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Effects of atorvastatin on progression-regression of renal injury in hyperlipidemic chickens

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Summary. Complex interrelationships exist between hyperlipidemia and the progression of renal injury. The aim of this study was to evaluate the impact of high plasma cholesterol and triglyceride levels on renal structure and the effects of atorvastatin on progression-regression of renal injury. One-hundred chickens were divided into five groups: Group A: Standard diet (SD) for 6 months; Group B: Hyperlipidemic diet (HD) for 6 months; Group C: HD for three months and SD during the next 3 months; Group D: HD for 3 months and SD during the next 3 months, when they received oral atorvastatin (3 mg/kg/d); Group E: HD for the whole 6 months, and atorvastatin (3 mg/kg/d) during the last 3 months. Increased α-actine immunostaining was found in glomeruli of groups B and C. An important decrease of immunostaining was observed in glomeruli of atorvastatin treated groups. Group D showed the lowest value for presence of lipids, and significant differences were found with respect to the rest of the groups. The glomeruli of group B presented the highest damage grades and those of group D showed the lowest grades and presented significant differences from the rest of the groups. The combination of atorvastatin therapy and proper diet proved to be effective in promoting renal disease regression. However, the study of several parameters indicates that neither only diet nor atorvastatin in the progression group resulted completely effective in decreasing the progression of the disease.

Key words: Atorvastatin, Hyperlipidemia, Renal injury, Chicken

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Introduction

Complex interrelationships exist between hyperlipidemia and the progression of renal injury. Several pieces of evidence indicate that hyperlipidemia is a common feature in renal failure, showing an association between hyperlipidemia and degree of glomerular injury (Gröne et al., 1994; Moorhead et al. 1997; Wanner et al., 1997a; Vázquez-Pérez et al., 2001). Furthermore, chronic kidney disease leads to the development of secondary abnormalities in kidney metabolism that contribute to increased cardiovascular morbidity and mortality (Hansson, 2005). Studies in experimental animals and man strongly suggest that many biochemical and histological features that accompany glomerulosclerosis are similar to those observed in the systemic vascular lesions of atherosclerosis (Grond et al., 1986; Avram, 1989). This kind of study may act as the basis of novel treatment strategies to prevent renal disease.

Thus, glomerulosclerosis can be classified as an extension of the atherosclerotic process into the glomerular capillary and is characterized by accumulation of lipid-rich foam-like cells within the mesangium and exaggerated expansion of the mesangial matrix, resulting in disturbances in structural and functional integrity of glomeruli (Kamanna et al., 1998).

Animals have been used as experimental models in atherosclerosis-related research since the turn of this century (Narayanaswamy et al., 2000). Avian models of atherosclerosis helped pioneer the study of vascular biology, and offer economic and technical advantages over mammalian models (Wang et al., 1999). The chicken fed with a hyperlipidemic diet is a good animal model for the study of atherosclerosis (Siller, 1961; Gosling et al., 1969; Valdés, 1976; García Pérez et al., 2003, 2005; Ayala et al., 2005). The chicken is small and suitable for prolonged laboratory investigation, able to develop spontaneous atherosclerosis and capable of producing atherosclerosis after cholesterol feeding with

elevated hypercholesterolemia (Wong, 1975).

There is scarce information on how hyperlipidemia can affect glomerular structure and renal disease, and even less, on the potential renoprotective effect of atorvastatin. Therefore, the aim of this study was to evaluate the impact of high plasma cholesterol and triglyceride levels on renal structure and the effects of atorvastatin on progression-regression of renal injury.

Materials and methods

Animals and diets

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 2 kinds of diet (they received a standard growth diet during the first 3 weeks of their life). Water was given *ad libitum*.

SD (standard diet): A standard growing mash. The weekly amount of this was increased with the age of the animals.

HD (hyperlipidemic diet): A standard growing mash with pure cholesterol (2% of the mixture) and 20% of the mixture of saturated oil (palm oil).

After a three-month induction period, ten 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 three-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 three 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.

Sampling

All animals were sacrificed after 6 months of receiving both diets and/or treatments. Blood samples (1 ml) were extracted after an overnight fasting period from the axillary vein of all chickens. In all cases, plasma samples were taken into 10 mM trisodium citrate-containing tubes. Serum was separated and analyzed for the determination of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, and C-reactive protein (CRP). Total cholesterol, LDL, HDL, and triglycerides were measured using a D-2400 analyzer (Hitachi Ltd., Tokyo, Japan) and commercially available assays from Roche Diagnostics (Manheim, Germany). The method described by Kostner et al. (1985) was used for precipitation of HDL.

Kidneys were removed and cleaned of surrounding tissue. 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.

Histology and immunohistochemistry

Kidney samples were fixed in 10% formaldehyde (in PBS) (0.1M phosphate-buffered saline, pH 7.4) for 10 h and embedded in paraffin; afterwards, 5µ-thick paraffin sections were cut and stained with haematoxylin and eosin (H&E), Periodic Acid-Schiff (PAS) and Masson's Trichrome staining techniques, dehydrated and mounted in Dpex mounting medium (Panreac, Spain). Other slides were used for inmunohistochemistry. Briefly, after being de-paraffinized and re-hydrated, slides were incubated for 30 min with 0.3% H_2O_2 in PBS to block endogenous peroxidase, washed with PBS, and blocked for 30 minutes at room temperature with 1:20 NRS (normal rabbit serum). Then, samples were incubated with mouse anti-α-actine (1:100, Dako, Barcelona, Spain) overnight at 4°C. After washing in PBS, sections were incubated with peroxidase conjugated rabbit anti-mouse Ig for 1h and peroxidase was developed with 3,3-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide. After washing in tap water, sections were counterstained with haematoxylin. In the control, α-actine antibody was substituted for PBS.

