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Atherosclerosis 197 (2008) 72–83

ATHEROSCLEROSIS

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Squalene in a sex-dependent manner modulates atherosclerotic lesion which correlates with hepatic fat content in apoE-knockout male mice

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Received 1 February 2007; received in revised form 23 July 2007; accepted 2 August 2007
Available online 12 September 2007

Abstract

Background: Squalene is an intermediate of cholesterol biosynthesis which can be obtained from the diet where it is abundant, for example, in olive oil. The effect of this isoprenoid on the development of atherosclerosis was investigated on apoE-knockout mice.
Methods and results: Two groups of animals, separated according to sex, were fed on standard chow diet: the control group receiving only vehicle and the second group an aqueous solution of squalene to provide a dose of 1 g/kg/day in male and female mice. This treatment was maintained for 10 weeks. At the end of this period, plasma lipid parameters, oxidative stress markers and hepatic fat were measured as well as cross-sectional lesion area of aortic root in both groups. Data showed that in males squalene feeding reduced atherosclerotic lesion area independently of plasma lipids and activation of circulating monocytes. In contrast, squalene intake did not decrease lesion area in females, despite reducing plasma cholesterol and triglycerides, isoprostane and percentage of Mac-1 expressing white cells. In males, atherosclerotic lesion area was positively and significantly associated with hepatic fat content and the plasma triglycerides were also strongly associated with liver weight.
Conclusions: These results indicate that administration of squalene modulates lesion development in a gender specific manner, and that accumulation of hepatic fat by liver is highly correlated with lesion progression in males. Hence, squalene administration could be used as a safe alternative to correct hepatic steatosis and atherosclerosis particularly in males.

Keywords: Squalene; Atherosclerosis; Lipoprotein; apoE-deficient mice

1. Introduction

Atherosclerosis is the primary cause of heart disease and stroke in westernized societies [1]. ApoE-deficient mouse is a good animal model because it develops severe atherosclerosis on a regular low-fat/low-cholesterol diet. The progression and histopathology of lesions in this animal model show similar features to those observed in humans and other species, including fatty streaks, necrotic cores, fibrous caps and even plaque rupture [2–3]. It is known that Mediterranean diet,

Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; FPLC, fast performance liquid chromatography; HDL, high density lipoproteins; HDL-C, high density lipoprotein cholesterol; LDL, low density lipoproteins; LDLr, low density lipoprotein receptor; PON-1, paraoxonase 1; SR-B1, scavenger receptor B1; TC, total cholesterol; TG, triglycerides; VLDL, very low density lipoproteins

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doi:10.1016/j.atherosclerosis.2007.08.008

characterized by an abundant intake of plant foods (fruits, vegetables, cereals, legumes), is associated with a lower incidence of coronary heart disease [4–5], being olive oil the main source of fat in this diet [6]. Recently, beneficial effects of the unsaponificable fraction of olive oil on atherosclerosis have been reported [7], but to advance in the understanding of these beneficial properties, analysis of the effects of specific components upon atherosclerosis is required.

Olive oil contains the largest amount of squalene among vegetable oils in the range of 2.5–9.2 g/kg [8–9]. Squalene is an isoprenoid metabolic precursor of cholesterol and other sterols. Serum squalene originates partly from endogenous cholesterol synthesis and partly from dietary sources, especially in populations consuming large amounts of olive oil or shark liver. In humans, 60–85% of dietary squalene is absorbed and transported in serum [10–13], while the efficiency of gastrointestinal squalene absorption has been estimated at 42% in animals [14]. Dietary squalene is found in postprandial lipoprotein fractions [15], generally in association with very low density lipoproteins, from which it is distributed to various tissues [11]. Some experimental models have shown that squalene feeding increases both tissue squalene and cholesterol contents, the latter reflecting stimulated cholesterol production from squalene [16–19]. However, several studies have also demonstrated that dietary squalene either has no effect or decreases serum triglyceride and cholesterol levels in humans [11–20] and animals [21]. These results have been attributed to increased elimination of cholesterol as fecal bile acids [11] and to inhibition of HMG Co A reductase by dietary squalene due to negative feed-back regulation [22].

The relationship between squalene and atherosclerotic lesion has not been previously studied. The aim of this study is to address the effect of high squalene ingestion on the development of atherosclerosis in apoE-deficient mice. Analyses of lipid, lipoproteins, hepatic fat, and atherosclerotic lesions were performed to uncover potential mechanisms involved in the response to squalene supplementation.

2. Material and methods

2.1. Animals

Homozygous apoE KO mice, hybrids of C57BL/6J × 129 Ola strains were bred in *The Unidad Mixta de Investigación*, Zaragoza. Seventeen males and 15 females, aged 2 months, were fasted overnight, anesthetized with isofluorane, and blood samples obtained by retroorbital bleeding to estimate initial plasma cholesterol and triglycerides. Two random groups, of equal plasma cholesterol and triglycerides for each sex, were housed in sterile filter-top cages. Animals had ad libitum access to food and water. The protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza. Body weights and food intake were recorded throughout the experiment.

2.2. Diet and treatment

The mouse chow, Teklad Mouse/Rat Diet no. 2014 from Harlan Teklad (Harlan Ibérica, Barcelona Spain), was provided daily. To avoid the potential confounding effects of variation among batches of chow, a single batch was reserved and used throughout the experiment. Two groups of study fed the same chow diet were established: the squalene group whose beverage was a 1% (v/v) glycerol solution supplemented in squalene to provide a dose of 1 g/kg/day and the control group that received the glycerol solution used as vehicle. Beverage was prepared and replaced every 2 days. The experimental period lasted 10 weeks and diet and treatment were well tolerated.

