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Original article

Effect of atorvastatin and diet on non-alcoholic fatty liver disease activity score in hyperlipidemic chickens

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ABSTRACT

Non-alcoholic steatohepatitis (NASH) is part of the spectrum of non-alcoholic fatty liver disease (NAFLD), which includes from simple steatosis and steatohepatitis, to the most severe cirrhosis and carcinoma, which develops in the absence of excessive alcohol intake. NAFLD is the most common liver disorder in affluent societies. There is no proven treatment for NAFLD/NASH. One of the most frequent adverse effects of statins is an increase in hepatic aminotransferases. Studies that evaluate if the benefits of statins overcome the risks in NASH are lacking. The present study was conceived to explore the effect of both atorvastatin and diet on regression of steatohepatitis, using a chicken experimental model induced by a hyperlipidemic diet (HD). Plasma lipid levels, liver enzymes and hepatic histopathology, as well as image analysis were performed to determine changes in liver lipid deposits and inflammatory infiltration. Features of steatosis, cell-ballooning, and inflammation were scored to obtain the NAFLD activity score (NAS). A severe level of steatosis was found in animals fed on HD. Atorvastatin treated groups showed smaller size of lipid deposits and a lower level of inflammation than non-treated groups. Atorvastatin therapy induced a significant reduction of hepatocellular damage, even though in the animals which continuously received a hyperlipidemic diet. The combination of atorvastatin therapy and a standard diet produced the lowest decrease of NAS. Our results show that atorvastatin therapy not only decreased plasmatic levels of cholesterol and triglycerides, but also induced a reduction of liver steatosis, inflammation and hepatocellular damage, without increasing plasmatic amynotransferase levels.

1. Introduction

Sedentary lifestyles and poor dietary choices are contributing to a weight gain epidemic in westernized societies. Recent epidemiological studies suggest an increased risk of cardiovascular disease and type II diabetes in overweight and obese individuals. Unfortunately, incidence of the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD), which can precede the development of cardiovascular disease and type II diabetes, are also increasing [1].

Non-alcoholic steatohepatitis (NASH) is part of the spectrum of NAFLD, which includes different lesion grades, from simple steatosis and steatohepatitis, to the most severe cirrhosis and hepatocellular carcinoma, which develops in the absence of excessive alcohol intake. NAFLD is the most common liver disorder in affluent societies, representing the hepatic metabolic consequence of relative overnutrition and reduced physical activity [2,3].

NAFLD is a complex disorder involving environmental factors and genetic predisposition. As a result of this complexity, animal models of the spectrum of NAFLD provide the necessary tools to overcome confounding variables, such as genetic heterogeneity, gender differences, and environmental factors, including diet and lifestyle [4]. Much is still unknown about the pathophysiology of steatohepatitis in humans. Studies in animal models might provide crucial insights in the pathogenesis and therapeutic options of this disease. Given the difficulty of studying all the factors involved in food intake in human populations, studies in animal models allow manipulation of dietary composition in order to research the role of diet in the pathogenesis of steatohepatitis.

Chickens are predisposed to fat deposition in the liver [5]. Furthermore, the chicken has been considered as a suitable model

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for studies on the comparative lipid metabolism because it is highly sensitive to dietary modifications [6,7]. Therefore, the chicken model offers technical advantages over mammalian models, and may help in the development of a more rational treatment strategy.

With no proven treatment for NAFLD/NASH, the focus of several investigations has been on the treatment of components of the metabolic syndrome (obesity, hypertension, dyslipidemia, and diabetes). Lipid lowering agents can also lower risks of the metabolic syndrome and NAFLD. It is well-known that statins combat dyslipidemia, a hallmark of the metabolic syndrome, by reducing serum triglycerides (TG) and increasing high-density lipoproteins (HDL) levels. However, one of the most frequent adverse effects of statins is an increase in hepatic aminotransferases and caution is needed when prescribing statins to patients with liver disease [8]. Furthermore, liver injury has been associated with these drugs [9]. Studies that evaluate if the benefits of statins overcome the risks in NASH are lacking. To our knowledge, experimental studies on the potential hepatoprotective effect of atorvastatin and diet in NASH have not been reported. Therefore, the present study in an animal model was conceived to explore the effect of both atorvastatin and diet on regression of steatohepatitis. Plasma lipid levels, liver enzymes and hepatic histopathology, as well as semiquantitative and quantitative assessment by image analysis were performed to determine changes in liver lipid deposits and inflammatory infiltration. Features of steatosis, cell-ballooning, and inflammation were scored to obtain the NAFLD activity score (NAS).

2. Materials and methods

2.1. Animals and treatments

One hundred male 3-week-old White Leghorn chickens (Pollos Pujante, Murcia, Spain) were housed under controlled conditions. Each room had air-conditioning and thermostatic control in order to minimize variations in temperature and humidity (approximately 23 °C and 60%, respectively). The chickens were randomly assigned to two kinds of diet (they received a standard growth diet during the first 3 weeks of their life). Water was given *ad libitum*:

- standard diet (SD): a standard growing mash. The weekly amount of this was increased with the age of the animals;
- hyperlipidemic diet (HD): a standard growing mash with pure cholesterol (2% of the mixture) and 20% of the mixture of saturated oil (palm oil).