For electron microscopy, samples were fixed in 2% glutaraldehyde in PBS for 2h at 4°C and embedded in LR White. Semithin sections were stained with toluidine blue and ultrathin sections were obtained and stained with uranyl acetate and lead citrate. Samples were photographed on the electron microscope Zeiss EM/10cR.

Image analysis

Semiquantitative and image analysis was performed to determine changes in lipid deposits, glomerular damage, immunoreactivity, glomerular basal membrane thickness and renal vascular changes. All histological and immunohistochemical quantifications were carried out by an expert pathologist blinded to diet and/or treatment.

Lipid deposit analysis

One-hundred square fields (134 mm^2) were semiquantitatively evaluated to assign a score. To evaluate the absence and presence of the lipid deposits, two items were used, 0: absence, 1: presence. To analyze

the type of deposits the following scoring was determined, 0: isolated lipid droplets in the cells, 1: large lipid deposits invading several continuous cells, 2: mixed type. Diameter of large lipid deposits were analyzed 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 microscopy (Zeiss Axioskop, Madrid) connected to a video camera (Sony 151-AP) and a control computer. After obtaining a digital image, fat deposits were chosen interactively by a graphic line.

Glomerular damage analysis

In order to assess the degree of glomerular damage, a modification of Boffa et al. (2003) classification was used. Injury scale: 1 means no exaggerated extracellular matrix deposition in glomeruli or 1 to 25%, and 2, 3 and 4 correspond to 26 to 50%, 51 to 75% and 76 to 100% of increased extracellular matrix deposition per glomerulus, respectively. A morphometric analysis of the renal corpuscles was also performed. Glomerular areas were selected by grey level while equivalent diameter of corpuscles was obtained interactively.

Immunohistochemical quantification

All images were captured in one session during which microscope illumination and camera settings were identical. Red-Green-Blue-filtered grey scale values from images were analyzed using MIP 4.5 software by an operator who was blinded to the experimental group. Green channel was used to the grey level analysis because it gave maximum contrast. The digital image consists of a 512x512 matrix of pixels, where each pixel consisted of a number between 0 (black) and 255 (white) representing the intensity of transmitted light or grey level at a point. Grey level was related with α-actine content (darkest corresponded to highest α-actine content). Analysis was performed in the inverted (negative) image in order to have the highest values corresponding to highest α-actine content. Regions containing α-actine in the glomeruli were selected and area and medium grey level in the negative image were measured. Reference corpuscle area was also measured. Relative α-actine content per corpuscle was estimated using the following equation: α-actine content = α-actine area x average α-actine grey level/corpuscle area.

Glomerular basal membrane thickness analysis

Digitized electron microscopy images were analyzed with image analysis software MIP4.5. The thickness of glomerular basal membrane was measured interactively.

Renal vascular analysis

Digitized images of intralobular arteries and renal arterioles were analyzed with image analysis software MIP 4.5. The following parameters were determined: external and internal diameter of vessel, wall/lumen ratio and wall thickness.

Statistical analysis

Results are expressed as mean ± standard error. Mann-Whitney non parametric test was used for semiquantitative analysis while statistical significance for quantitative analysis was evaluated by ANOVA or Welch and the corresponding post-hoc test. Statistics were performed using SPSS v 11. A P-value <0.05 was considered as statistically significant.

Results

Effects of hyperlipidemia on circulating lipid levels (Table 1)

Chickens fed an HD (group B) showed significantly higher plasma levels of cholesterol than animals fed on SD (group A). Atorvastatin treatment attenuated this increase in plasma cholesterol (groups D and E); lower values were also observed in the SD regression group C. Animals fed on SD (group A) had comparatively lower levels of CRP ($p < 0.05$) than those fed on the HD. No HD diet and/or atorvastatin treatment significantly decreased these parameters, but group D showed the lowest values.

Light microscopy (Fig. 1)

Light microscopy evaluation showed the following histological characteristics:

Group A (healthy control): neither fat accumulation,

Table 1. Values of the main lipids and CRP measured in serum from animals of all different experimental groups (mean ± standard error).

Experimental group	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	CRP (REU/ml)
Group A	104.4±5.5***	51.7±18.8***	67.9±6.1***	26.1±2.6***	1.07±0.29***
Group B	980.3±141.3	351.8±18.0	253.4±32.5	656.5±112.6	2.75±0.26
Group C	204.2±40.8***	253.4±90.9***	95.4±20.3***	85.5±27.9***	2.55±0.91
Group D	197.0±74.3***	31.6±7.2***	88.5±19.3***	77.6±26.4***	2.00±0.38
Group E	413.8±93.6	356.9±145.6	99.4±22.1	242.9±79.3	2.01±0.33

Statistical analysis was performed vs HD-fed animals (group B). *: P<0.05; ***: P<0.001. HDL: high-density lipoprotein; LDL: low-density lipoprotein; CRP: C reactive protein; REU: relative ELISA units.

