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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.