2.3. Plasma determinations

After the experimental period, animals were sacrificed by suffocation in CO_2 and blood was drawn from their hearts. Total plasma cholesterol and triglyceride concentrations were measured in a microtiter assay, using commercial kits from Sigma Chemical Co. (Madrid, Spain). HDL cholesterol was determined in a similar manner after phosphotungstic acid—$MnCl_2$ (Roche, Barcelona, Spain) precipitation of apoB containing particles [23]. Measurement of the total isoprostane 8-iso-$PGF_{2\alpha}$ was carried out by immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Paraoxonase was assayed as arylesterase activity as previously described [24] and results were expressed as μmol phenylacetate hydrolyzed $min^{-1}\,L^{-1}$ ($IU\,L^{-1}$). Apolipoproteins A-I and A-V were quantified by ELISA with specific polyclonal antibodies (Biodesign, Saco, ME, and Santa Cruz Biotechnology, Santa Cruz, CA, respectively) as previously described [25–26]. All assays were completed in triplicate and all samples were analyzed in the same day. The intra-assay CV in all cases was lower than 4%. To analyze plasma lipoprotein profiles, 100 μL of pooled plasma samples within each treatment group were subjected to fast protein liquid chromatography gel filtration using a Superose 6B column (GE Healthcare, Barcelona, Spain), as previously described [27].

2.4. RNA preparation and analysis

At the moment of sacrifice, livers were immediately removed and frozen in liquid nitrogen. RNA was isolated using Trigent reagent MRC (Cincinatti, OH, USA) following the manufacturer's instructions. DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from AMBION (Austin, TX, USA). RNA was quantified by absorbance at $A_{260/280}$ (the $A_{260/280}$ ratio was greater than 1.75). The integrity of the 28 S and 18 S ribosomal RNAs was verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining.

Equal amounts of RNA from each mouse were used in quantitative real-time RT-PCR analyses. First-strand cDNA

synthesis and the PCR reactions were performed using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Madrid, Spain), according to the manufacturer's instructions and as previously described [26]. The following primers were used in real-time PCR: for *Apoa1* - sense, 5′-GCT GAA CCT GAA TCT CCT GGAA-3′, antisense 5′-ACT AAC GGT TGA ACC CAG AGT GTC-3′; for *Apoa5* - sense, 5′-TTG GAG CAA AGG CGT GAT GG-3′, antisense 5′-TGA GGA GCG ACA CTG CGG TG-3′; for *Ldlr* - sense, 5′-TTT TGG AGG ATG AGA ACC GG-3′, antisense, 5′-GGC ACT GAA AAT GGC TTC GT-3′; for *Srb1* -sense, 5′-CTC CCA GAC ATG CTT CCC ATA A-3′, antisense, 5′-CCA TTT GTC CAC CAG ATG GAT C-3′; and for *cyclophilin B* - sense, 5′-GGA GAT GGC ACA GGA GGA A-3′, antisense, 5′-GTA GTG CTT CAG CTT GAA GTT CTC AT-3′. Real-time PCR reactions were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. *Cyclophilin B* mRNA was used as the invariant control.

2.5. Evaluation of atherosclerotic lesions

The heart was perfused first with phosphate-buffered saline and later with phosphate-buffered formalin (4%, pH 7.4 Panreac, Barcelona, Spain) under physiological pressure. Hearts were dissected out and the aortic base of the hearts were taken and transferred to liquid OCT (Bayer Diagnostic, Germany). Serial cryosections of the proximal aorta and the aortic sinus were made and stained with Sudan IV B (Sigma Chemical Company), and counter-stained with hematoxylin and eosin (Sigma Chemical Company) as previously described [27]. Average lesion sizes were used for morphometric evaluations based on the method of Paigen et al. [28]. Images were captured and digitized using a Nikon microscope equipped with a Cannon digital camera. Morphometric analyses were evaluated blindly using Scion Image software (Scion Corporation, Frederick, Maryland, USA).

2.6. Blood cell analysis of surface molecule expression

After 10 weeks, the mice were fasted overnight, anesthetized using isofluorane, and blood samples were collected using retro-orbital bleeding. Approximately 1×10^6 white blood cells were re-suspended in PBS supplemented with 0.1% (w/v) BSA and 10 mmol/L sodium azide and analyzed for the expression of Mac-1 (Anti-CD11b from Becton-Dikinson, Madrid, Spain) using fluorescence-activated cell sorter analysis. The results are expressed as the proportion (%) of the marker-positive cells recovered in the region corresponding to monocytes.

2.7. Histological analysis

Pieces of liver were stored in neutral formaldehyde and included in paraffin. Sections (4 μm) were stained with hematoxylin and eosin and observed with a Nikon microscope. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of total liver section.

2.8. Analysis of hepatic squalene and lipids

For hepatic squalene and fat determinations, lipids were extracted from 100 mg of tissue, using 2,6-di-tert-butyl-*p*-cresol as antioxidant. The extracted lipids were redissolved in 1 mL of chloroform: methanol (2:1, v/v) and preserved at −20 °C until analysis. The lipid composition was determined by HPLC as described by Perona and Ruiz-Gutierrez [29]. Lipids were transmethylated and the resulting fatty acid methyl esters analyzed by gas chromatography as described by Ruiz-Gutiérrez et al. [30] using a model 5890 series II gas chromatograph (Hewlett-Packard Co., Avondale, USA) equipped with a flame ionization detector and a capillary silica column Supelcowax 10 (Supelco Co., Bellefonte, USA) of 60 m length and 0.25 mm internal diameter.

2.9. Western-blot

Fifteen micrograms of hepatic homogenate proteins were loaded onto 10% SDS-polyacrylamide gels. Electrophoresis and protein transfer to PVDF membranes (Millipore, Madrid) were carried out as previously described [31]. To detect LDLr or SR-B1 (scavenger receptor B1) protein expression, goat or rabbit polyclonal antibodies against mouse LDLr (Santa Cruz Biotechnology, Santa Cruz, CA) or SR-B1 (Abcam, Cambridge, UK) receptors were used. Equal loadings were confirmed by using an anti-Hsc70 antibody obtained from Santa Cruz Biotechnology. Detection was carried out using a secondary antibody anti-goat or rabbit IgG conjugated to horseradish peroxidase (Sigma Chemical Co., Madrid, Spain) and ECL as chemiluminiscent substrate (GE Healthcare, Madrid, Spain). Membranes were exposed to ECL film (GE healthcare, Madrid) for several time periods to achieve signal intensity within the dynamic range of quantitative detection, and films scanned at a 600 dpi resolution. Intensity of bands for each condition, taken as volume of pixels per mm^2, was calculated using Quantity One® software version 4.5.0 (Bio-Rad, Madrid, Spain) and normalized to that corresponding to Hsc70 signal.