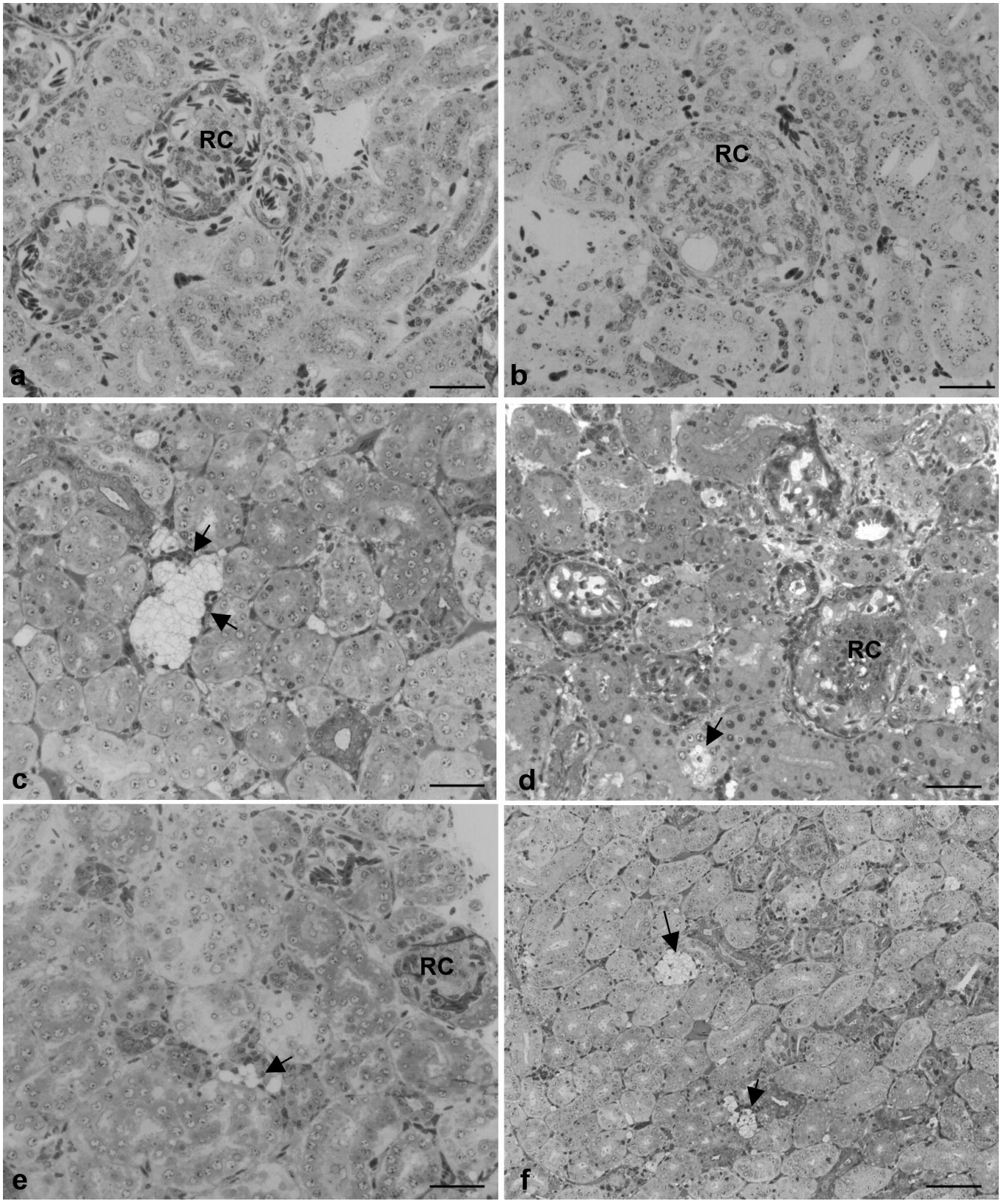

Fig. 1. Semithin sections. Toluidine blue staining; **a.** Group A, healthy control sample. Normal morphology is observed. **b-c.** Group B, hyperlipidemic control sample. A high level of glomerulosclerosis was detected in the renal corpuscles. Lipid deposits invaded completely several adjacent cells. **d.** Group C, spontaneous regression sample. Different levels of glomerulosclerosis are observed in corpuscles. **e.** Group D, pharmacological regression sample. Note the small size of lipid deposits. **f.** Group E, progression sample. A moderate size of lipid deposits is observed. RC: renal corpuscles, arrows: lipid deposits. Bars: a-e, 30 µm; f, 60 µm

