEM ($n = 6$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

important AFSA decreases (53% and 66%, respectively) while hamsters fed with E3 exhibited an 80% AFSA reduction compared to HF controls. Aortic cholesterol behaved in an identical pattern (Table 1) and reduced from 49% (E1) to 62% (E2) and 85% (E3). Lean hamsters (standards) did not exhibit any histologic evidence of hepatic steatosis and fibrosis (Fig. 4a). In contrast, steatosis was observed in hamsters fed with HF diet (Fig. 4b) compared to standards. No marked reduction in the degree of steatosis was noted in the livers from HF-fed hamsters treated with the lowest doses of Extramel® (0.7 and 2.8 mg/d, not shown) and fatty changes only appeared at the highest dose, i.e. 5.6 mg/d (Fig. 4c). Histologic steatosis behaved like liver lipids (Table 1) and body weight suggesting the importance of body weight regulation on the development of hepatic steatosis. No necrotic cells or histologic evidence of hepatotoxicity was observed in standard or HF-fed hamsters. Hepatic inflammation was absent in all hamsters and there was no histologic evidence of fibrosis or any visible

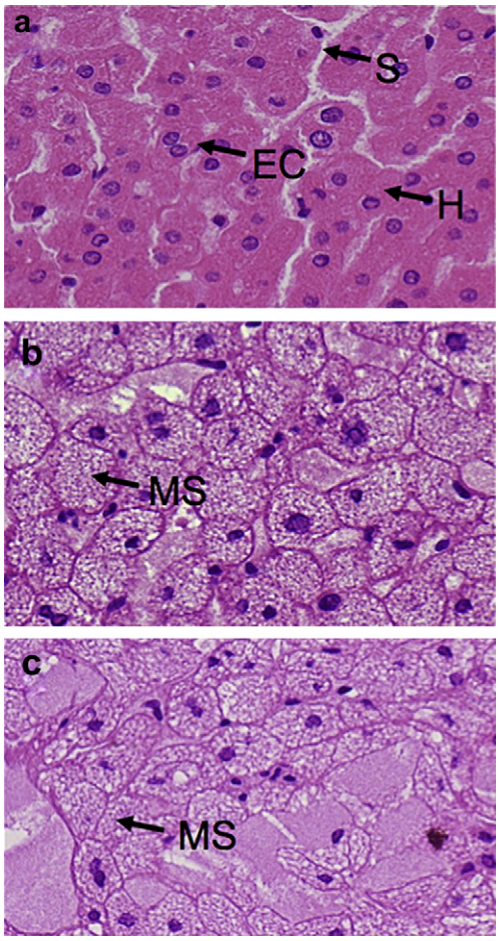

Figure 4 Histologic evaluation of hepatic steatosis in standard (a), atherogenic (b) or Extramel®-treated (5.6 mg/d) (c) hamsters for 12 weeks. Representative liver sections (original magnification ×200) are illustrated. (a) Liver section from a hamster fed with the standard diet. (b) Atherogenic hamsters fed with no Extramel® illustrate the development of hepatic steatosis. (c) Slight reduction in hepatic steatosis in 5.6 mg/d Extramel®-fed hamsters. Sinusoidal capillary (S); endothelial cell (EC); hepatocyte (H); microvascular steatosis (MS).

evidence of Extramel® altering any of these variables, and no increase in Kupffer cells or macrophages was found.

Discussion

This study provides evidence for the first time that dietary supplementation of a melon juice concentrate rich in SOD protects against diet-induced oxidative stress and atherosclerosis in hypercholesterolemic hamsters, and no toxicity or evidence of other unwanted pharmacological effects of Extramel® was noted at either levels of supplementation. This indicates that at the low doses used here Extramel® is a safe nutraceutical supplement.

The combination of high dietary saturated fat and cholesterol is commonly used to promote atherosclerosis in golden Syrian hamster and atherosclerotic lesions similar to those found in humans, i.e. fatty streak, can be found after prolonged feeding periods [15].

