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Human-equivalent Dose of Atorvastatin Can Reduce Non-alcoholic Fatty Liver Disease in Two Mice Models with No Impact on Endoplasmic Reticulum Stress Proteins

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Abstract

In the last decade, non-alcoholic fatty liver disease (NAFLD) has become the most frequent liver disease, surpassing viral diseases, which were the main causes of fibrosis and cirrhosis. There is no treatment for the more advanced NAFLD forms, and it is considered the world's second leading cause of liver transplant. Risk factors for the development and progression of NAFLD include being overweight or obese, type 2 diabetes and insulin resistance. NAFLD has a spectrum ranging from simple steatosis to steatohepatitis (NASH) and cirrhosis, and approximately 2% may progress to hepatocellular carcinoma. About 25% of people with hepatic steatosis progress to NASH; however, a proportional increase in associated diseases is expected due to the increasing rates of obesity among the population, including children. Notably, an inadequate/persistent endoplasmic reticulum (ER) stress due to alterations in the cellular redox state or excessive protein synthesis is a process that appears to be central to NAFLD progression. Thus, the present study evaluated the impact of atorvastatin (AT) administration on high-fat diet-induced NAFLD and NASH in wild-type and ApoE -/- mice and on the hepatic ER response. The administration of a human equivalent dose of AT improved liver fat content and AKT serine phosphorylation with no changes in ER stress-related protein expression (ATF4, BIP, IRE1a, and CHOP). Thus, statin therapy may reduce NAFLD and NASH in mice, but this effect is unrelated to attenuated ER stress.

Keywords: Atorvastatin; Steatohepatitis; Hepatic steatosis; Endoplasmic reticulum stress

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a disorder generally associated with obesity characterized by fat infiltration (steatosis) into the liver, accumulating to greater than 5%, without the presence of secondary factors such as alcohol consumption [1]. In developed countries, such as the United States, there is a prevalence of more than 30% of the population with NAFLD [2]. This high percentage of people affected by this pathology is linked to its frequent association with obesity, with 57-98% of these individuals having NAFLD [3].

The progression of NAFLD to non-alcoholic steatohepatitis (NASH) is associated with inflammation, apoptosis and fibrosis [4], increasing the risk for developing liver carcinoma and the number of liver transplant candidates [5]. In general, NASH is the hepatic expression of the metabolic syndrome associated with diseases such as type 2 diabetes mellitus, dyslipidemia and insulin resistance.

The complete installation and/or worsening of NAFLD is associated with a malfunction of the endoplasmic reticulum (ER) [6]. In the liver, the ER has a great capacity to adapt to intra and extracellular events. Drugs/medications, inflammation, obesity, hyperglycemia, and other conditions and disorders can attenuate this organelle's adaptability leading to hepatic lipid metabolism alterations [6]. This change in liver homeostasis triggers what is known as ER stress (ERS).

A relationship between ERS and obesity has been reported in mice submitted to a high-fat diet (HFD) [7], and similar observations were observed in humans diagnosed with NASH [8]. This relationship was further confirmed with the overexpression of the GRP78/BiP chaperone in Ob/Ob mice, which reduces ERS and improves insulin sensitivity and steatosis parameters [9].

Statins act directly on the HMG-CoA reductase enzyme, reducing its action through competitive inhibition. These compounds decrease plasma LDL-cholesterol concentrations, minimizing the risks of cardiovascular diseases due to hypercholesterolemia and hypertriglyceridemia. Statin therapy is considered safe with few adverse events [10]. Thus, since dyslipidemia-associated cardiovascular diseases are a major cause of death in people with NAFLD, statin therapy seems to be a viable strategy [10]. The indication of statins for NAFLD/NASH is not established, but their lipid-lowering action could attenuate lipid dysregulation presented by many patients with these conditions. In the present study, we administered atorvastatin (AT) at human equivalent doses and monitored the effects on disease progression and ERS in two fatty liver animal models.