After a 3-month induction period, 10 chickens in each group were sacrificed to evaluate the hyperlipidemic effect. Afterwards, the chickens fed on HD were randomly divided into four groups and were kept for another 3-month period with different diets. Thus, the groups of our study were as follows:

- group A ($n = 16$): SD for 6 months (healthy control);
- group B ($n = 16$): HD for 6 months (hyperlipidemic control);
- group C ($n = 16$): HD for 3 months and SD during the next 3 months (spontaneous regression group);
- group D ($n = 16$): HD for three months and SD during the next 3 months, when they received oral atorvastatin at clinical doses (pharmacological regression group);
- group E ($n = 16$): HD for the whole 6 months, and oral atorvastatin at clinical doses during the last 3 months (progression group).

Atorvastatin was orally administered at doses of 3 mg/kg/day. Animals were weekly body-weighed in order to calculate the doses. Medications were administered (force-fed) daily at 8 a.m.

2.2. Blood sampling

Blood samples (1 ml) were extracted after an overnight fasting period from the axillary vein. In all cases, blood was collected into 10 mM trisodium citrate-containing tubes. Plasma was separated and analyzed for the determination of total cholesterol, low-density lipoprotein (LDL), HDL, TG, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl-transferase (γ-GT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), creatine kinase (CCK), C-reactive protein (CRP) and fibrinogen. Total cholesterol, LDL, HDL, triglycerides, AST, ALT, γ-GT, AP, LDH, and CCK were measured using a D-2400 and P800 analyzers (Hitachi Ltd., Tokyo, Japan) and commercially available assays from Roche Diagnostics (Manheim, Germany). The method described by Kostner et al. [10] was used for precipitation of HDL.

2.3. Tissue collection

All animals were sacrificed by intraperitoneal administration of pentobarbital, after 6 months of receiving both diets and/or treatments. Livers were removed for histological examinations.

All experimental procedures were approved by the University of Murcia institutional Animal Care Committee, in accordance with the guidelines for ethical care of experimental animals of the European Union.

Liver samples were fixed in 10% formaldehyde in phosphate-buffered saline (0.1 M PBS, pH 7.4) for 10 h and embedded in paraffin; afterwards, 5 μ-thick paraffin sections were cut and stained with haematoxylin and eosin (H&E) and Verhoeff Van Giesson staining techniques. A histological assessment of the tissue was performed for each animal by a pathologist who was blinded to the study.

2.4. Steatosis analysis

Lipid deposits were evaluated semiquantitatively in 10 animals (100 fields (×400) in each experimental group). Liver samples were classified assigning a score relative to the level of lipid deposits in the sample, according to the histologic classification by Brunt et al. [11] and modified by Angulo [12]:

- 0 corresponds to normal, with absence of lipid deposits or a level lower than 5%;
- 1 or mild, with lipid deposits lower than 33%;
- 2 or moderate, with lipid deposits between 33% and 66%;
- 3 or severe, with lipid deposit levels over 66%.

Percentages of samples within each semiquantitative score were determined for each experimental group and statistical analysis was performed.

A more detailed evaluation of lipid deposits was carried out by quantification of the percentage of steatosis area in liver parenchyma: lobular and centrilobular zones in 10 microscopic fields (square fields of 134 μm^2), obtaining 100 determinations for experimental group and zone. Mean and standard error were determined for each group and zone, and a comparative statistical analysis was also carried out. These parameters were quantified by image analysis using the MIP 4.5 (Microm, Image Processing software, Consulting Image Digital, Barcelona). Briefly, the image analysis system consisted of a light microscope (Zeiss Axioskop, Madrid) connected to a video camera 151-AP (Sony, Madrid) and a control computer. After obtaining a digital image, fat deposits were chosen interactively by a graphic line, and percentages of steatosis were measured.

2.5. Inflammatory infiltration analysis

Number of inflammatory foci was assessed microscopically (200×) in 10 fields for each animal. Appearance of inflammatory foci was classified as 1, or low density; 2, moderate; 3, high density. Furthermore, area and maximal diameter of inflammatory foci were evaluated in 10 microscopic fields (square fields of 267 μm^2), obtaining 100 determinations for each experimental group, by image analysis using the MIP 4.5 (Microm, Image Processing software, Consulting Image Digital, Barcelona). Inflammatory density was calculated with the following ratio: area of inflammatory infiltration (obtained by image analysis)/area of the entire field. Measurements were made in five square fields of 267 μm^2 for each animal.

2.6. Hepatocyte ballooning analysis

Ballooning classification (0–2) was made following a histological scoring system [13]:

- 0: none;
- 1: few balloon cells, i.e. rare but definite ballooned hepatocytes being present, as well as cases that are diagnostically borderline;
- 2: many cells or prominent ballooning.

Evaluations were made in five square fields of 134 μm^2 for each animal.

2.7. NAFLD activity score

Features of steatosis, cell-ballooning, and inflammation were scored as above and single grades were summed up to obtain the NAS, ranging from 0 to 8. A semiquantitative-NAS was obtained by measured of semiquantitative steatosis, number of foci per microscopic field (200×) and frequency of ballooning [13]. A NAS $\geq$5 was considered diagnostic of NASH, NAS $\leq$2 excluded NASH (simple steatosis), and NAS in between was considered indeterminate [13]. Besides this qualitative score, we also obtained a quantitative NAS, based on results of the same parameters and image analysis (steatosis percentages, the same ballooning classification (0–2), and lobular inflammatory density: 1, or infiltrate <2.5%; 2, with infiltrate between 2.5% and 5.2%; and 3, with infiltrate over 5.2%).