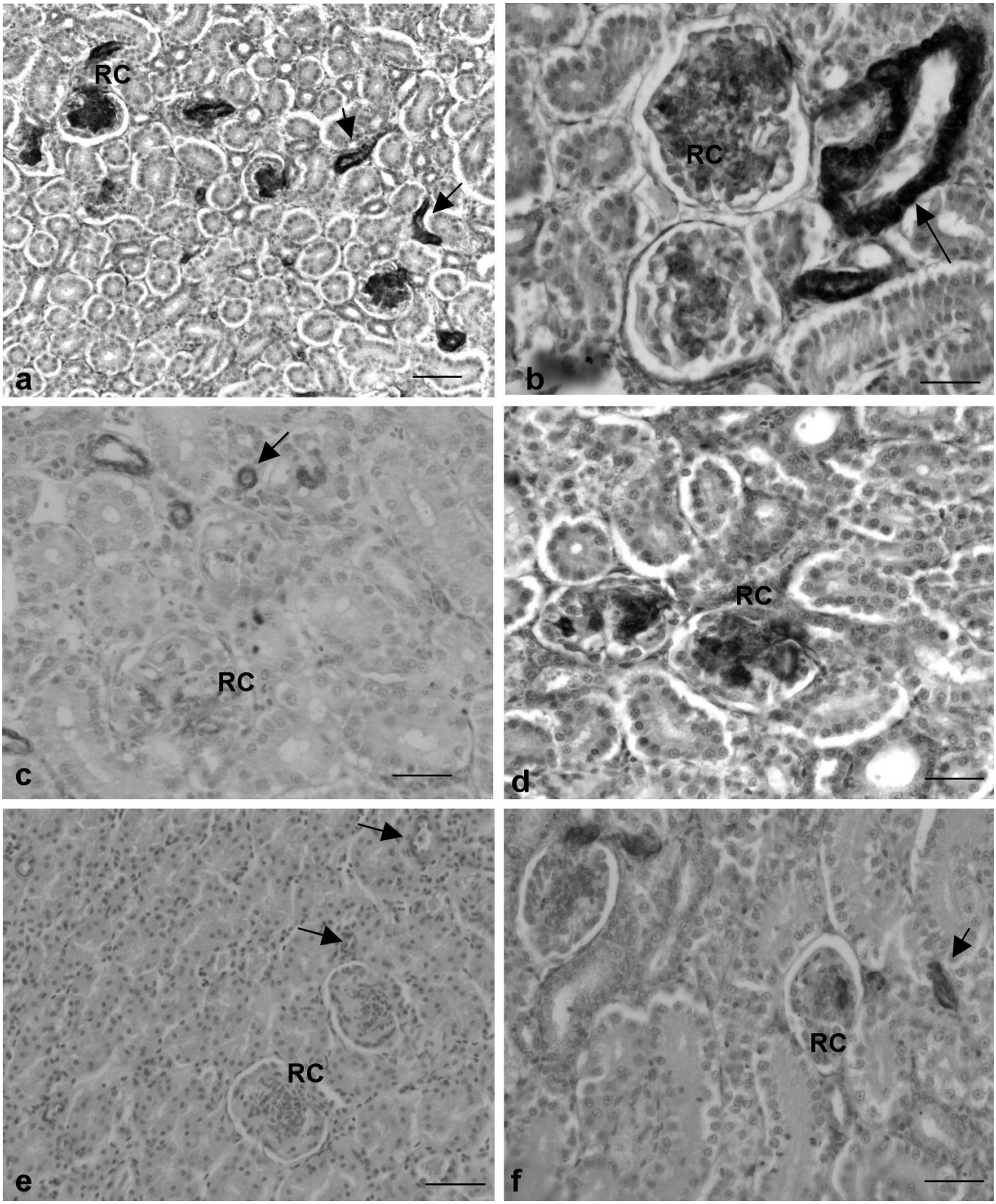

Fig. 2. α-actine immunorreactivity was observed in glomeruli and vessels. **a-b.** Group B, hyperlipidemic control sample. Note a strong reactivity in glomeruli and vascular smooth muscle cells. **c.** Group A sample. Slight reactivity is observed. **d.** Group C, spontaneous regression sample, a strong α-actine immunoexpression is detected. **e.** Group D, pharmacological regression sample. Slight reactivity is showed. **f.** Group E, progression sample. Moderate reactivity is observed. RC: renal corpuscle, arrows: vessels. Bars: a, 50 µm; b-f, 30 µm