Material and Methods

Animals and study design

Male Wild Type (WT) and Apolipoprotein E knockout (ApoE -/-) C57BL/6 mice were obtained from the animal facility at the University of São Paulo Medical School and maintained in a temperature-controlled room at 25 ± 2 ºC. The animals had free access to food and water. There were five animals in each experimental group. The control group was fed regular rodent chow containing 19% protein, 56% carbohydrate, 3.5% lipids, 5% cellulose and 4.5% vitamins and minerals, providing 3.2 kcal / g (Nuvilab - CR-1, Colombo, Paraná, Brazil). The second group was fed a pelletized high-fat diet (HFD) chow, containing 15.8% protein, 27% carbohydrate and 57.2% lipids and vitamins and minerals, providing 5.5 kcal/g (PragSoluções Biosciences, Jau, SP, Brazil), for 12 consecutive weeks to induce steatosis (i.e., the NAFLD model). The third group was fed a pelletized western diet (WD) containing 17% protein, 43% carbohydrate, 40% lipids, 0.15% cholesterol and vitamins and minerals, providing 4.6 kcal / g (Research diet, New Brunswick, New Jersey, USA) for seven consecutive weeks to induce steatohepatitis (i.e., NASH model) [11]. All procedures with animals were previously approved by the Ethics Committee on the use of animals at the USP-ICB (Protocol: No. 9621291117).

Treatment

Dose-response experiments were performed with AT in animals with high-fat diet-induced NAFLD. Three doses (5, 10 and 15 mg/kg/day) of the drug were administered in a single daily dose administered orally via gavage for 15 days. This treatment period is equivalent to 1.5 years of treatment in humans [12]. The 5 and 15 mg/kg AT doses in mice are equivalent to the minimum and maximum (10 and 80 mg/kg/day, respectively) indicated doses for humans, based on the formula by Reagan-Shaw et al. [13].

Evaluation of body mass and fat

Total body mass, lean mass and fat were analyzed using an LF50 minispec (Bruker, Boston, MA, USA).

Glucose tolerance test (GTT)

The tests were performed in the afternoon after fasting for 6 hours [14,15]. Animals received a 1 mg kg-1 body weight dose of glucose via intraperitoneal injection. Blood samples were collected from the tail vein and applied to reactive strips (Roche, São Paulo, Brazil). Blood glucose concentrations were measured 0, 15, 30, 60, 90 and 120 minutes after glucose delivery using an Accu-chek Active apparatus (Roche, Mannheim, Germany).

Liver enzymes

After euthanasia, the animal's blood was collected and centrifuged (2000 rpm, 15 minutes) to separate the plasma. Then, the LabTest kit (Lagoa Santa, Minas Gerais, Brazil) was used to analyze hepatic pyruvic transaminase (ALT) and oxaloacetic transaminase (AST) enzyme activity.

Liver histology

Hematoxylin & Eosin and Oil Red O staining

For hematoxylin & eosin staining, the liver was fixed in a 10% formaldehyde solution for 8 hours. Subsequently, the fixed samples were kept in 70% alcohol overnight. The samples were then dehydrated in baths of 95% alcohol, 100% alcohol and xylene. After dehydration, the tissue samples were embedded in paraffin at 60ºC. A microtome (Zeiss, Jena, Germany) cut the samples into six-micron slices. Then the slices were stained with hematoxylin and eosin.

For oil red O staining, liver samples were embedded in tissue-tek (Thermo Scientific, MA, USA), placed in isopropanol alcohol and frozen in liquid nitrogen. Twelve-micron slices were prepared using a cryostat (Micron H560, Thermo). Three slices from different parts of the samples were positioned on each slide. The slides were stained with ORO and Mayer's hematoxylin. Twenty images from each animal were obtained using a microscope with 20× objective magnification. The ORO-stained area was identified using the ImageJ program (National Institutes of Health, MD, USA).

Immunoblotting

After the animals were anesthetized and the loss of corneal reflexes assessed, the abdominal wall was opened to visualize the liver and portal vein. Some mice received a bolus saline infusion through the portal vein with or without 1U Humulin R insulin (Eli Lilly of Brazil, SP, Brazil) to evaluate the intracellular insulin action. Thirty seconds later, the liver was removed and homogenized in lysis buffer (100 mM Tris, 10% SDS, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, pH 7.4). The tissue extracts were centrifuged at 15,294 × g, at 4°C, for 40 minutes. The protein content of the supernatants was measured by the Bradford method. Aliquots of the supernatant, containing 50 µg total protein, were treated with Laemmli buffer supplemented with 200 mM dithiothreitol, loaded onto 8% and 10% polyacrylamide gels, and subjected to SDS-PAGE. The proteins were then transferred from the gels to nitrocellulose membranes using a Bio-Rad mini trans blot Apparatus (Hercules, CA, USA). The membranes were incubated in TBST-B blocking buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, and 5% skimmed milk) for two hours at room temperature to prevent any nonspecific binding to the membrane. The nitrocellulose membranes were incubated with the specific primary antibodies overnight at 4°C and subsequently incubated with a secondary antibody conjugated to horseradish peroxidase for one hour at room temperature. The immunoblots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo). The immunoblots were visualized using an Amersham TM Imager 600 and quantified using the ImageJ software.