2.8. Statistical analysis

Results are expressed as mean ± standard error. Mann-Whitney and Kruskal-Wallis non parametric tests were used for assessment of statistical significance in semiquantitative analysis, while statistical significance for quantitative analysis was evaluated by ANOVA or Welch test, and Bonferroni or Games-Howell post-hoc tests. Statistics were performed using SPSS v14. A p-value <0.05 was considered as statistically significant.

3. Results

3.1. Effects of hyperlipidemia on circulating lipid levels and hepatic function test.

Animals fed on the hyperlipidemic diet for 6 months (group B) showed an increase in all lipid parameters in the serum when compared to those of chickens fed the standard diet (group A) (Table 1). The return to the SD for 3 months reverted partly this effect (groups C and D, $p < 0.001$ in all cases) (Table 1). Moreover, animals fed on HD (group B) had comparatively higher levels of CRP ($p < 0.001$) than those fed on the SD. No diet and/or treatment significantly decreased these parameters. In our model there was no significant increase in concentrations of the analyzed enzymes (AST, ALT, γ-GT, AP or CCK).

3.2. Histology

Histological analysis showed that the liver samples of healthy control chickens (group A) presented neither fat accumulation, nor inflammatory infiltration, nor significant hepatocyte ballooning (Figs. 1 et 2). On the contrary, animals fed on HD (group B) developed steatosis with abundant fat deposition. It was predominantly macrovesicular, with isolated single droplets that resulted in nuclear eccentricity because they occupied the entire cell cytoplasm, and involved up to 66% of the lobules, although some hepatocytes showed also microvesicular steatosis. Ballooning degeneration of hepatocytes resulting from accumulation of intracellular fluid was characterized by swollen cells, often closely associated with the most distended hepatocytes by the esteatosis. A moderate grade of inflammation (based on observations of the number of foci) was found in lobular and portal zones; it was more evident in intra-acinar location. The return to the SD (group C) partially ameliorated histological findings, reaching minimum criteria for the diagnosis of steatohepatitis. Steatosis was usually lower than 33% of the sample with some degree of lobular and portal mild inflammation. Microgranulomas and lipogranulomas were occasionally found. Cell-ballooning was scarce in this group. Microvesicular steatosis (clusters of hepatocytes with intracytoplasmatic septations) was found in group D (return to the SD with atorvastatin) but to a minimal extent (<5%). Scarce or none inflammation was observed in lobular zone, whereas it was mild in portal location. Cell-ballooning was absent. Administration of atorvastatin to chicks fed on HD (group E) did not improve histological parameters as in animals fed on SD: steatosis (macrovesicular and microvesicular), lobular and portal inflam-

Table 1
Values of the main lipids, enzymatic and hepatic proteins measured in the serum from animals of all different experimental groups.

Experimental Groups	A	B	C	D	E
Cholesterol (mg/dl)	104.4 ± 5.5[a]	980.3 ± 141.3	204.2 ± 40.8[a]	197.0 ± 74.3[a]	413.8 ± 109.6
Triglycerides (mg/dl)	51.7 ± 18.8[a]	351.8 ± 18.0	253.4 ± 90.9[a]	31.6 ± 7.2[a]	356.9 ± 145.6
HDL (mg/dl)	67.9 ± 6.1[a]	353.4 ± 32.5	95.4 ± 20.3[a]	88.5 ± 19.3[a]	99.4 ± 22.1
LDL (mg/dl)	26.1 ± 2.6[a]	656.5 ± 112.6	85.5 ± 20.3[a]	77.6 ± 26.4[a]	242.9 ± 79.3
AST (IU/l)	206.8 ± 36.2	267.3 ± 37.2	231.8 ± 47.8	371.5 ± 158.1	372.4 ± 74.2
ALT (IU/l)	3.4 ± 1.0	17.8 ± 5.1	11.1 ± 4.5	4.1 ± 1.1	18.3 ± 6.8
γ-GT (IU/l)	15.4 ± 2.7	9.3 ± 2.4	16.67 ± 4.1	12.3 ± 2.1	20.8 ± 9.8
AP (IU/l)	634.7 ± 161.7	406.3 ± 94.8	142.4 ± 50.2	275.2 ± 123.8	385.4 ± 36.9
LDH (IU/l)	568.6 ± 91.4	1115.0 ± 133.6	499.4 ± 109.6	617.9 ± 182.1	1069.0 ± 268.9
CRP (REU/ml)	1.07 ± 0.29[a]	2.75 ± 0.26	2.55 ± 109.6	2.00 ± 0.38	2.01 ± 0.33

HDL: high-density lipoprotein; LDL: low-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GT: gamma-glutamyl transpeptidase; AP: alkaline phosphatase; LDH: lactate dehydrogenase; CRP: C reactive protein; IU: international units; REU: relative ELISA units.
[a] Statistical analysis was performed vs. HD-fed animals (group B). $p < 0.001$.