2.10. Statistical analysis

Unless otherwise stated, results are expressed as mean ± S.D. The Kolmogorov and Smirnov's test was used to verify whether the variables were normally distributed and the Levenne *F*-test to assess the homogeneity of variances. When both analyses were accepted, comparison between variables was carried by two-tailed unpaired *t*-test. When the variables did not exhibit a Gaussian distribution or failed to show homology of variances, groups were compared using Mann–Whitney *U*-test. The value of probability obtained in

Table 1
Effects of squalene on apoE-knockout mice plasma parameters

	Males				Females			
	Chow ($n=8$)	Squalene ($n=9$)	Type 1 α	Type 2 β	Chow ($n=7$)	Squalene ($n=8$)	Type 1 α	Type 2 β
Plasma cholesterol (mmol L^{-1})	11.95 ± 2	11.6 ± 2.5	0.67	0.1	8.6 ± 0.6	7.0 ± 1	0.03*	0.6
HDL cholesterol (mmol L^{-1})	0.18 ± 0.05	0.15 ± 0.05	0.16	0.3	0.16 ± 0.04	0.16 ± 0.05	0.46	0.1
Plasma triglycerides (mmol L^{-1})	1.2 ± 0.2	1.1 ± 0.2	0.24	0.1	0.85 ± 0.07	0.62 ± 0.1	0.03¶*	0.3
ApoA-I (AU L^{-1})	292 ± 48	294 ± 35	0.92	0.1	280 ± 26	244 ± 31	0.05*	0.1
ApoA-V (AU L^{-1})	17 ± 4	20 ± 10	0.34	0.1	21 ± 8	20 ± 8	0.84	0.1
8-Isoprostaglandin $F_{2\alpha}$ (pg mL^{-1})	207 ± 9	170 ± 23	0.01*	0.05	202 ± 15	53.3 ± 18	0.01*	0.01
Arylesterase activity (UI/L)	20.3 ± 5	17.7 ± 6	0.35	0.1	19.6 ± 4	21.1 ± 5	0.55	0.1

Results are expressed as means ± S.D. Mice were fed chow or squalene diet for 10 weeks and fasted overnight before blood collection. Statistical analysis to evaluate dietary response was done using two-tailed unpaired *t*-test except for ¶that Mann–Whitney *U*-test was used.
* $P<0.05$ vs. chow diet.

both cases was considered as α or type 1 error and was calculated using Instat 3.02 for Windows software (GraphPad, S. Diego, CA, USA). The type 2 or β error was calculated using Statmate 2.0 for Windows (GraphPad). To test the association among all variables, the Spearman's rank-order correlation coefficient (r_s) or the Pearson correlation test was used using SPSS software, version 11.0 (SPSS Inc., Chicago, IL). Differences were considered significant when α or $P<0.05$ and the power of the analysis was robust when $\beta \leq 0.2$.

3. Results

3.1. Effect of squalene on plasma lipoproteins

As shown in Table 1, after 10 weeks of experimental period, the intake of squalene significantly decreased plasma cholesterol, triglycerides and apoA-I in females, while there were no changes in these parameters in males. The power of this statement was quite strong for males due to the low values of type 2 errors found. In females, plasma cholesterol and triglycerides displayed not such clear cut as the type 2 error was higher suggesting more overlap between groups of animals. No significant change with high power of prediction in plasma levels of apoA-V were observed by the administration of squalene in either sex.

Fig. 1 shows the distribution of cholesterol among the different plasma lipoproteins of pooled plasma separated by FPLC. Minimal changes were observed in cholesterol distribution of all lipoproteins in both groups of males (Fig. 1a and c). In females, squalene intake decreased cholesterol carried by very low density lipoproteins without changes in other lipoprotein fractions (Fig. 1b and d), corresponding with the reduction of plasma cholesterol and triglycerides observed in this sex.

3.2. Changes in body weight and liver parameters

There were no significant differences in body weight gain between control and squalene groups in both sexes, as shown in Table 2. However, liver weight was found significantly decreased in males consuming squalene while this effect was not observed in females (Table 2). The power of both findings was quite high due to the low values of type 2 errors observed.

To test whether squalene accumulated in liver, we measured the amount of this compound in this organ. There was

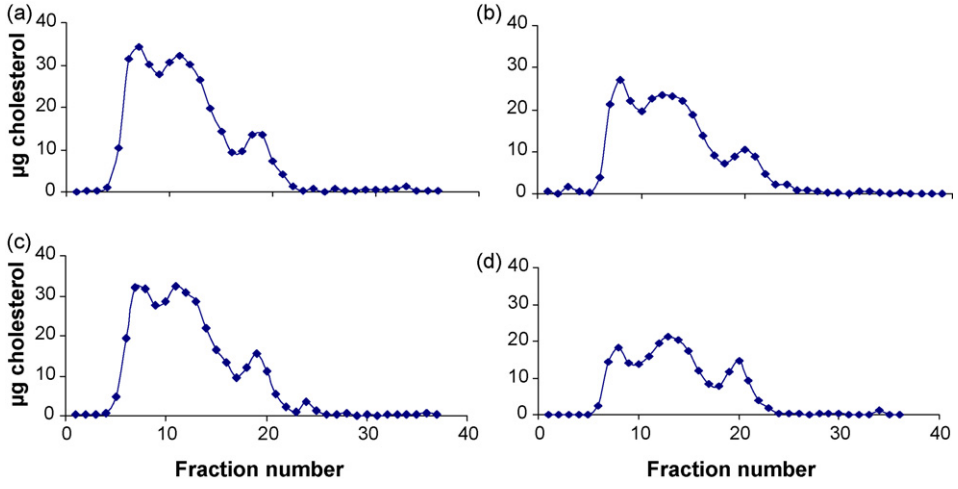

Fig. 1. FPLC plasma lipoprotein profile from apoE-knockout mice following the different experimental diets. Results are shown as µg of cholesterol per fraction. (a) and (c) male on chow and squalene diet; (b) and (d) female on chow and squalene diet, respectively.