The primary antibodies used were pSer473AKT (catalog number 7985-R) from Santa Cruz (Dallas, TX, USA) and CHOP (catalog number 2895), ATF4 (catalog number 11815), BIP (catalog number 3183), IRE1α (catalog number 3294), β-actin (catalog number 4970) all from Cell Signaling

Technology (Danvers, MA, USA). Protein loading into SDS-PAGE was normalized by ponceau staining or β-actin intensity.

Statistical analysis

The results were analyzed using the GraphPad Prism version 9.0.0 (121) program (GraphPad Software, La Jolla, CA, USA). The minimum sample size per group for each parameter analyzed was defined by an n sufficient to analyze samples' distribution through the D'Agostino and Pearson omnibus normality test recommended using the same software. All samples were evaluated for normal distribution and subjected to a one-way or two-way ANOVA ($p<0.05$) followed by the post-hoc Tukey's multiple comparisons tests. The results are expressed as the mean ± standard deviation (mean ± SD) using scattered dot plots (each sample corresponds to one mouse). We considered the three "Rs" for the usage of animals in experimental science. Reduction was achieved by choosing only three doses at baseline to determine the optimal dose for follow-up studies, and some analyses were performed using liver fragments from the same animals.

Results

10 mg/kg/day is the optimal atorvastatin dose to reduce liver enzyme activities and improve insulin-stimulated AKT phosphorylation

As expected, the mice fed the HFD for 12 consecutive weeks displayed about a two-fold increase in blood ALT activity compared to those that consumed regular rodent chow (Figure 1A). The 5 and 10 mg/kg/day AT treatment protocol during the last two weeks of the diet protocol decreased this activity to values similar to regular chow-fed mice. The 15 mg/kg/day dose had no impact on the augmented ALT activity but induced an approximately 30% increase in the AST activity (Figure 1B). We also analyzed the insulin-induced AKT serine phosphorylation in the livers of the HFD mice treated with the three AT doses to determine which dose to utilize for subsequent experiments. Figure 1C shows a representative immunoblot of samples perfused with insulin and then probed with an anti-pSerine AKT antibody. Compared with the basal condition, without the acute insulin perfusate and hormone stimulus, it is possible to see the increased AKT serine phosphorylation in the liver homogenates from AT10-treated mice. Based on these results, we opted to administer the 10 mg/kg/day dose in the subsequent experiments.