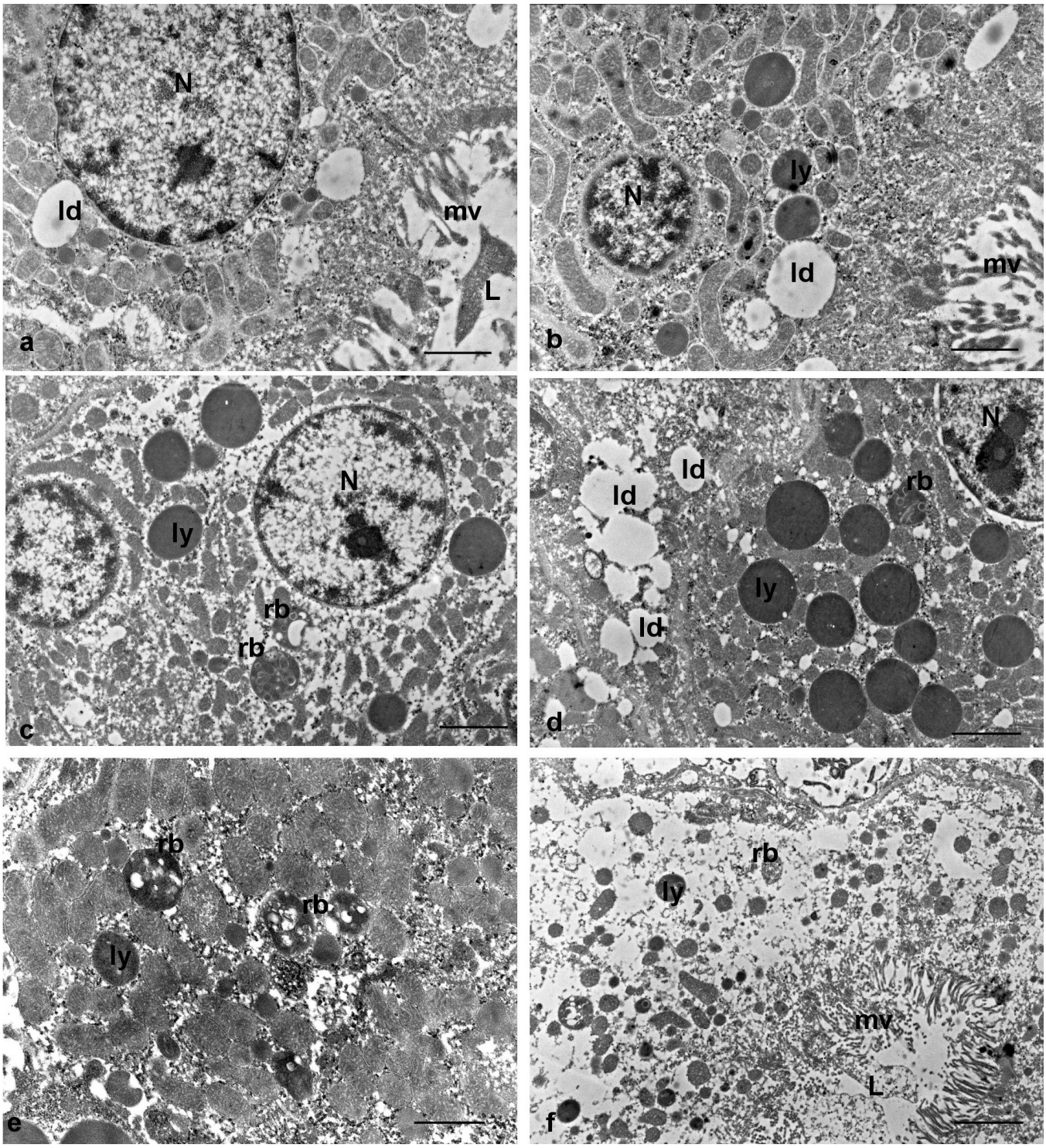

Fig. 3. Proximal tubular cells. **a-b.** Group A, healthy control. Normal morphology. Scarce lipid droplets and lysosomes. **c-f.** Group B, hyperlipidemic sample. Note the increased number of lysosomes and residual bodies in degenerative cells. N: nucleus, ld: lipid droplets, ly: lysosomes, rb: residual bodies, mv: microvilli, L: lumen. Bars: a, b, 1.25 µm; c, d, 1.58 µm; e, 1 µm; f, 2.5 µm

nor inflammatory infiltration or significant extracellular matrix proliferation were observed. Furthermore, no hialinosis or fibrosis was seen.

Group B (hyperlipidemic control): numerous fat deposits were observed, commonly occupying the whole cytoplasm, in several adjacent interstitial cells. They appeared like swollen cells and constituted great fat accumulations in the renal parenchyma. In some sections, inflammatory foci, usually diffuse, were seen, which surrounded the fat accumulations. Interstitial fibrosis was not observed Thickening of mesangial matrix was observed in most sections, and the capillary lumen was sometimes occluded.

Group C (spontaneous regression group): numerous fat accumulations were observed, but in lower number and smaller size than those of group B. Inflammatory foci were not significant and interstitial fibrosis was not observed. Glomeruli showed an increased mesangial matrix, but lower than group B.

Group D (pharmacological regression group): fat accumulations were scarcely observed, in a similar way to the description of group A. Inflammatory foci were not observed. Interstitial fibrosis was not significant. Mesangial matrix had an expanded appearance.

Group E (progression group): great fat accumulations and small lipid droplets were found. Small inflammatory