Table 2
Effects of squalene on apoE-knockout mice body weight and liver parameters

	Males				Females			
	Chow ($n=8$)	Squalene ($n=9$)	Type 1 α	Type 2 β	Chow ($n=7$)	Squalene ($n=8$)	Type 1 α	Type 2 β
Body weight change (g)	4.6 ± 1.3	5.3 ± 1.1	0.33	0.2	5.4 ± 1.2	6.2 ± 1.5	0.5	0.2
Liver weight (g)	0.9 ± 0.1	0.7 ± 0.06	0.04*	0.15	0.7 ± 0.1	0.7 ± 0.1	0.6	0.1
Liver fat (%)	11.3 ± 6.1	5.8 ± 3.5	0.02¶*	0.1	3.6 ± 1.3	5.2 ± 1.7	0.1	0.4
Liver squalene (μg/kg)	314 ± 81	435 ± 192	0.2	0.2	546 ± 202	610 ± 224	0.6	0.1

Results are expressed as means ± S.D. Mice were fed chow or squalene diet for 10 weeks and fasted overnight before blood collection. Statistical analysis to evaluate dietary response was done using two-tailed unpaired *t*-test except for ¶that Mann–Whitney *U*-test was used.
* $P<0.05$ vs. chow diet.

a basal difference in the content of this compound between both sexes being higher in females than in males ($P<0.006$, $\beta=0.2$). Administration of squalene did not induce significant accumulation of squalene in the liver of either sex with a high degree of confidence as values of type 2 error indicated (Table 2).

When hepatic fat content was measured; a significant sex difference also appeared in mice consuming chow diet ($P<0.012$, $\beta=0.2$) with liver fat content being higher in males than in females. Male mice receiving squalene showed a significant decrease of fat in liver with a low type 2 error (Table 2), an effect which was corroborated by histological analysis (Fig. 2a–c). In contrast, although females showed a trend to increase hepatic fat, this difference was not statistically significant and underpowered (Table 2). A correlation study was carried out to establish whether concentration of liver squalene may be associated with hepatic fat accumulation in males. As reflected in Fig. 2d, an inverse and statistically significant association was found in males. In females, this association although weak and not significant also existed ($r=-0.53$, $P<0.06$). These results suggest that squalene is implicated in fat movement in the liver and this effect is sex-dependent.

3.3. Quantification of lesion area

Fig. 3 shows atherosclerotic lesion area in male (a) and female (b) mice. Lesions observed in all animals killed at 18 weeks of age were at the stage of foam cell infiltration into the intima (data not shown). Quantification of aortic lesion area revealed a different pattern between sexes due to squalene intake. Thus, male mice receiving squalene showed a significant decrease in lesion, while female mice did not change their lesion area. The power of both observations was quite high due to low type 2 errors observed. To test whether or not aortic atherosclerotic lesions were associated with hepatic fat or hepatic squalene concentration in either sex, we carried out correlation studies whose male results are shown in Fig. 4. A statistically significant direct association ($r_s=0.64$; $P<0.02$) between lesion area and hepatic fat accumulation was observed in males (Fig. 4a). The same panel indicates that there were two types of responses in males, in one group hepatic fat accumulation induced a pronounced increase in lesion while in other group the increase was more moderate and even saturable. No such association was observed in females ($r_s=0.08$; $P<0.8$). In males, a high statistically significant direct correlation ($r_s=0.81$; $P<0.000$) between hepatic weight and plasma triglycerides was observed (Fig. 4b). In females, this association was not present at all ($r_s=0.07$; $P<0.8$). This finding suggest a different management of hepatic fat between sexes and the relevance of plasma triglyceride levels as a surrogate marker of hepatomegaly in males.

3.4. Paraoxonase activity and isoprostane levels

The effect of squalene on the antioxidative defenses was studied by means of the paraoxonase activity and the plasma levels of 8-isoprostaglandin $F_{2\alpha}$. The arylesterase activity of paraoxonase in the different experimental groups is shown in Table 1. According to the results, squalene administration did not induce significant changes in the activity of the enzyme in either sex with low β error in this statement. In contrast, squalene intake significantly decreased plasma levels of 8-isoprostaglandin $F_{2\alpha}$ in both sexes with a high power of prediction according to the low values of type 2 error observed. The effect was more pronounced in females (Table 1).

3.5. Mac-1 analysis

We analyzed Mac-1 expression to monitor the degree of activation of circulating white blood cells. Mac-1 (CD11b) is a monocyte integrin molecule, reported to be a ligand for ICAM- 1 and 2, and involved in monocyte recruitment during atherosclerosis development [32]. As data shows, in males squalene did not change the proportion of cells expressing Mac-1 (Fig. 5a). However, in females the proportion of cells expressing Mac-1 was significantly lower in the squalene compared to the control group (Fig. 5b) with a strong value of prediction due to a low β error.

3.6. Hepatic apolipoprotein and receptor gene expression

In order to verify whether or not squalene was involved in hepatic apolipoprotein and lipoprotein receptor mRNA expressions, we assayed *Apoa1*, *Apoa5*, *Ldlr* and *Srb1* message levels in liver by quantitative real-time PCR. Data in