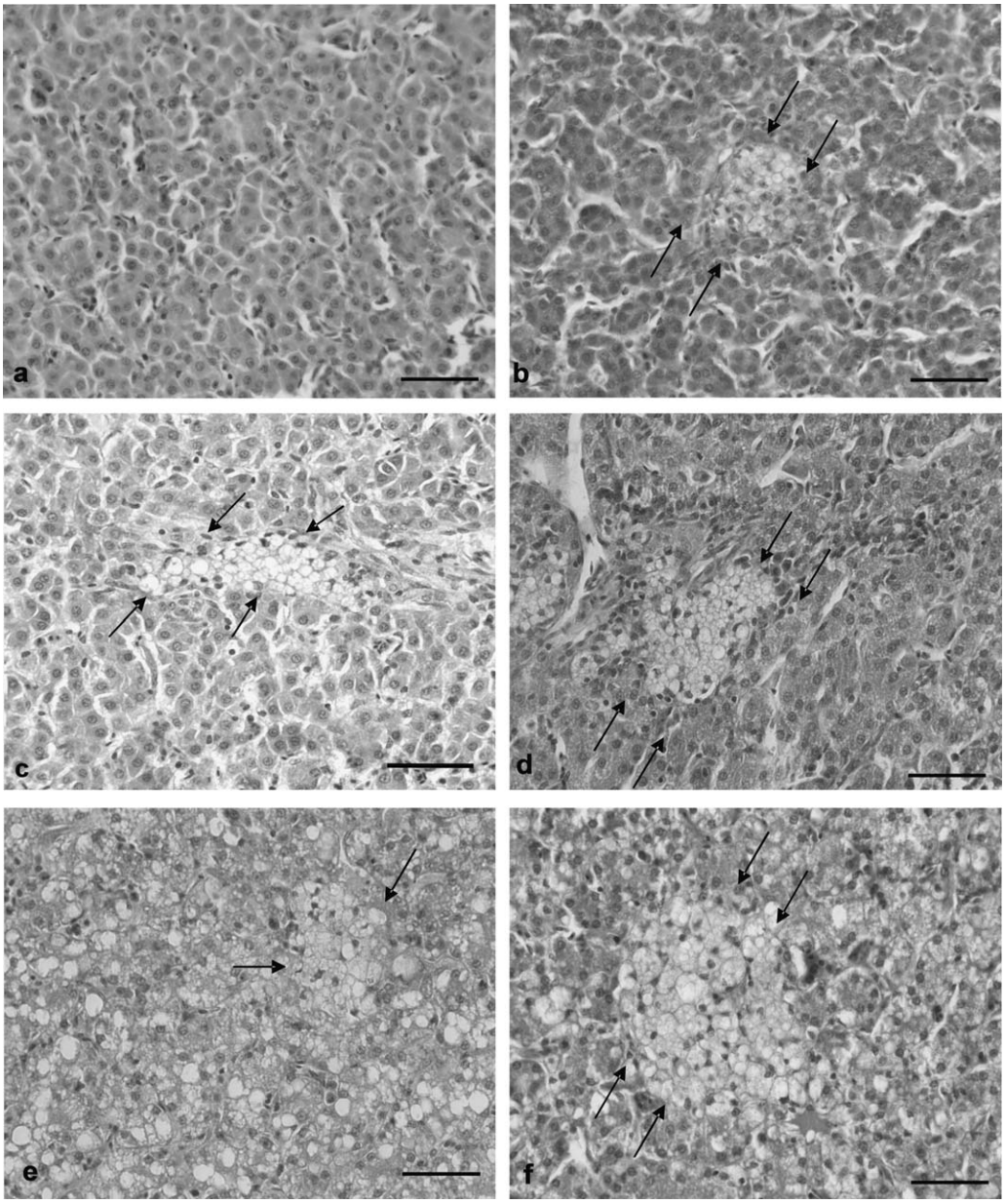

Fig. 1. Development of hepatic steatosis in the different experimental groups. (a) Group A, healthy control; no lipid deposits were observed. Fatty changes ranges from vacuolation of a few liver cells in groups D (pharmacological regression) (b, c) and C (spontaneous regression) (d) to severe steatosic changes in groups E (progression) (e) and B (hyperlipidemic control) (f). Note the big extension of the steatosis region in hyperlipidemic and progression samples (e, f). Arrows: lipid deposits. Haematoxylin-eosin staining. Bars: 40 μm.

mation (higher than in group C), presence of lipogranulomas and microgranulomas, and hepatocyte ballooning were found. No histological signs of cirrhosis were found under any diet/treatment in any group of chicks.

3.3. Steatosis analysis

Semiquantitative analysis of steatosis showed a score 0 in the group A (healthy control) (Table 2). Statistically significant differences existed between groups C (spontaneous regression group; score 2) and D (pharmacological regression group; score 1), whereas a severe level of steatosis was found in groups B (hyperlipidemic control) and E (progression group), without significant differences between them.

Besides, quantitative analysis of steatosis was carried out. Decreasing percentages of lobular steatosis were found from group B (with the maximum percentage) to groups E, C, D, and A (healthy control, absence of steatosis). Atorvastatin treated groups (D and E) showed smaller size of lipid deposits than non-treated groups (C and B, respectively). No significant differences were found between lobular and centrilobular zones for each experimental group.