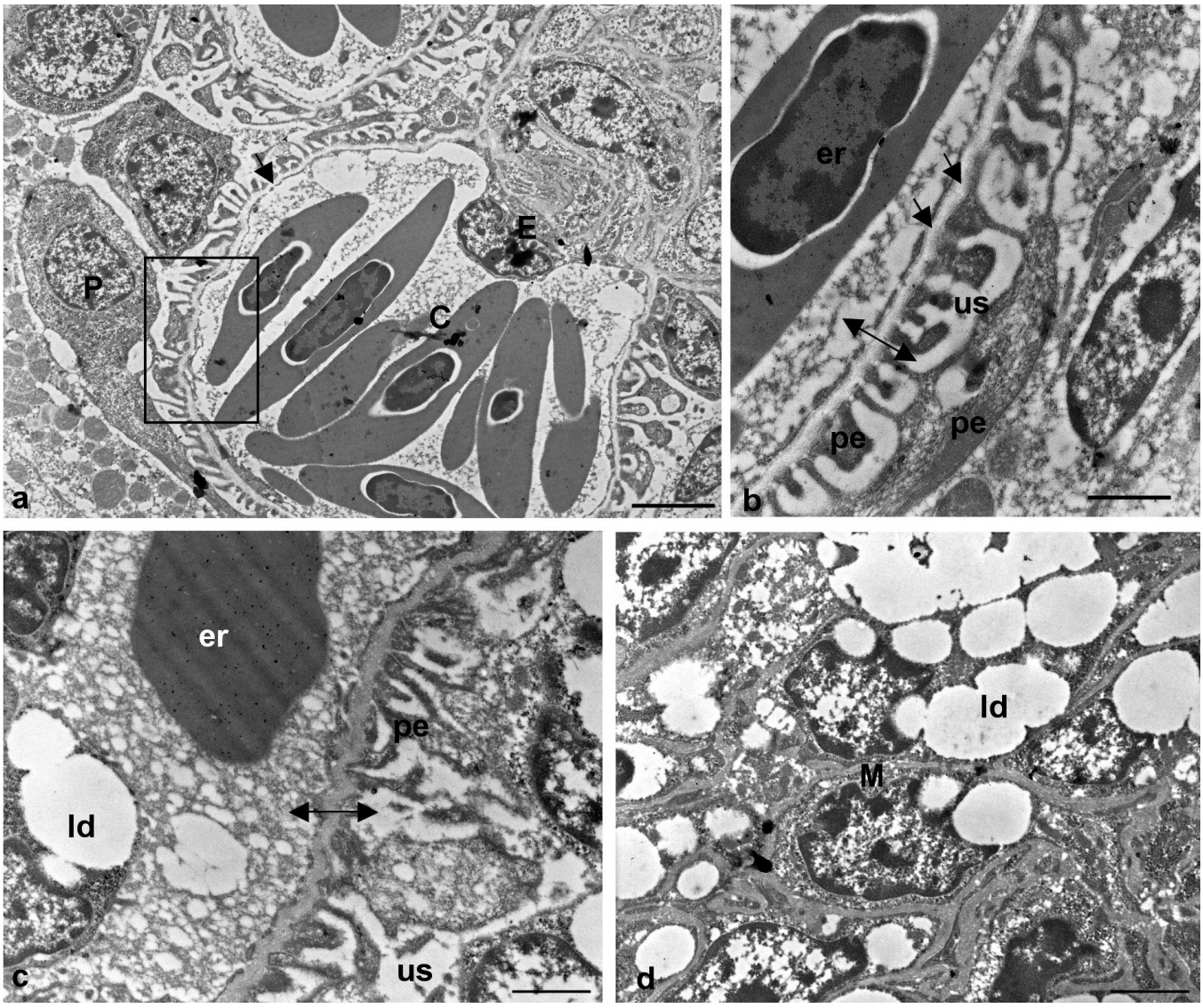

Fig. 4. Renal corpuscle. **a-b.** Group A, healthy control. A normal ultrastructural morphology is showed. **c-d.** Group B, hyperlipidemic control. Note the high content of lipid droplets and the thickness of basement membrane. Neither denudation of basement membrane nor podocyte injury are observed. C: capillary, P: podocyte, E: endothelial cell. Er: eritrocyte, us: urinary space, pe: pedicels, ld: lipid droplets, M: mesangial cell. Bars: a, 3.2 µm; b, c, 1 µm; d, 1.58 µm

foci were also observed and interstitial fibrosis was not significant. Mesangial proliferation was seen in glomeruli.

In the renal parenchyma, no interstitial fibrosis was observed in any experimental group. Scarce inflammatory foci were found in group B and E.

Immunohistochemical analysis

Immunoexpression of α-actine was observed in glomeruli and media layer of vessels in each experimental group. Increased α-actine immunostaining was found in glomeruli of groups B and C. An important decrease of immunostaining was observed in glomeruli of atorvastatin treated groups (Fig. 2).

Electron microscopy

A normal ultrastructure was observed in groups A and D. Increased deposits of lipids were observed in groups B and E. Lipid droplets were found in the cytoplasm of proximal and distal tubules cells. An increased number of lysosomes and residual structures, as a result of lipid metabolism, were also found in these tubules. Cellular degenerative processes were occasionally observed, due to the high level of lipids (Fig. 3). Mesangial cells with lipid droplets were found in the same groups, B and E, but they were absent in group A. The described changes in group B were greater than in group E. Glomeruli of these groups presented an increased thickness of basement membrane (0.34±0.01 vs 0.19±0.00 in group A, mean±standard error, mm), and a greater size of mesangial matrix deposit. These features were predominant in group B, although neither basement membrane denudation nor podocyte injury was observed (Fig. 4).

Image analysis

By means of quantitative or semiquantitative methods we measured several parameters related to renal damage: lipids, glomerular damage, immunohistochemistry and renal vascular changes.

Lipid analysis (Table 2)

A semiquantitative analysis of absence/presence of lipids showed that no lipid accumulation was observed in group A, while an important presence of lipids was seen in kidneys of groups B, C and E, without significant differences among them. Group D showed the lowest value for presence of lipids, and significant differences were found with respect to the rest of groups.

The percentage of each type of lipid deposit was determined. 100% of group B samples was classified as mixed type, which was significantly different from the rest of the groups. In group C, the percentages of small and large lipid deposits were very similar; in group D, the highest percentage was that of small lipid deposits. In group E, large lipid deposits were predominantly

Table 2. Renal lipid analysis.

Experimental group	Absence/presence of lipids[1]	Small lipid deposits (%)	Large lipid deposits (%)	Mixed type (%)	Diameter of large lipid deposits (μm)
Group A	00±0.00*	---	---	---	---
Group B	0.72±0.04	---	---	100%	37.7±1.4
Group C	0.75±0.06	37.5	35	27.5%	17.7±1.2
Group D	0.39±0.06*	55.1	27.5	17.2%	12.1±0.7
Group E	0.66±0.07	26.6	60	13.3%	17.9±0.9

[1]: Values expressed as mean ± standard error. *: P<0.05. Significant differences with the rest of groups.

Table 3. Grade of glomerular damage, percentages of each grade of lesion, glomerular area and α-actin immunoexpression.

Experimental group	Grade of glomerular damage[1]	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	Glomerular area (mm^2)	α-actin content (%)
Group A	1.77±0.08	43.4	36.3	18.1	2.0	4541.6±240.2	12±1*
Group B	3.58±0.06*	0.0	7.0	28.2	64.6	5957.1±334.9	31±5
Group C	2.70±0.07	3.0	39.3	42.4	15.1	4451.0±306.1	26±2
Group D	2.03± 0.08	27.2	47.4	20.2	5.0	4392.6±267.2	16±4*
Group E	2.52±0.09	17.1	31.3	37.3	14.1	5028.3±62.0	3±6*

[1]: Grade of glomerular damage on the basis of modified Boffa et al. (2003) classification (1-4). Values expressed as mean ± standard error. *: P<0.05. Significant differences with the rest of groups.

observed. The largest lipid deposits were found in group B while the smallest were in group D.

Glomerular damage (Table 3)

The semiquantitative analysis showed that the mean value of healthy control animals glomeruli was between 1-2 grades. Glomeruli of group B presented the highest damage grades (3-4). The mean value of group C samples was between 2-3, while most glomeruli in group D were grade 2. Those in group E showed a grade 2-3. The most important and significant lesions were found in group B, with respect to the rest of groups. No significant differences were observed between groups A and D which presented the lowest grade of glomerular lesion and presented significant differences from the rest of the groups.

After morphometric analysis of corpuscles, no differences were found in the diameter in the different experimental groups. The mean diameter was 111mm. Glomerular area values of group B were significantly higher than those of groups A, C and D, but no significant differences were found between values of group B and E for this parameter.

Immunohistochemical analysis

Significant changes in the immunoexpression of α-actin, indicator of mesangial cell activation, were found in glomeruli, both for increases of area and grey colour intensity.