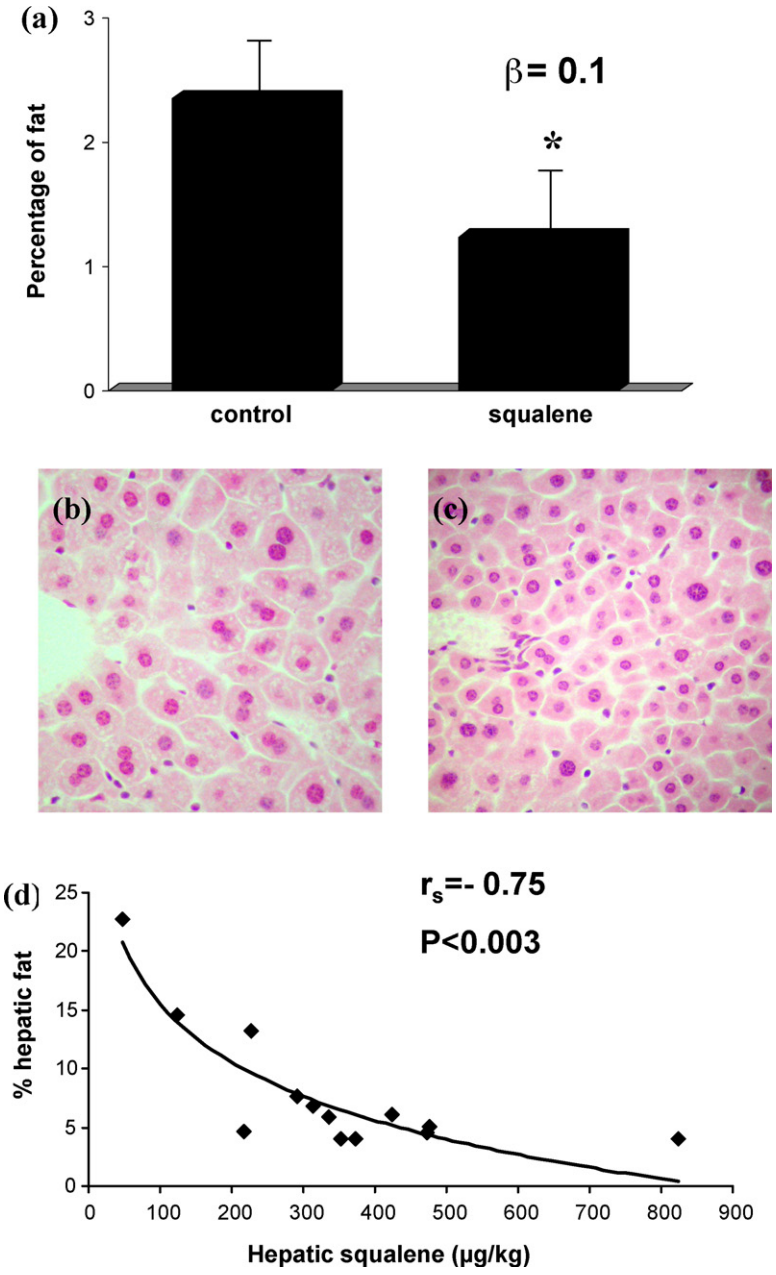

Fig. 2. Effects of squalene on hepatic steatosis in apoE-knockout male mice. (a) Morphometric changes of hepatic fat content. Data are means ± S.E.M. for each group. Statistical analyses were done according to Mann–Whitney *U*-test. $^{*}P < 0.01$ vs. control and β error was calculated as described in Section 2. (b) and (c) representative micrographs of control and squalene-treated livers. (d) Relationship between hepatic fat and liver squalene content of control and squalene-treated in mice. Correlation was done according to Spearman's test.

Fig. 6 are expressed as arbitrary units referred to the level of *Cyclophilin B*. No significant change in hepatic *Apoa1* mRNA levels with high power of prediction was observed in males (Fig. 6a) or in females (Fig. 6b). However, sex-dependent differences were observed with respect to hepatic *Apoa5* mRNA expression. Thus, while male mice receiving squalene showed significantly increased *Apoa5* expression with high power of prediction (Fig. 6c), no significant change upon squalene feeding was observed in female livers with a reasonable value of type 2 error (Fig. 6d). Sex differences were also noted regarding *Ldlr* and *Srb1* expressions. In this respect, livers of males showed no significant change for the former (Fig. 6e) and a significant increase for the latter (Fig. 6g) in both cases with a high power of prediction. The opposite trend was observed for females regarding both receptor expressions, a significant increase for the *Ldlr* (Fig. 6f) upon squalene administration, although with a low power of prediction, and no statistical significant change with high power of prediction in *Srb1* (Fig. 6h) expression by the administration of this agent. These results demonstrate that administration of squalene induces sex-dependent changes on hepatic apolipoprotein and lipoprotein receptor mRNA

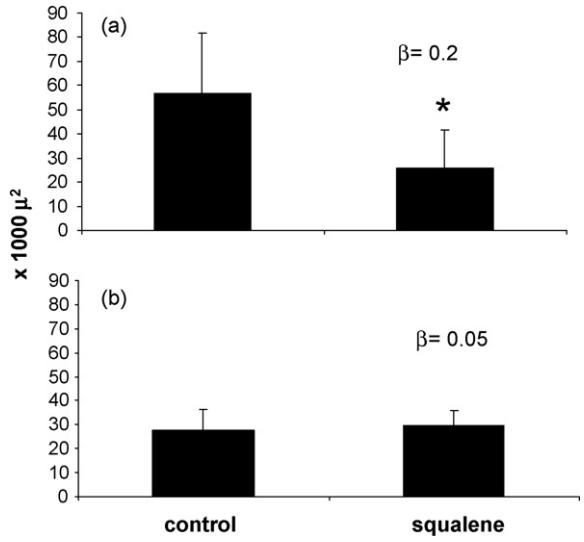

Fig. 3. Effects of squalene on atherosclerotic lesions in apoE-knockout mice. Cross-sectional analysis of aortic lesions in males (a) and females (b). Data are means ± S.E.M. for each group. Statistical analyses were done according to Mann–Whitney *U*-test. $^{*}P < 0.01$ vs. control. β Error was calculated as described in Section 2.

expressions. To verify whether the mRNA changes were reflected on LDLr and SR-B1 protein levels, Western analysis was carried out. As shown in Fig. 7a, squalene administration induced an increase in protein levels of LDLr in males. In females (Fig. 7b), this effect was not observed. No changes were observed in hepatic SR-B1 protein levels in males and females (Fig. 7c and d). In all cases a good power of prediction accompanied the results according to the β error observed.

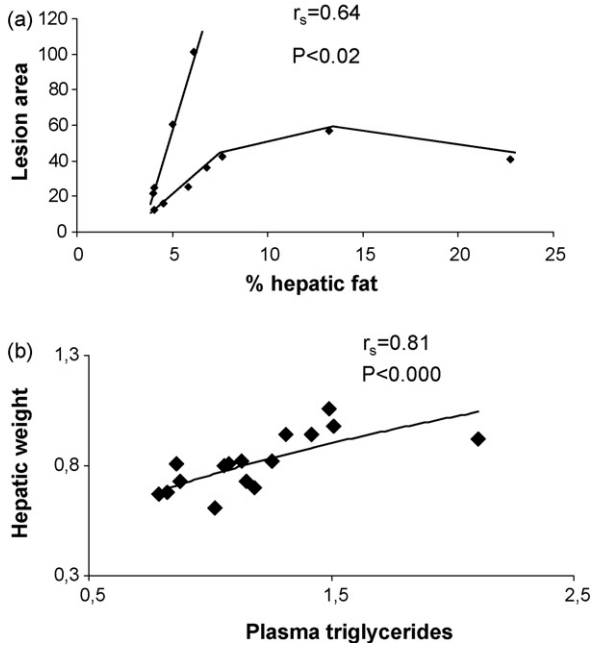

Fig. 4. Relationship between lesion area and hepatic fat (a) and hepatic weight and plasma triglycerides (b) in males. Correlation was done according to Spearman's test.