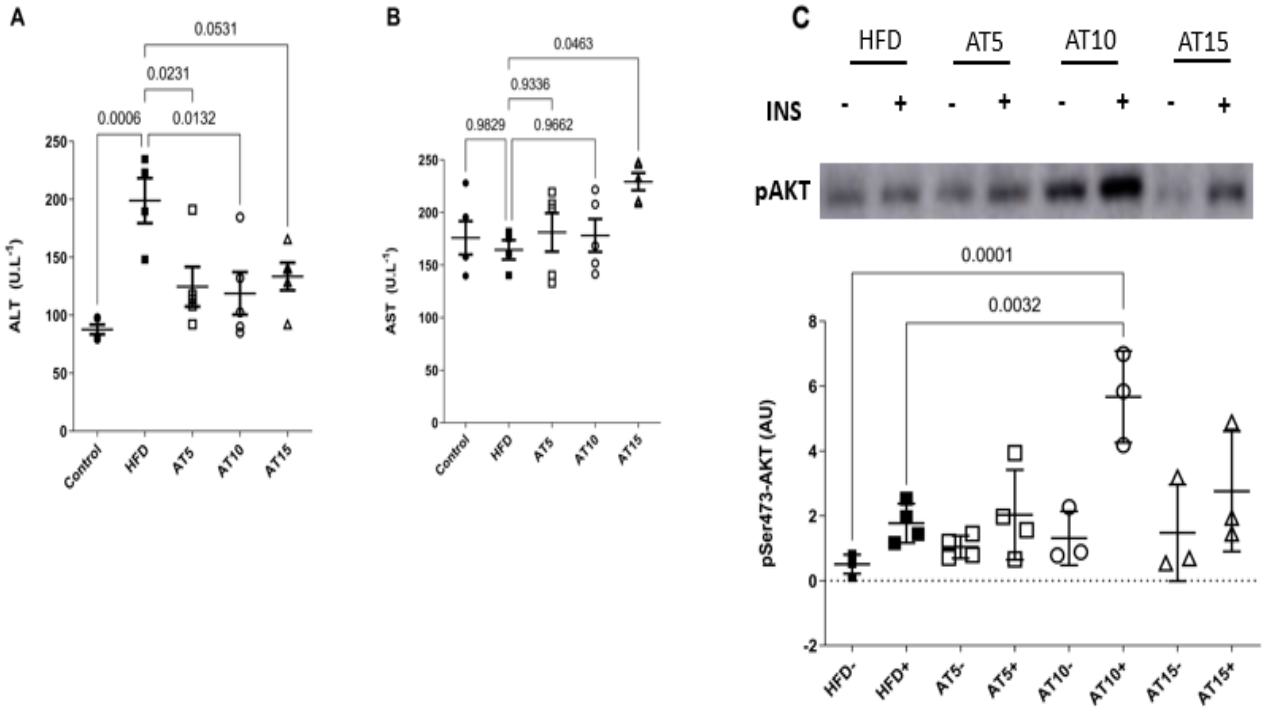

Figure 1: Effects of atorvastatin on liver enzyme activities and AKT phosphorylation. C57BL/6 male mice were fed with either a high-fat diet (HFD) for 12 weeks or regular chow (Control) and received 5, 10 or 15 mg/kg/day (AT5, AT10, AT15, respectively) or tap water (Control; HFD) once a day for 15 consecutive days via oral gavage. (A, B) Hepatic enzymes: ALT and AST; (C) Typical immunoblotting with anti-phosphoserine 473 AKT antibody in liver samples from mice that received a bolus infusion of insulin (+) or saline (-) 30 seconds previously the liver extraction. Data are presented as the mean ± SD. Normalization was done by Ponceau staining. The statistical differences detected by one-way ANOVA are indicated.

Administration of atorvastatin changes fat mass composition in mice with NAFLD but not with NASH

In both mouse models, total body mass and fat and lean mass were evaluated on the 15th day of AT treatment. Despite the expected enhancement in body mass due to the HFD in WT mice (Figures 2A-2C), the AT treatment had no impact on the body or lean mass (Figures 2A, 2B). In contrast, a percentual reduction in the fat mass was detected in these animals (Figure 2C). On the other hand, there were no modifications in the body mass, fat and lean mass of the ApoE -/- mice (Figures 2D-2F).