The α-actin content in group A showed significant differences from the rest of the groups. No significant differences were found between groups B and C. The lowest α-actin content was found in group D, which showed significant differences from the rest of the groups, except for group E.

Vascular system analysis (Table 4)

Both renal arteriolae and intralobular arteries were analysed separately. Internal and external diameters, wall thickness and wall/lumen ratio were measured, in order to detect potential vessel changes.

External diameter of intralobular arteries was found to be significantly higher in groups B and C than those of groups A and D, while no statistically significant differences were observed for the lumen diameter between the different groups.

Wall thickness of intralobular arteries was found to be significantly lower in groups A and D than those of groups B, C and E. No significant differences were found between A and D, or between B, C and E. For the wall/lumen ratio no significant differences were observed between groups A and D; however, samples of group A showed significant differences from the rest of the groups.

External diameter of renal arterioles was significantly lower in group A than in groups B and E; samples of group B showed significant differences from groups A, D and E.

The lumen diameter showed no significant differences between the studied groups. Analysis of the wall thickness of arteriolae in groups A and D showed significant differences from the rest of the groups. Similar results were observed for wall/lumen ratio.

Table 4. Analysis of intralobular arteries: external and lumen diameters, wall thickness and wall/lumen ratio (values expressed as mean ± standard error).

Experimental groups	External diameter (mm)	Lumen diameter (mm)	Wall thickness (mm)	Wall / lumen ratio
Group A	28.37±1.07	19.29±1.04	9.08±0.51	0.52±0.05
Group B	35.61±1.33	20.38±0.93	15.23±0.69	0.78±0.03
Group C	36.66±1.30	20.90±0.98	15.76±0.67	0.78±0.04
Group D	28.50±1.25	17.50±0.85	11.00±0.73	0.68±0.07
Group E	32.00±1.62	17.44±0.43	14.55±0.83	0.85±0.04

Table 5. Analysis of renal arteriolae: external and lumen diameters, wall thickness and wall/lumen ratio (values expressed as mean ± standard error).

Experimental groups	External diameter (mm)	Lumen diameter (mm)	Wall thickness (mm)	Wall / lumen ratio
Group A	11.59±0.46	5.82±0.26	5.55±0.40	0.97±0.09*
Group B	18.35±1.04	6.06±0.43	11.84±0.55	2.09±0.13
Group C	15.00±1.12	5.07±0.43	9.85±0.68	1.68±0.13
Group D	11.92±0.74	6.72±0.58	4.51±0.31	0.04±0.14*
Group E	15.71±0.80	5.56±0.35	9.72±0.44	1.82±0.11

* $P<0.05$. Significant differences with the rest of groups.

Discussion

The results show that diet-induced hypercholesterolemia and hypertriglyceridemia is associated with renal damage, supporting the potential role of lipids in renal injury.

The chicken experimental model has proved itself useful for this drug intervention study. One of the advantages of using chickens versus other species is short atherosclerosis regression time (Ayala et al., 2005); besides, paradoxical observations raise an important issue relating to interpretation of the results of drug intervention studies in genetically derived mouse models (Bocan, 1998). For example, the impairment of triglycerides transport through the secretory pathway of hepatocytes in apolipoprotein E deficient mice has been described (Mensenkamp et al., 2004).

Semiquantitative lipid analysis showed the beneficial effect of combination of diet and atorvastatin treatment (group D) on the decrease of lipid content in renal parenchyma. Neither only diet (group C) nor atorvastatin in progression group (E) induced significant decreases of this parameter. Differences found between groups for the type of lipid deposit could be due to the degree of renal lipid infiltration. In this way, an early stage of the disease induces the formation of small lipid deposits in isolated cells; a further degree of disease gives rise to larger lipid accumulations, which occupy a great portion of cytoplasm, in several adjacent interstitial cells. The effect of SD and atorvastatin therapy (group D) induces a decrease of the size and quantity of the lipid deposits, and also causes a greater dispersion of them in isolated cells.

CRP is commonly used as a useful indicator of systemic inflammation, but also in cardiovascular disease. We did not find a clear correlation between CRP values and histological analysis, since inflammatory foci were scarcely observed, mainly in group B. It could be due to the effect of diet and/or atorvastatin, since only group B presented significant inflammatory foci. Furthermore, no interstitial fibrosis was observed by trichromic Masson staining histological analysis. It must be taken into account that a moderate level of disease was developed in our experimental model, and a longer duration of the experiment could have led to a great number of animals discharged from the study. Other diet-induced experimental models have developed fibrosis, and simvastatin reduced both inflammation and fibrosis in pigs (Wilson et al., 2003) and rats (Vieira et al., 2005). Besides, extensive foam cell plaque formation was observed in the aortae of chickens maintained on a prolonged atherogenic diet, and cessation of cholesterol feeding was followed by fibrotic changes in several studies (Ayala et al., 2005). Probably, fibrosis of renal tissues may be observed later in chickens than in pigs or rats, and the induction method determines the degree of renal damage.

The combined effect of diet and atorvastatin induced a significant regression of glomerular damage, since we did not find statistically significant differences between groups A (healthy control) and D (atorvastatin regression). Accumulation of plasma components, such as macrophages and low-density lipoprotein (LDL), as well as production of cytokine and reactive oxygen species could be some of the mechanisms underlying glomerular injury (Kim et al., 1995; Rohrmoser and Mayer, 1996; Wanner et al., 1997b).

Atorvastatin not only induced regression of glomerular damage, but also decreased progression of mesangial proliferation. Our data are in agreement with experimental and clinical studies showing that statins reduce the severity of glomerular injury (Rubin et al., 1994; Wheeler, 1998; Oda and Keane, 1999; Vázquez-Pérez et al., 2001), but also highlight the potential role of statins for achieving renal disease regression. The mechanisms underlying this beneficial effect could involve inhibition of monocyte infiltration, LDL oxidation, extracellular matrix accumulation and mesangial cell proliferation (Kim et al., 1995; Wheeler, 1998; Oda and Keane, 1999).