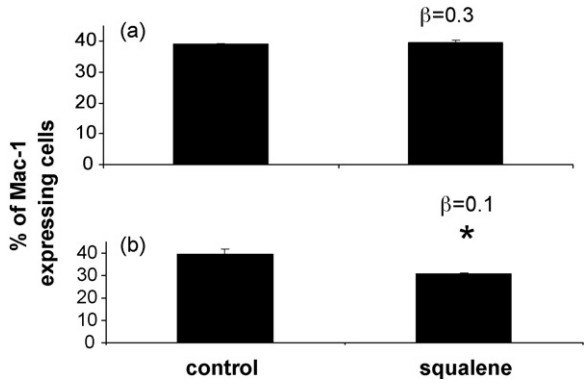

Fig. 5. Effects of squalene on blood cell activation. Leukocyte Mac-1 expression in males (a) and females (b). Flow cytometry analysis of blood Mac-1 (CD11b) positive cells was made. Two pools from each group (four to five animals per group) were prepared and analyzed. Data are means ± S.E.M. for each group. Statistical analyses were done according to Mann–Whitney *U*-test. $^{*}P < 0.05$ vs. control. β Error was calculated as described in Section 2.

4. Discussion

Mediterranean diet is associated with a lower incidence of coronary heart disease [5]. The main source of fat in this diet is olive oil [6]. It is unknown which components of olive oil provide its protective effects. The present study was designed to evaluate the influence of squalene, the main hydrocarbon in olive oil, on lipid metabolism and atherosclerosis development of apoE-knockout mouse. Our results show a differential response to squalene intake between males and females when administered for 10 weeks. In males, the reduced atherosclerotic lesion area was independent of plasma lipid levels. In females, this decrease of lesion area did not occur despite the fact that squalene reduced plasma cholesterol and triglycerides levels. Hepatic fat content was also modulated by squalene differently depending on sex. Thus, while squalene administration decreased hepatic fat in males, it resulted in increased hepatic fat content in females. In both sexes, lesion changes were significantly correlated with hepatic fat although this association was particularly significant in males.

Squalene administration dose used in the present study was 1 g/kg/day which is five times lower than the lethal dose 50 (5 g/kg/day) [33]. Assuming an average mouse weight of 25 g, each animal received 25 mg of squalene per day. In previous studies, we have used extra virgin olive oils of 9 g/kg squalene content and diets contained 20% (w/w) of olive oil [24], assuming an average solid intake of 2.5 g per mice that would represent an intake of 4.5 mg of squalene. In the present study, a dose five times higher than provided in nutritional approaches and five times lower than the toxicological threshold was considered a reasonable starting point to test the potential effect of this isolated olive oil component. Interestingly, hepatic squalene content was found significantly different between males and females consuming the chow diet (Table 2). The prolonged oral squalene administration

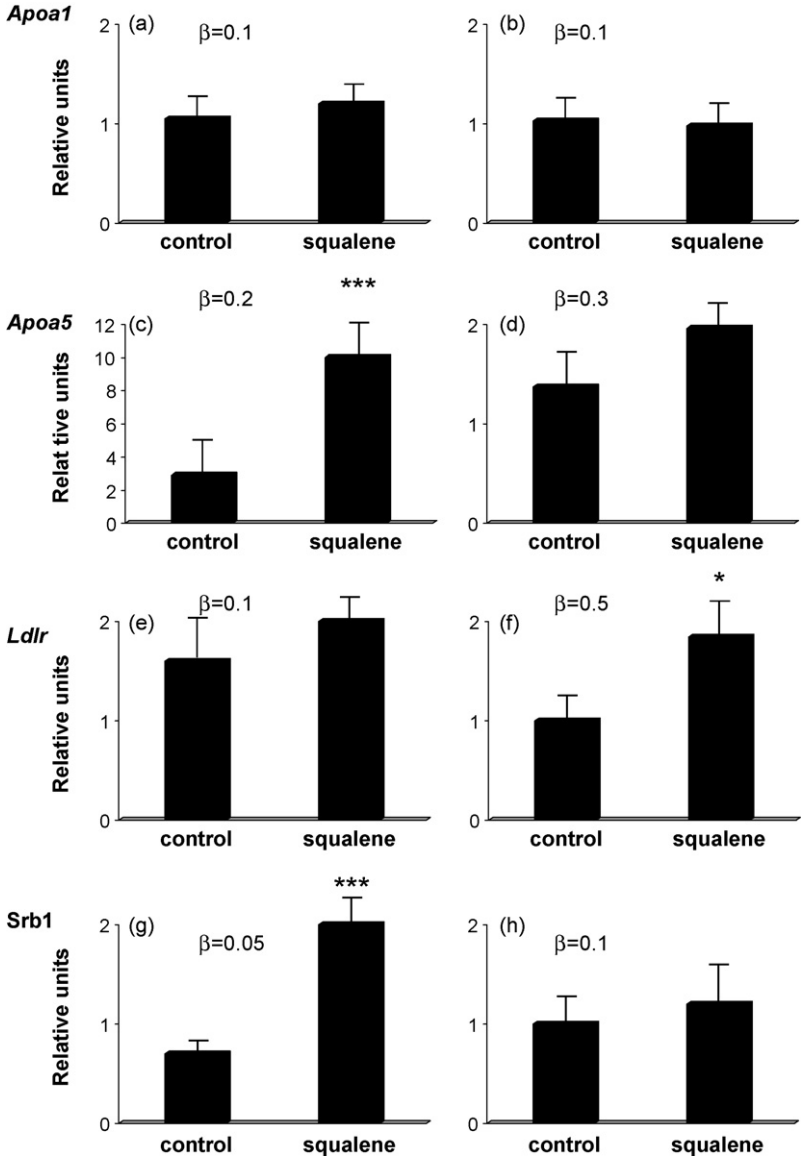

Fig. 6. Effects of squalene on hepatic *Apoa1*, *Apoa5*, *Ldlr* and *Srb1* mRNA expressions. Results are expressed as mean ± S.E.M. of arbitrary absorbance units normalized to the *Cyclophilin B* gene expression with the qRT-PCR analysis as described in Methods. Statistical analyses were done according to Mann–Whitney *U*-test. $^{***}P<0.01$ vs. control and $^{*}P<0.05$ vs. control. (a), (c), (e), (g) males and (b), (d), (f), (h) females. β Error was calculated as described in Section 2.

showed a trend to increase hepatic levels of this compound but it did not abolish the sex difference. In contrast, the intake of squalene was accompanied by a reduction in male liver steatosis. These observations indicate that the amount of liver fat content can be modulated by nutritional interventions, being squalene intake an interesting agent for male animals.