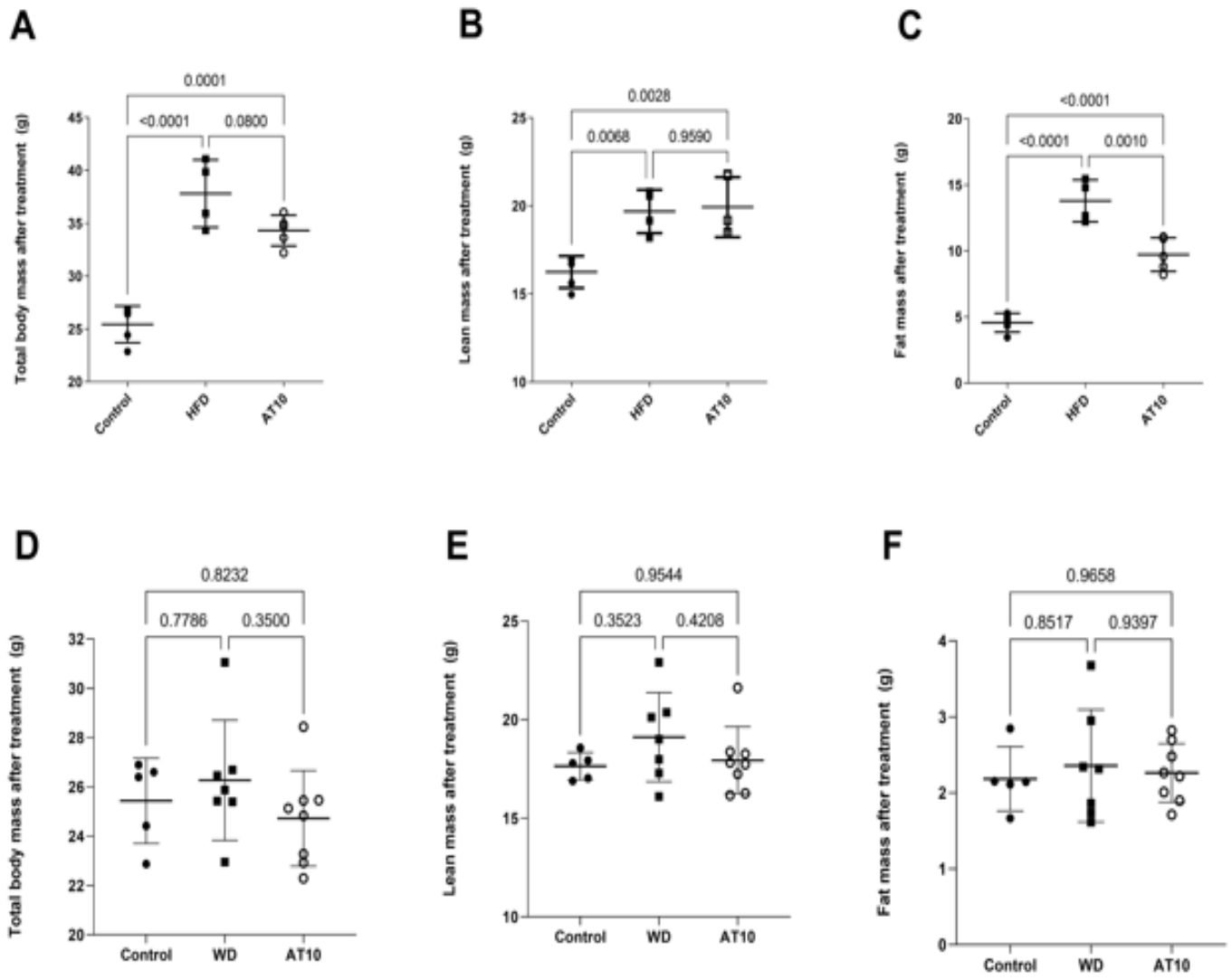

Figure 2: Atorvastatin administration changes body composition in mice with NAFLD. C57BL/6 male mice were fed with either a high-fat diet (HFD) for 12 weeks, a western diet (WD) for seven weeks or regular chow (Control) and received 10 mg/kg/day (AT10) or tap water (Control; HFD; WD) once a day for 15 consecutive days via oral gavage. (A, D) Total body mass after treatment in mice with NAFLD and NASH; (B, E) Lean mass after treatment in mice with NAFLD and NASH; (C, F) Fat mass after treatment in mice with NAFLD and NASH. Data are presented as the mean ± SD. The statistical differences detected by one-way ANOVA are indicated.

Atorvastatin does not influence glucose tolerance in both models but improves ALT in the early stages.

Figures 3A, 3D shows that glucose tolerance was not altered by AT treatment. However, basal glucose in the NASH model was lower than in the WD group (Figure 3F). The NAFLD animals that received AT10 showed improved blood ALT activity (Figure 3G), but in the AT15 group, the AST activity was higher. The treatment did not impact these enzymes in the NASH model (Figures 3I, 3J).

Atorvastatin reduces histological triacylglycerol content in both models

Figure 4A and 4C represents the histological pattern of NAFLD and NASH in mice treated with AT. Both models presented hepatocyte vacuolization that could be attenuated by the treatment (Figure 4B, 4D).

Human-equivalent dose of atorvastatin does not change protein expression related to endoplasmic reticulum stress in either model

Expression of ERS-related proteins, ATF4, BIP, IRE1a, and CHOP, were assessed in both models (Figure 5A, 5F). The 10 mg/kg/day of AT did not impact the protein expression patterns in either model (Figure 5).