3.4. Inflammatory infiltration and cell ballooning

Inflammatory infiltration in lobular zone was present in all the experimental groups, except in group A (Table 3). Statistically significant differences ($p < 0.05$) were observed for the number of foci between groups A and B, and B (hyperlipidemic control, with the highest number of foci) and the rest of groups (C, D, and E).

Morphometric analysis of the inflammatory foci (i.e. area and maximal diameter) showed a lower level of inflammation in atorvastatin treated groups (D and E) than in non-treated groups (B and C, respectively). Significant differences ($p < 0.05$) for lobular

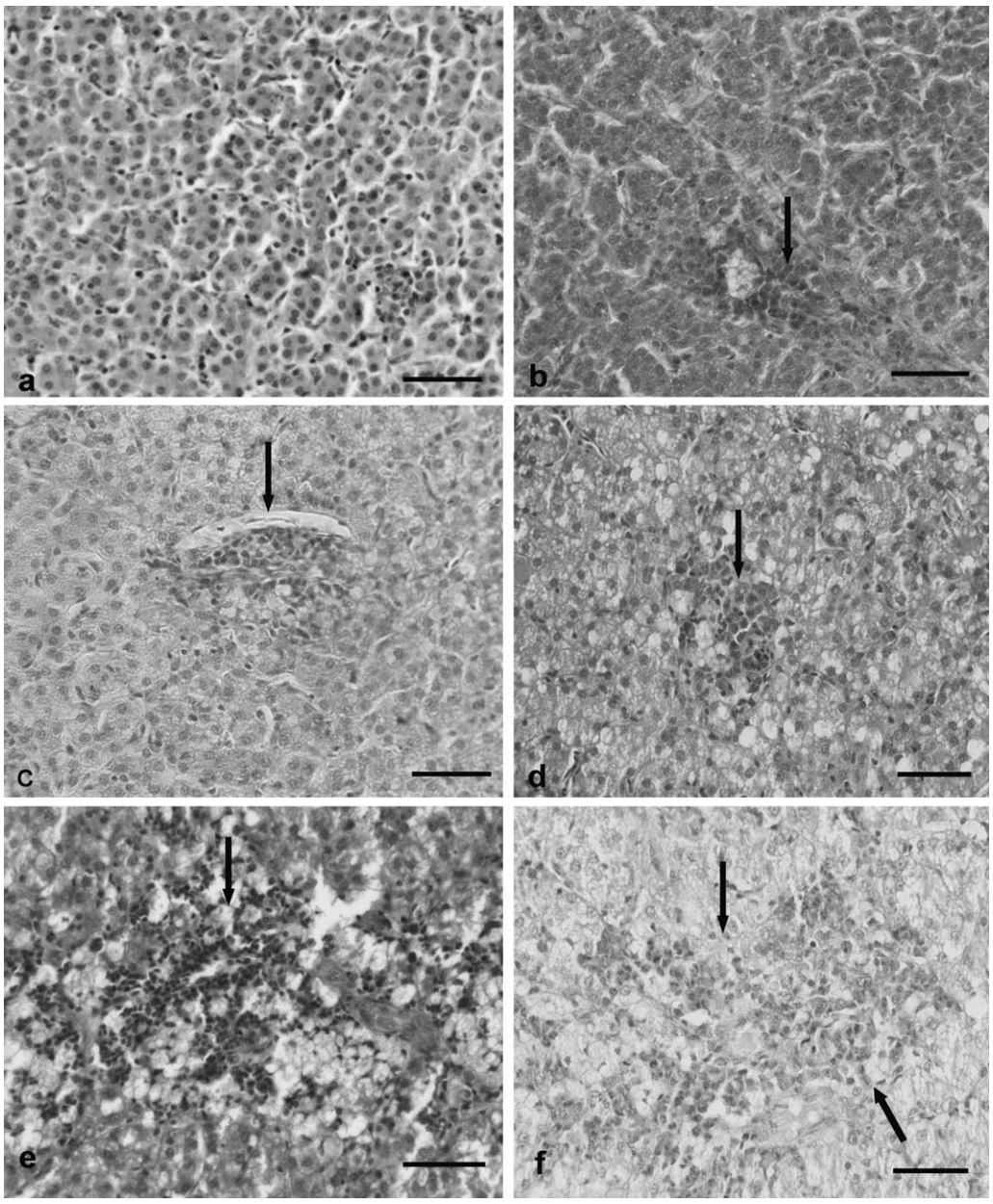

Fig. 2. Lobular inflammation in liver samples from chicks of the different experimental groups. a: group A, healthy control; no inflammatory infiltrate was observed; b: group D, pharmacological regression; c: group C, spontaneous regression. Scattered and mild lobular inflammation is observed in C and D groups (b, c); d: group E, progression, which contains a moderate dense infiltrate of inflammatory cells; e–f: group B, hyperlipidemic control, which shows a high lobular inflammatory infiltrate and associated hepatocyte ballooning degeneration. Arrows: lobular inflammation. Haematoxylin-eosin staining. Bars: 40 μm.

inflammatory density were found between group B (with the highest values) and the rest of groups, between D and E, and between B and E, but there were no statistically significant differences between C and D, or C and E. The lowest value for portal inflammatory density was found in group D (return to SD, with atorvastatin). Significant differences existed between group D and the rest of groups, but no differences were observed between groups B, C, and E.