There are few studies regarding the effects of squalene on plasma lipid parameters. We have found that squalene has a marked different effect in male and female apoE-knockout mice. Thus, in males squalene did not influence plasma cholesterol, its distribution among the different plasma lipoproteins, or triglyceride levels (Table 1 and Fig. 1) with high power of prediction. In contrast, administration of squalene to females, although reducing total cholesterol and triglyceride levels (Table 1), had a low statistical power of prediction. This fact may raise two possibilities either the sample number was low or there is a phenomenon of polymorphic genetic response with responder and non-responder subjects. Indeed, studies in animals and humans reveal that diet squalene supplementation has no effect or reduces plasma cholesterol and triglyceride levels [11–34]. Our results are in agreement with those observed in humans and in other animal species but introduce sex as a discerning variable to uncover differential responses and even the potential of differential genetic responses in females. As shown by the distribution of cholesterol among the different plasma lipoproteins (Fig. 1), the reduction of total cholesterol in females by squalene administration could be due to a reduction of VLDL cholesterol levels. This VLDL decrease also explains the

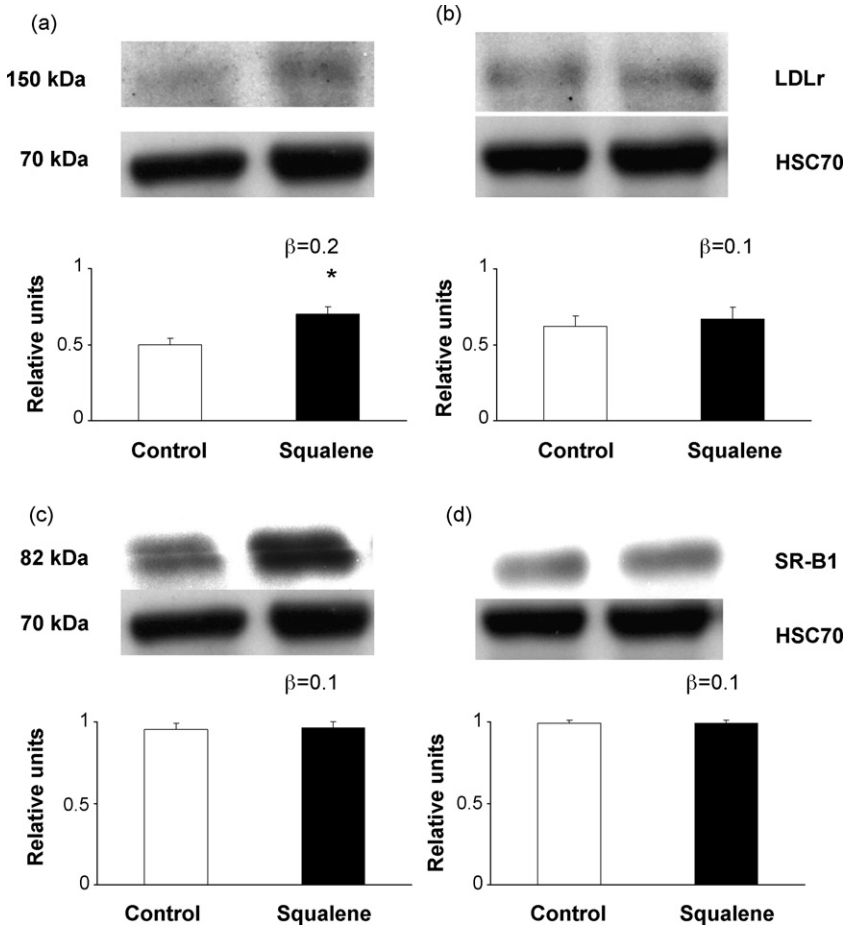

Fig. 7. Effects of squalene on hepatic LDLr and SR-B1 protein expressions. Representative blots and data expressed arbitrary absorbance units normalized to the HSC70 expression (mean ± S.E.M.) are given. Western analysis was carried out as described in Methods. Statistical analyses were done according to Mann–Whitney *U*-test. $^{*}P < 0.05$ vs. control. (a), (c) males and (b), (d) females. β Error was calculated as described in Section 2.

hypotriglyceridemia found in the squalene female group. It has been proposed that apoA-V lowers plasma triglyceride levels by modulating hepatic VLDL assembly and secretion [35–36] rather than modulating the removal of these particles [37]. Our findings provide further evidence for an intrahepatic role of this apolipoprotein as suggested by Olofsson [37] since there is no change of plasma apoA-V in plasma in both sexes (Table 1), no correlation with plasma triglycerides ($r_s = 0.16$, $P < 0.6$ and $r_s = -0.27$, $P < 0.35$ for females and males, respectively), and increased expression of *Apoa5* (Fig. 6d). In addition, the subcellular location of apoA-V in endoplasmic reticulum has conveyed to propose a not well-defined role in modifying lipidation of apoB-100. Our data suggest that squalene could be influencing the action of this apolipoprotein in a sex-dependent manner. Thus, in males the administration of squalene increased *Apoa5* mRNA liver expression (Fig. 6c), reduced hepatic fat content (Table 2), which may explain the decrease in liver weight of males fed on squalene (Table 2), and did not alter plasma triglycerides (Table 1). In this scenario, it seems that increased male *Apoa5* mRNA expression is not efficient enough to promote action. Several possibilities may arise: translation of mRNA is not efficient, apoA-V is not properly included in the reticulum, the cooperation with microsomal transfer protein in lipidation may be out of control, etc. In addition, the hepatic decrease of fat content in males strongly modified by hepatic squalene content ($r_s = -0.75$, $P < 0.003$) could be indicative that the metabolic use of fatty acid should be also enhanced provided that plasma triglycerides are not modified. On the other hand, in females, the observed actions although cautiously taken due to a lesser statistical power are clearly suggestive of different actions of squalene according to sex. In fact, the decrease in plasma triglycerides (Table 1) together with the decrease in circulating VLDL (Fig. 1) may explain the lack of change in hepatic fat in squalene fed females (Table 2) and a more efficient action of apoA-V whose hepatic expression was slightly increased (Fig. 6d). The different accumulation in hepatic fat between sexes is in agreement with previous results in mice from other authors [38]. The administration of squalene also induced significant opposite effects on hepatic expressions of receptors involved in lipoproteins such as *Ldlr* and *Srb1* at the mRNA level. However, these changes were not translated into protein variations (Fig. 7). These results are suggesting interesting and complex mechanisms