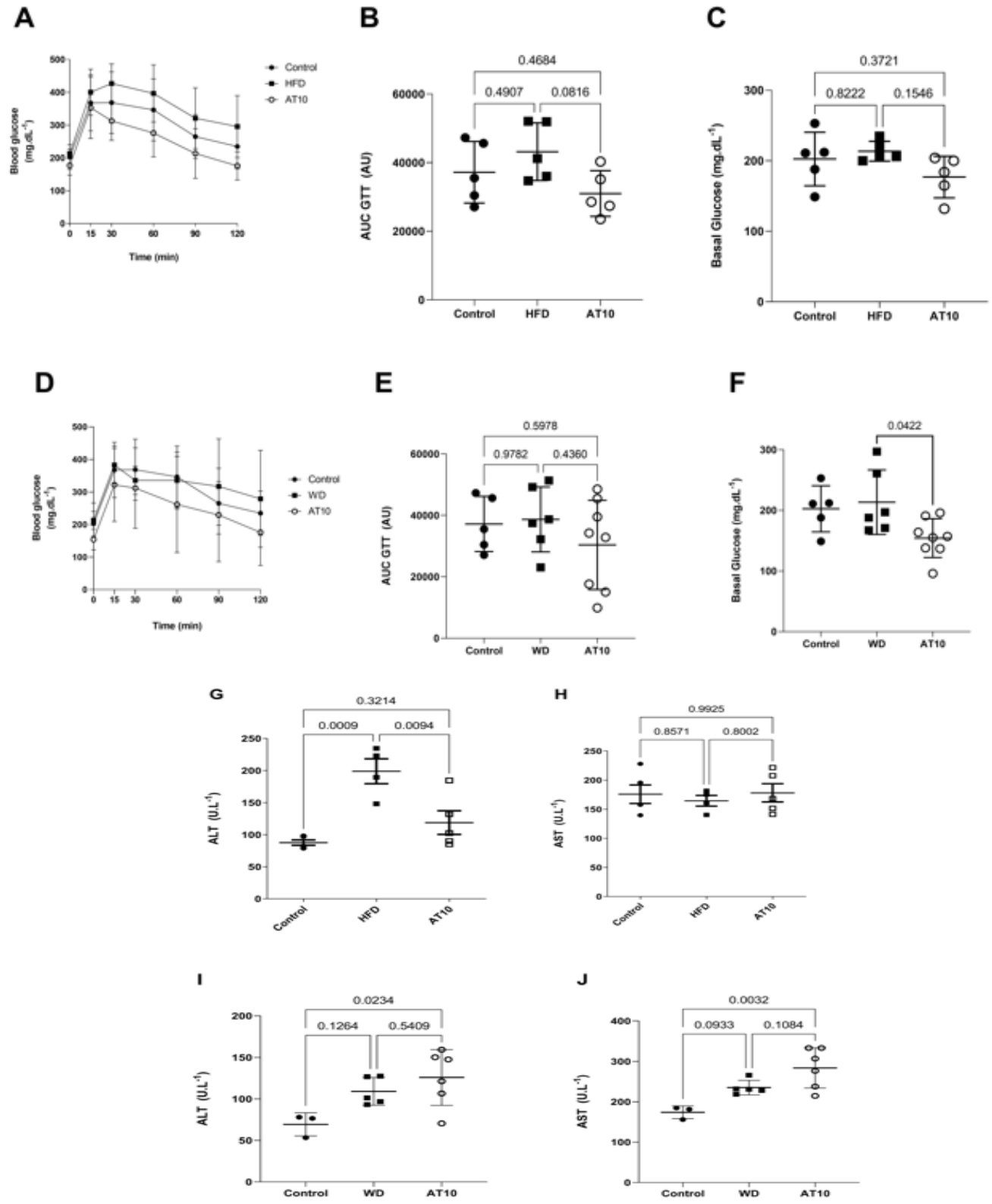

Figure 3: Effects of atorvastatin on glucose tolerance and hepatic enzymes in mice fed high-fat or western diets. C57BL/6 male mice were fed with either a high-fat diet (HFD) for 12 weeks, a western diet (WD) for seven weeks or regular chow (Control) and received 10 mg/kg/day (AT10) or tap water (Control; HFD; WD) once a day for 15 consecutive days via oral gavage. (A, D) Plasma glucose levels during intraperitoneal GTT; (B, E) GTT area under the curve analysis; (C, F) Six-hour fasted blood glucose; (G, H) ALT and AST activities in NAFLD model; (I, J) ALT and AST in NASH model. Data are presented as the mean ± SD. The statistical differences detected by one-way ANOVA are indicated.