Yoshida et al. (1989) and Adamczack et al. (2003) observed a positive correlation between glomerulosclerosis degree and glomerular volume in the early stages of glomeruloesclerosis. In later stages, glomerular volume decreases as a consequence of fibrosis and sclerosis. By image analysis we found significantly higher glomerular areas in group B, than those of the rest of groups, except for group E. This finding suggests that our study developed an early stage of injury, where no interstitial fibrosis was found. Therefore, a beneficial effect could be attributed to the diet (SD vs HD), which only by itself (group C) induces a decrease in glomerular hypertrophy. Atorvastatin increases this effect. Probably, a longer period of atorvastatin therapy could induce a significant decrease of glomerular volume in the progression group (E). These results are similar to those reported by Vázquez-Pérez et al. (2001), who found a preventive effect of atorvastin on glomerular hypertrophy. Furthermore, Maddox et al. (2002) described in obese Zucker rats, that dietary food restriction prevents, and potentially reverses, glomerular hypertrophy in the early stages of disease. Our results differ with those of Vázquez-Pérez et al. (2001), since they found that atorvastatin reduces but does not normalize, glomerular hypertrophy. In fact, we found similar values of glomerular areas for groups A and D. Thus, in our study, atorvastatin normalizes glomerular hypertrophy. Adamczack et al. (2003) demonstrated the reversibility of glomerular damage, whenever no basement membrane denudation exists. We also found regression of renal damage in group D, and no denudation of basement membrane was observed in the most affected group (B). Ultrastructural analysis also showed a normal morphology of podocytes.

Immunoexpression of α-actin was determined in the present study, as an indicator of mesangial cell activation. Changes in immunohistochemical parameters were associated with mesangial cell hypertrophy. Joles et

al. (2000) only found minimum increases of α-actin immunoexpression, and concluded that renal damage induced by hyperlipidemia affected podocytes to a major extent, and therefore affected mesangial proliferation only to a lesser extent. We did not find this kind of change in the podocytes. Furthermore, the hyperlipidemic group (B) showed the highest α-actin immunoreactivity, which was found to be significantly different to the rest of the groups. Joles et al. (2000) reported that both hypercholesterolemia and hypertrigliceridemia aggravate renal injury primarily via podocyte rather than via mesangial cell damage. Probably, nephrectomy accelerated degenerative mechanisms and therefore produced direct ultrastructural changes in podocytes, without time to develop mesangial proliferation. In our experiment, developed in a 6-month period, probably pathological changes were more progressive, and gave rise to lipid accumulation and mesangial proliferation. A longer period was not advisable because of high mortality of the chickens at later stages of disease. The lowest a-actin content was found in group D, which showed significant differences from the rest of groups, except for group E. Therefore, atorvastatin therapy effectively inhibited mesangial proliferation; thus, we could conclude that atorvastatin accelerated regression and decreased progression of mesangial proliferation.

Zager et al. (2001) found increases of lipid deposits in proximal tubules of obese rats. In our study, lipid deposits were present in the cytoplasm of proximal and distal tubules of most affected experimental groups. Moreover, a high metabolic activity was observed in tubular cells of groups B and E, producing an increase of lysosomes and residual structures in cytoplasm.

Vascular system analysis showed that no significant differences for external diameter of intralobular arteries existed between groups A (healthy animals) and D (atorvastatin regression group) and E (atorvastatin progression group), both treated with atorvastatin. Therefore, atorvastatin therapy has shown vascular benefits related to media layer growing and reversal of vascular damage. We did not find significant differences for the lumen diameter between the different groups, perhaps because vascular remodeling was at initial stages. Analysis of wall thickness of intralobular arteries showed similar results to external diameter. No significant differences were found between groups A and D, showing the benefit of combining atorvastatin therapy and a proper diet to increase regression of disease. However, the lack of significant differences between groups B and E indicate the ineffectiveness of atorvastatin to decrease progression of the disease. Vascular remodelling implies structural changes due to hyperplasia, hypertrophy and extracellular matrix alteration; other implied mechanisms are altered smooth muscle cell growth, increase of adhesive molecules, and inflammation (Heagerty, 1993).

Although Dominguez et al. (2000) only analysed diet-induced nephropathy, without a drug intervention study as in our experiment, our results are in line with them, who found that a hyperlipidemic diet induced an increase of wall thickness in renal arteries and of external diameter, without affecting lumen area, thus, indicating an initial stage of atherosclerosis. In fact, Glagov et al. (1987) found that lumen stenosis may be delayed until the atherosclerotic lesion occupies a certain percentage of the internal elastic lamina area. In the final stages of the disease the lumen area diminishes markedly.

In the same way as intralobular arteries, lumen diameter of renal arteriolae did not show significant changes between groups, perhaps because vascular remodeling was at initial stages. Atorvastatin seems to be effective in increasing regression, because no significant differences were found between groups A and D. Moreover, atorvastatin also seems to decrease progression, because significant differences were observed between groups E (lower values) and B.

In summary, all these data suggest that hyperlipidemia in chickens is associated with renal damage, and induces glomerulosclerosis, glomerular hypertrophy, mesangial proliferation, changes in α-actin immunoexpression, and vascular remodeling. A combination of atorvastatin therapy and proper diet proved to be effective in promoting renal disease regression. However, the study of several parameters indicates that neither only diet nor atorvastatin in the progression group resulted completely effective to decrease progression of disease. Additional long-lasting studies will be needed to confirm the role of atorvastatin in decreasing progression of renal disease.

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