Table 2
Semiquantitative and quantitative steatosis analysis.

Group	Semiquantitative analysis (%)[a]				Quantitative analysis (%)[b]	
	Grade 0 <5%	Grade I 5–33%	Grade II 33–66%	Grade III >66%	Lobular	Centrilobular
A	100^{a}	0^{a}	0^{a}	0^{a}	0.0 ± 0.0^{a}	0.01 ± 0.01^{a}
B	0^{b}	0^{b}	33^{b}	67^{b}	55.11 ± 1.21^{b}	53.76 ± 2.10^{b}
C	0^{c*}	27^{c*}	72^{c*}	0^{c*}	7.10 ± 0.51^{c}	8.99 ± 0.51^{c}
D	9^{d}	63^{d}	27^{d}	0^{d}	2.92 ± 0.31^{d}	2.98 ± 0.38^{d}
E	0^{b}	$11{,}1^{b}$	$44{,}4^{b}$	$44{,}4^{b}$	32.30 ± 1.03^{e}	20.55 ± 1.29^{e}

[a, b, c, d, e] Ddifferent upper-case letters show significant differences between groups ($p < 0.05$; except * $p < 0.06$; $n = 100$ for each experimental group).
[a] Kruskal-Wallis and Mann-Whitney tests.
[b] ANOVA and Bonferroni tests (mean ± standard error).

Table 3
Analysis of inflammatory infiltration and cell-ballooning (values expressed as mean ± standard error).

Group	Number of foci/field[f]	Appearance[g,h]	Foci area (μm^2)[f]	Maximal diameter (μm)[f]	Lobular inflammation density (%)[f]	Portal inflammation density (%)[f]	Cell ballooning[g,i]
A	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.44 ± 0.15^{a}	0 ± 0^{a}	0 ± 0^{a}
B	3.71 ± 0.16^{b}	2.37 ± 0.78^{b}	8754.42 ± 643.32^{b}	130.27 ± 6.57^{b}	7.88 ± 0.37^{b}	6.25 ± 1.02^{b}	1.85 ± 0.02^{b}
C	1.4 ± 0.09^{c}	2 ± 1^{c}	8754.42 ± 643.32^{c}	104.18 ± 6.27^{c}	$3.42 \pm 0.22^{c,d}$	4.07 ± 0.43^{b}	0.42 ± 0.04^{c}
D	1.13 ± 0.09^{c}	1 ± 1^{d}	3031.32 ± 338.39^{d}	76.86 ± 5.53^{d}	2.98 ± 0.18^{d}	2.91 ± 0.33^{c}	0.17 ± 0.02^{d}
E	1.42 ± 0.11^{c}	2 ± 1^{c}	$3869.68 \pm 311.66^{c,d}$	$95.77 \pm 4.96^{c,d}$	3.76 ± 0.24^{c}	3.93 ± 0.33^{b}	1.01 ± 0.05^{e}

[a, b, c, d, e] Different upper-case letters show significant differences between groups ($p < 0.05$; $n = 100$ for each experimental group).
[f] Welch and Games-Howell tests.
[g] Kruskal-Wallis and Mann-Whitney tests.
[h] Appearance of inflammatory foci was classified as: 1, or low density; 2, moderate; 3, high density.
[i] Cell-ballooning classification: 0: none; 1: few balloon cells; 2: many cells or prominent ballooning.

Table 4
Non-alcoholic fatty liver activity score (NAS) (values expressed as mean ± standard error).

Groups	A	B	C	D	E
Qualitative-NAS	0 ± 0^{a}	$5{,}6 \pm 0{,}09^{b}$	$3 \pm 0{,}08^{c}$	$2 \pm 0{,}08^{d}$	$4{,}4 \pm 0{,}09^{e}$
Quantitative-NAS	0 ± 0^{a}	$7{,}13 \pm 0{,}12^{b}$	$2{,}90 \pm 0{,}14^{c}$	$1{,}83 \pm 0{,}11^{d}$	$4{,}58 \pm 0{,}16^{e}$

[a, b, c, d, e] Different upper-case letters show significant differences between groups ($p < 0.05$; $n = 100$ for each experimental group). Anova and Bonferroni tests. Range from 0 to 8.

Analysis of cell-ballooning showed decreasing values from group B, to groups E, C, D, and A. Statistically significant differences existed between all of them.

3.5. NAFLD activity score

Both qualitative and quantitative NAS analysis showed statistically significant differences between all the experimental groups (Table 4). The highest values were observed in animals fed on HD (group B) followed by the groups E, C, D, and A. Higher values of NAS were observed in animals fed on HD (B and E) than in those fed on SD (C and D, respectively); besides, lower values were observed in atorvastatin treated animals (D and E) than in non-treated ones (C and B, respectively). Higher differences between groups were found for quantitative NAS than for qualitative NAS.

4. Discussion

Non-alcoholic fatty liver disease, especially non-alcoholic steatohepatitis, forms a definite threat to human health. With the increase in obesity, an increase in NAFLD can be expected. Studies in animal models could provide significant information on therapeutic options for this disease. The present study shows that chickens fed high cholesterol/high fat diets develop a severe and reproducible steatohepatitis in 3–6 months, with pathological findings similar to those of human NASH. These facts confirm the potential role of the hyperlipidemic chicken as a model of non-alcoholic steatohepatitis [14], and its use for therapeutic trials. In fact, we use an animal model to overcome confounding variables, such as genetic heterogeneity, gender differences, and environmental factors, including diet and lifestyle [4].