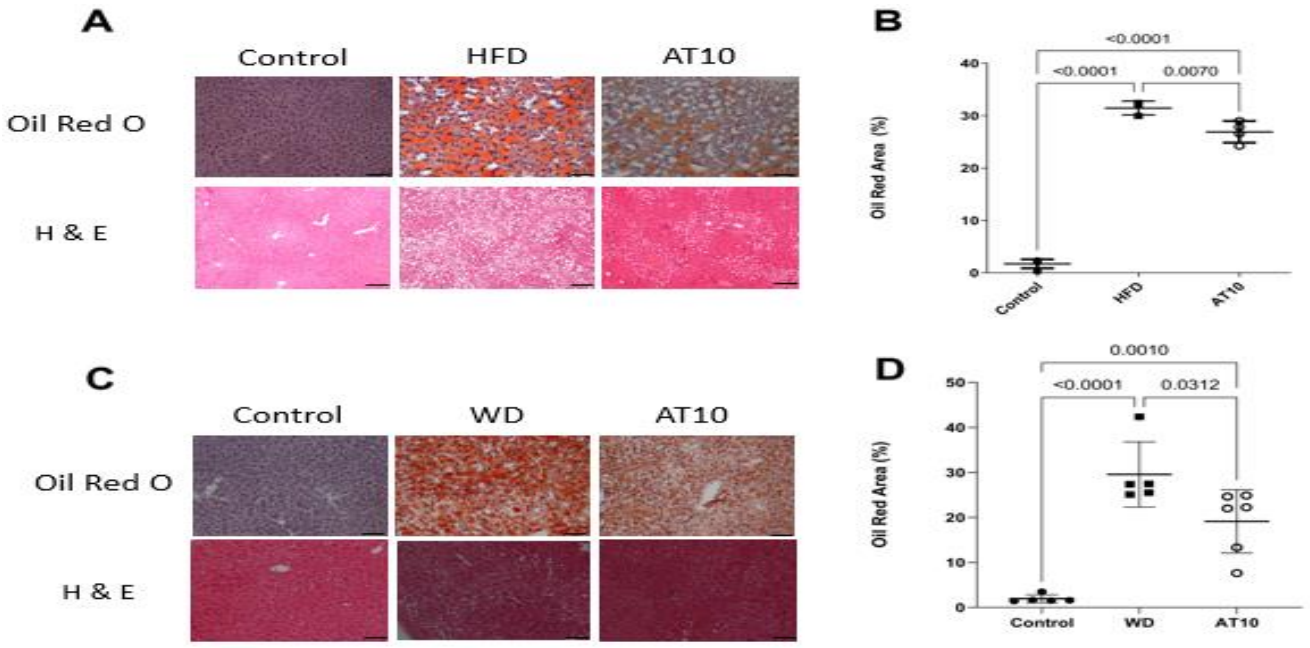

Figure 4: Effect of atorvastatin on histological liver steatosis patterns. C57BL/6 male mice were fed with either a high-fat diet (HFD) for 12 weeks, a western diet (WD) for seven weeks or regular chow (Control) and received 10 mg/kg/day (AT10) or tap water (Control; HFD; WD) once a day for 15 consecutive days via oral gavage. (A, C) Representative photomicrographs of liver samples stained with Hematoxylin & Eosin (HE) and Oil Red O (ORO) from all groups. All images correspond to a 20× magnification. (B, D) Percentage of ORO area in both models. Data are presented as the mean ± SD. The statistical differences detected by one-way ANOVA are indicated.