of regulation of LDLr and SR-B1 receptors modulated by squalene in a sex-dependent manner.

When we analyzed cross-sectional aortic lesions, we also found differences between males and females. In males, squalene intake significantly reduced atherosclerotic lesion area (Fig. 3a) whereas this effect was not observed in females (Fig. 3b). We found that lesion area was positively associated with hepatic fat content in males ($r = 0.64$, $P < 0.02$). The concomitant presence of increased hepatic lipids and atherosclerosis has been previously described in dogs and rabbits [39–41], but not in Mongolian gerbils [42]. Furthermore, an association between both parameters following different interventions has been observed [43–44]. Ferrochelatase deficient mice showed increased hepatic lipids, hyperlipidemia and development of atherosclerosis. In humans, this deficiency has been also associated with liver disease, hypertriglyceridemia and a low level of high density lipoprotein cholesterol [45]. Recently; nonalcoholic fatty liver disease (NAFLD), a feature of the metabolic syndrome and encompassing a wide variety of liver damage, ranging from steatosis to steatohepatitis, fibrosis and cirrhosis, has been found associated with advanced carotid atherosclerosis in several populations [46–48]. Indeed hepatic steatosis, the hallmark of NAFLD, regardless of its "metabolic" or "viral" etiology, has also been proposed to contribute to the progression of liver disease, arterial hypertension and type 2 diabetes mellitus [49]. In this way, the excessive accumulation of triglycerides within the liver could contribute to expand the relevance of the metabolic syndrome (syndrome X, insulin resistance syndrome) [50] and its clinical complications. This is becoming the major health problem of our times, conspiring to shorten life span by increasing coronary heart disease and stroke [51]. More severe expression of the frequent metabolic syndrome is laminopathies, which combine insulin resistance, android distribution of adipose tissue, dyslipidemia, predisposition to early atherosclerosis and hepatic steatosis [52]. Our study provides further support for a relationship between hepatic fat and development of arteriosclerosis, but it equally exemplifies a role for squalene in modulating hepatic fat content in a sex-dependent manner.

In addition, squalene also modulated the regulation of apolipoprotein A-I in a sex-dependent manner. Our study shows that, in males, squalene did not modify *Apoa1* mRNA expression (Fig. 6a) nor the plasma apolipoprotein A-I levels (Table 1) while in females it decreased plasma apolipoprotein A-I levels (Table 1) without change in hepatic *Apoa1* mRNA expression (Fig. 6b). In this sex, *Apoa1* mRNA expression was negatively correlated with liver weight ($r_s = -0.61$, $P < 0.02$). These results indicate that *Apoa1* mRNA expression could be coordinated with liver fat content and that the contribution of hepatic mRNA expression to the plasma response is the consequence of an interaction of sex and squalene intervention.

Squalene intake did not influence paraoxonase activity in either sex (Table 2). Administration of squalene decreased F_2-isoprostanes in both sexes (Table 2). Analysis of F_2-isoprostanes has emerged as a specific and reliable marker of oxidant stress in vivo [53]. On the other hand, circulating monocytes of squalene fed females expressed less Mac-1, without changes in males. Our results are in agreement with the data from Fontana et al. [54] that found that F_2-isoprostanes activate Mac-1 (CD11b) through a very restricted signaling pathway. From our female data, we can conclude that a reduction of isoprostane levels and a decrease in Mac-1 expressing circulating monocytes do not decrease lesion area, perhaps as a consequence of the decreased plasma apoA-I levels in presence of fatty livers. Conversely in males, a reduction of atherosclerotic lesion area is not associated with a reduction of Mac-1 expression, but is associated with decreases in liver steatosis and no change in plasma apolipoprotein A-I. These data would indicate that hepatic production of apoA-I could counteract other pro-atherosclerotic changes such as increased monocyte activation. In fact, apolipoprotein A-I has been shown to inhibit macrophage conversion into foam cells and thus restricting the growth of atherosclerotic plaques [55]. The results in squalene in both sexes regarding lesion are suggestive that apoA-I is playing a role besides the change in inflammatory and oxidative parameters, an interpretation which is in agreement with other authors [56].

In summary, our data demonstrate, for the first time, that high intake of squalene modulates atherosclerotic lesion area in apoE-knockout mice in a sex specific manner. Thus, squalene reduced lesion area in males without changes in females and lesion areas were positively associated with hepatic fat content in males. These data show that a unique diet component can modulate the development of atherosclerosis in the apoE-knockout experimental model eliciting different responses in males and females. Our results also provide some evidence that squalene administration could be used as a safe alternative to alleviate hepatic steatosis and atherosclerosis development particularly in males.

Acknowledgments

This research was supported by grants FEGA-FEOGA (CAO99-014), FEDER-CICYT (SAF2007-60173 and AGL2002-00495), FISS 01/0202, *Redes* DGA (B-69) and *FISS de investigación cooperativa* C03- 01 and G03-140 and by *Fundación Española del Corazón*. R.C., S.A., M.A.N. and N.G. were recipient of DGA, FEGA-FEOGA and *Fundación Cuenca Villoro* fellowships. We thank Angel Beltrán, Jesús Cazo, Jesús Navarro, Carmen Navarro and Clara Tapia from *Unidad Mixta de Investigación* for their invaluable help in maintaining animals.

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