An increase in all lipid parameters measured in the plasma of chickens fed a cholesterol and fat enriched diet, was found, when compared to those of chickens fed the standard diet. A similar dyslipidemia, with high increases of tryglicerides and LDL, which is associated with NAFLD, has been described in humans [15]. It is well-known that statins combat dyslipidemia by reducing serum triglycerides and increasing HDL levels in humans. Both return to the SD, and atorvastatin therapy produced a significant decrease of lipidic parameters, but not of CRP, considered as an inflammation biomarker. CRP values were significantly higher in animals fed on HD (group B) than those fed on SD, but probably CRP is not as good inflammation biomarker in chickens, as it is in humans, because no diet and/or treatment significantly decreased these parameters. We did not found significant increases of hepatic enzymes in the chickens fed on HD, as previously shown for spontaneously hypertensive and hyperlipidemic rats [16], and normal mice [17] and rabbits [18]. Besides, other plasmatic parameters such as biliary acids could have a higher sensitivity in chickens than hepatic enzymes [19].

Currently, the liver biopsy is considered the Gold Standard for the direct measurement of hepatic fat and is the only reliable method for diagnosing fatty liver and/or NASH. Statistically significant differences were found for lipid deposits between the several experimental groups. A similar effect of statins was observed by Egawa et al. [20] when studying hepatic steatosis in aromatase-deficient (Ar−/−) mice. Elkin et al. [21] found a decrease of hepatic cholesterol and triglycerides with atorvastatin therapy in hereditary hyperlipidemic, non-laying hens. Atorvastatin administration also reduced cholesterol and triglycerides content in livers of Sprague-Dawley rats, by means of agonist action on hepatic PPARα and mitochondrial β-oxidation [22].

Statistically significant differences were found for morphometric analysis of the inflammatory foci between atorvastatin treated and non-treated groups. Neither Egawa et al. [20], nor Elkin et al. [21], when studying pitavastatin effect on liver steatosis in mice or atorvastatin therapy in hyperlipidemic chickens respectively, evaluated the inflammatory infiltrates, although they described a reduction of liver steatosis and of hepatic enzymes levels. Human clinical studies on atorvastatin or simvastatin therapy did not show significant differences in inflammatory infiltration when analyzing the number of inflammatory foci [23,24]. However, we observed a significant reduction of the number of inflammatory foci, as well as of inflammatory density in atorvastatin treated groups of our experimental model. Atorvastatin therapy could play an important role in NASH pathogenesis, since once hepatic steatosis is present, other factors, such as inflammation and oxidative stress, are thought to promote progression to NASH, fibrosis, and necrosis [1]. The role of statins in reduction of inflammation is thought to be due to inhibition of neutrophils chemotaxis and decrease of pro-inflammatory cytokines [25].

Ballooning degeneration is a recognized form of liver cell injury and is thought to be a significant feature of NASH [26]. In fact, the most important diagnostic criterion for distinguishing steatohepatitis from simple steatosis is the presence of hepatocyte ballooning [27]. Hepatocyte ballooning was observed throughout

the whole liver parenchyma in all the experimental groups, except healthy control. Besides, statistically significant decreases (near 50%) were observed between animals fed on HD and those fed on HD with atorvastatin therapy; and between those fed on SD and animals receiving SD and atorvastatin. Eckstedt et al. [23] found a stabilization of hepatocellular damage in patients treated with atorvastatin or simvastatin during 6 years, when comparing with non-treated patients. However, other authors did not observe such differences after atorvastatin treatment [24].

NAFLD activity score specifically includes only features of active injury that are potentially reversible in the short term. The advantages of such kind of scores is that they allow to establish comparisons in natural history studies and therapeutic trials, are simple to apply, and reproducible among observers. Higher differences between groups were found for quantitative NAS than for qualitative NAS, therefore quantitative NAS shows itself as more sensitive to discriminate small differences between groups. In our study, atorvastatin therapy induced a significant reduction of hepatocellular damage, even though in the animals which continuously received a hyperlipidemic diet (group E). The combination of atorvastatin therapy and a standard diet produced the lowest decrease of both qualitative and quantitative NAS. Ekstedt et al. [23] did not find significant differences in NAS, when studying atorvastatin-treated patients. However, Georgescu and Georgescu described a significant reduction of NAS, after a 4-year therapy with atorvastatin [24].

In summary, our results show that atorvastatin therapy did not only decreased plasmatic levels of cholesterol and triglycerides, but also induced a reduction of liver steatosis, inflammation and hepatocellular damage, without increasing plasmatic amynotransferase levels. Furthermore, the combination of atorvastatin and diet produced the lowest decrease on the several parameters studied.