As previously reported HF diet led to an increase in total and non-HDL cholesterol after 12 weeks of diet leading to lipid deposition on aortic arch. Interestingly, the diet-induced hypercholesterolemia is in parallel accompanied by a tendency of superoxide anion ($O_2^{\cdot -}$) overproduction in agreement with previous works in rat [17] or hamster [16] models of atherosclerosis. Thus, according to the oxidative hypothesis of atherosclerosis, it could be postulated that NADPH oxidase expression and activity work with high non-HDL cholesterol level to induce foam cells fatty streak [18] and subsequent atherosclerosis [19]. Here, the reduction of both $O_2^{\cdot -}$ production and AFSA by Extramel® further supports this hypothesis. Moreover, $O_2^{\cdot -}$ production in the liver was closely dependent on the overexpression of NAD(P)H oxidase subunit $p22^{phox}$, and in agreement with that, its liver expression was decreased by 80% after feeding Extramel® at 5.6 mg/d (i.e. 80 IU SOD).

The activity of the liver antioxidant defense system as a marker of the putative protective role of dietary Extramel® showed some interesting differences in our experimental conditions. No modification of the GSHPx activity appeared in liver homogenates from the experimental groups. However, the activity of SOD, the other main enzyme involved in the antioxidant defense mechanism, was gradually increased in erythrocytes according to the level of Extramel®. This occurred differently in the liver where Extramel® only worked at 5.6 mg/d, exhibiting an increase of 11% in erythrocytic SOD activity after 12 weeks of supplementation, as seen in Table 1. The increased blood and hepatic SOD activities by Extramel® consumption could in part counteract the increase in $O_2^{\cdot -}$. These findings agree with Vouldoukis et al. [7,8] who did show that feeding mice for 4 weeks with a melon extract exhibiting SOD, catalase and residual GSHPx activities led to an increase in circulating and liver SOD activity. Since Extramel® strongly prevented $O_2^{\cdot -}$ production and inducted SOD activity, we can assume that it favors the antioxidative balance by decreasing the oxidative stress, thus exhibiting beneficial effects on preventing early atherosclerosis. Such an increase of liver and blood SOD activity shown here as well as by Vouldoukis et al. [7,8] is not observed with other kinds of dietary antioxidants and seems to result from this antioxidant enzyme supplementation: when encapsulated by gliadin, the same melon extract rich in SOD triggers an increase in liver and circulating SOD activity after oral administration to rats not subjected to an oxidative stress. When the melon extract rich in SOD is not protected by gliadin, no more increase in circulating and hepatic SOD activity appeared [7,8]. Elsewhere, an oral supplementation of pure SOD encapsulated in liposomes promoted the circulating SOD activity; however, if not encapsulated in liposomes, and therefore destroyed at least in part during the digestive process, no longer increased circulating SOD activity can be observed [20]. Such an increase in circulating SOD activity also appeared in cats infected with FIV given the extract coated with gliadin [21]. In the same way, SOD mimics, synthesized molecules mimicking the effect of SOD and resisting to digestive conditions, were used successfully in the treatment of steatohepatitis in murine and they also induced an increase of SOD activity in liver [22]. An effect of the coating may be ruled out since an increased endogenous SOD activity appeared regardless of the type of coating used (liposomes, gliadin, palm oil). All these findings showed that increased endogenous SOD activities resulted from melon SOD and not from an active issuing peptide since nothing occurred without coating. Finally, such an induction of the cell antioxidant status is not observed with other kinds of dietary antioxidants [23,24] suggesting a specific effect of melon SOD. It should be pointed out that circulating erythrocytes, which do not contain nuclei, are not able to induce new synthesis of SOD once they enter the bloodstream. Since their circulating life span is about 120 days, thus, during the 12-week course of the experiment about 70% of the erythrocytes would not have been still replaced by maturing reticulocytes from the bone marrow. This 11% increase therefore represents the SOD inducted by supplementation. As expected in liver, a more important induction appeared, as all cells in this tissue have nuclei and ongoing synthesis of new protein. However, as pointed out by McCord and Edeas [25] overexpression of SOD could be lethal and dose–response curves have been established after isolated hearts were subjected to ischemia–reperfusion injury and protected by exogenous SOD [26]. SOD was very protective up to a point, beyond which protection was lost and injury was even exacerbated, with increased lipid peroxidation. This was corroborated by Levy et al. [27] who found that high overexpression of SOD increased ischemia–reperfusion injury to the brain. Even at the higher dose of supplementation used here, Extramel® did not lead to such upper limit dose dependent ending events, strengthening its safety as nutraceutical supplement. Finally, even though involved mechanisms remain unclear, it is known that SOD can be absorbed despite its high MW ($\approx$35 kDa). This was shown in the liver of rats after intravenous administration of SOD conjugated with colloidal gold [28] and more recently in HT-29 cells using liposomal SOD and the SOD mimic tempamine [29].