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References

[1] Rector RS, Thyfault JP, Wei Y, Ibdah JA. Non-alcoholic fatty liver disease and the metabolic syndrome: An update. World J Gastroenterol 2008;14:185–92.
[2] George J, Liddle C. Nonalcoholic fatty liver disease: pathogenesis and potential for nuclear receptors as therapeutic targets. Mol Pharmacol 2008;5:49–59.
[3] Schreuder TCMA, Verwer BJ, van Nieukerk CMJ, Mulder CJJ. Nonalcoholic fatty liver disease: An overview of current insights in pathogenesis, diagnosis and treatment. World J Gastroenterol 2008;14:2474–86.
[4] London RM, George J. Pathogenesis of NASH: animal models. Clin Liver Dis 2007;11:55–74.
[5] Cherian G, Holsonbake T, Goeger M, Bildfell R, Dietary. CLA alters yolk and tissue FA composition and hepatic histopathology of laying hens. Lipids 2002;37:751–7.
[6] García-Fuentes E, Gil-Villarino A, Zafra MF, García-Peregrín E. Differential changes in the fatty acid composition of the main lipid classes of chick plasma induced by dietary coconut oil. Comp Biochem Physiol B 2002;133:269–75.
[7] García Pérez B, Ayala I, Castells MT, Sánchez Polo MT, García Partida P, Valdés M. Effects of nifedipine, verapamil and diltiazem on serum biochemical parameters and aortic composition of atherosclerotic chickens. Biomed Pharmacother 2005;59:1–7.
[8] Anfossi G, Massucco P, Bonomo K, Trovati M. Prescription of statins to dyslipidemic patients affected by liver disease: a subtle balance between risks and benefits. Nutr Metab Cardiovasc Dis 2004;14:215–24.
[9] Chitturi S, George J. Hepatotoxicity of commonly used drugs: nonsteroidal anti-inflammatory drugs, antihypertensives, antidiabetic agents, anticonvulsivants, lipid-lowering agents, psychotropic drugs. Semin Liver Dis 2002;22:169–83.
[10] Kostner GM, Molinari E, Pichler P. Evaluation of a new HDL2/HDL3 quantitation method based on precipitation with polyethylene glycol. Clin Chem Acta 1985;148:139–47.
[11] Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467–74.
[12] Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002;346:1221–31.
[13] Kleiner DE, Brunt EM, Van Natta M, et al. Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histologic scoring system for non-alcoholic fatty liver disease. Hepatology 2005;41:1313–21.
[14] Ayala I, Martín Castillo A, Adánez G, Fernández-Rufete A, García Pérez B, Castells MT. Hyperlipidemic chicken as a model of non-alcoholic steatohepatitis. Exp Biol Med 2009;234:10–6.
[15] Cortez-Pinto H, Camilo ME, Baptista A, De Oliveira AG, De Moura MC. Non-alcoholic fatty liver: another feature of the metabolic syndrome? Clin Nutr 1999;18:353–8.
[16] Amagasa H, Okazaki M, Iwai S, Kumai T, Kobayashi S, Oguchi K. Enhancement of the coagulation system in spontaneously hypertensive and hyperlipidemic rats. J Atheroscler Thromb 2005;12:191–7.
[17] Okazaki M, Morio Y, Iwai S, Miyamoto K, Sakamoto H, Imai K, et al. Age-related changes in blood coagulation and fibrinolysis in mice fed on a high-cholesterol diet. Exp Anim 1998;47:237–46.
[18] Ichino K, Okazaki M, Usami S, Oguchi K. Involvement of enhanced coagulation and fibrinolysis system in induction of atherosclerosis in hyperlipidemic rabbits fed on a high cholesterol diet. In Vivo 1997;11:115–23.
[19] Elkin RG, Wood KV, Hagey LR. Biliary bile acid profiles of domestic fowl as determined by high performance liquid chromatography and fast atom bombardment mass spectrometry. Comp Biochem Physiol B Biochem Mol Biol 1990;96:157–61.
[20] Egawa T, Toda K, Nemoto Y, et al. Pitavastain ameliorates severe hepatic steatosis in aromatasedeficient (Ar−/−) mice. Lipids 2003;38:519–23.
[21] Elkin RG, Zhong Y, Donkin SS, Hengstschläger-Ottnad E, Schneider WJ. Effects of atorvastatin on lipid metabolism in normolipidemic and hereditary hyperlipidemic, non-laying hens. Comp Biochem Physiol B Biochem Mol Biol 2006;143:319–29.
[22] Sanguino E, Roglans N, Alegret M, Sánchez RM, Vazquez-Carrera M, Laguna JC. Atorvastatin reverses age-related reduction in rat hepatic PPARα and HNF-4. Br J Pharmacol 2005;145:853–61.
[23] Ekstedt M, Franzén LE, Mathiesen UL, Holmqvist M, bodemar G, Kechaqias s. Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: A histopathological follow-up study. J Hepatol 2007;47:135–41.
[24] Georgescu E, Georgescu M. Therapeutic options in non-alcoholic steatohepatitis (NASH). Are all agents alike? Results of a preliminary study. J Gastrointestin Liver Dis 2007;16:39–46.
[25] Dunzendorfer S, Rothbucher D, Schratzberger P, Reinisch N, Kahler CM, Wiedermann CJ. Mevelonate-dependent inhibition of transendothelial migration and chemotaxis of human peripheral blood neutrophils by pravastatin. Circ Res 1997;81:963–9.
[26] Lee RG. Nonalcoholic steatohepatitis: tightening the morphological screws on a hepatic rambler. Hepatology 1995;21:1742–3.
[27] Hübscher SG. Histological assessment of non-alcoholic fatty liver disease. Histopathology 2006;49:450–65.