The steatosis observed here was a simple fatty overload corresponding to non-alcoholic fatty liver disease (NAFLD). Schwimmer et al. [30] hypothesized that fatty liver is a risk factor for early onset of atherosclerosis independent of other prognostic risk factors. This suggests a more complex picture about the intertwined interrelationships between NAFLD and atherosclerosis. The possible biological mechanisms linking NAFLD and accelerated atherosclerosis are

still poorly known. NAFLD in its more advanced forms might act as a stimulus for further increased whole-body insulin resistance and dyslipidemia, leading to accelerated atherosclerosis. Unfortunately, insulin resistance was not measured here. This hypothesis is supported by the close, linear, relationship between liver fat content and direct measures of hepatic insulin sensitivity [31]. Another possible underlying mechanism linking NAFLD and atherosclerosis may be represented by an increased oxidative stress and chronic, subclinical inflammation, which are thought to be causal factors in the progression to more advanced forms of NAFLD [32]. Steatosis-derived ROS, which could be due to an overexpression and activity of NADPH oxidase [33] (as measured here in the HF-fed group), stimulated fatty acid oxidation, attendant hepatocyte injury and cytokine release are likely to perpetuate the liver damage of NAFLD and add further atherogenic stimuli to the already high oxidative status. Patients with NAFLD have higher plasma markers of oxidative stress [34] than those without NAFLD, most of them being associated with the severity of histological features of NAFLD independent of classical risk factors. Due to the central role of oxidative stress in liver diseases' pathogenesis and progression, the use of antioxidants proposed as therapeutic agents to counteract liver damage [35] emphasizes the potential use of Extramel® as dietary supplement.

The prevented progression of atherosclerosis by Extramel® is also due in part to decreased total and non-HDL cholesterol. Elsewhere, Extramel® acts by improving the antioxidant defenses as demonstrated by increased tissue SOD activity. Furthermore, our findings suggest for the first time that Extramel® prevents both NAD(P)H oxidase expression and $O_2^{\cdot-}$ overproduction in the liver from hypercholesterolemic hamster. This could be involved in the prevention of LDL oxidation and further atherosclerosis steps. HF-feeding activated and Extramel® reduced NAD(P)H activity (heart and liver) and expression (liver) reinforce the hypothesis of a nutritional modulation of ROS enzymatic producing systems. Finally, all these features due to Extramel® were associated with a huge prevention of AFSA. The relative contribution of each parameter is difficult to establish although it is tempting to speculate on a specific role of tissular oxidative stress. Moreover, it could be that these effects are not restricted to SOD activity that could act synergistically or additively with other antioxidant components to prevent atherosclerosis in the hamster model.

These promising results give rise to the use of Extramel® as potential dietary therapy and to further clinical studies. Investigation is warranted to define the mechanisms by which Extramel® protects.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

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