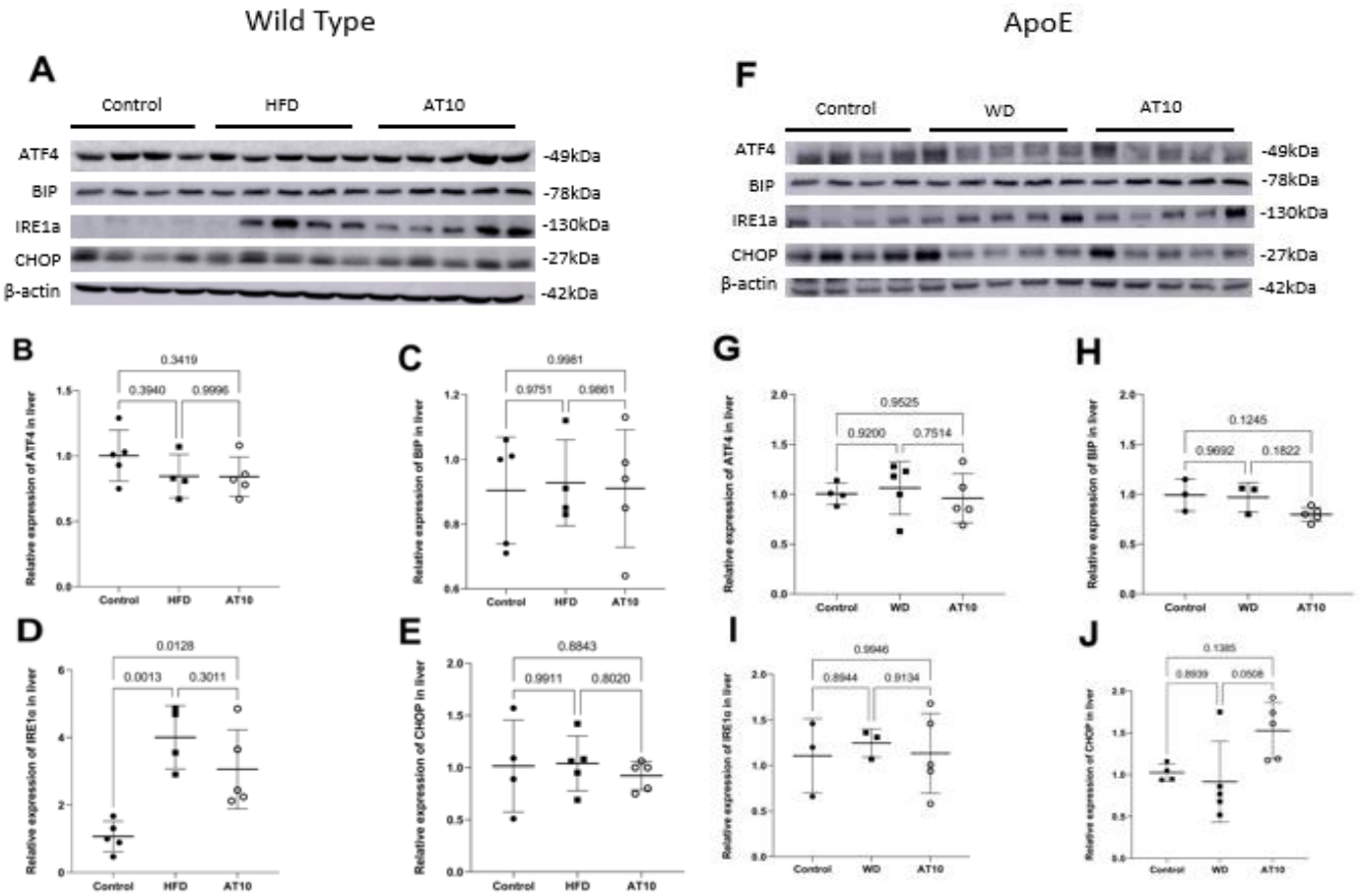

Figure 5: Analysis of protein expression of endoplasmic reticulum stress in both models. C57BL/6 male mice were fed with either a high-fat diet (HFD) for 12 weeks, a western diet (WD) for seven weeks or regular chow (Control) and received 10 mg/kg/day

(AT10) or tap water (Control; HFD; WD) once a day for 15 consecutive days via oral gavage. (A-E) Protein expression of ERE in mice with NAFLD; (F-J) Protein expression of ERE in mice with NASH. Data are presented as the mean ± SD. The statistical differences detected by one-way ANOVA are indicated.

Discussion

In this study, we evaluated the effect of AT on body mass composition, insulin-stimulated AKT phosphorylation, hepatic enzyme activities and ERS response-related protein expression. Fifteen days of treatment with AT effectively reduced total body mass and fat mass in mice with NAFLD, but this was not observed in animals with more advanced liver disease (i.e., NASH). As expected, HFD-fed animals gained significant weight at the end of the experimental period compared to control mice. The minispec analysis detected fat mass reductions of approximately 150% in the HFD group treated with AT. The same analysis revealed a significant increase in lean mass by about 30% in the HFD animals, with the pharmacological treatment having no impact on this parameter. This observation is important because of the myotoxic potential of statins [16].

Previously, SCHIERWAGEN et al. [17] reported that the body weight of ApoE-/- mice fed a WD did not increase compared to ApoE-/- mice fed a standard diet. Our study confirmed this finding, and it was also found that AT had no effect.

Lipid ectopic accumulation in the liver is directly associated with hepatic insulin resistance [18], and its reversal is associated with improved insulin resistance [18-20]. As expected, the HFD promoted glucose intolerance and impaired AKT's acute insulin-induced serine phosphorylation. While 10 mg/kg/day of AT improved insulin-stimulated AKT phosphorylation in the NAFLD model, it did not elicit a response in the NASH model. Along these lines, other studies demonstrated an increase in AT-induced AKT phosphorylation [21,22]. The discrepancy in increased AT-induced AKT activation deserves further attention, but could be due to increased insulin receptor binding and phosphorylation [22]. Additionally, the lack of an observable effect in the NASH model could be because the employed dose was too low to treat the more advanced disease state.

It is well established that animals fed an HFD or WD exhibit inflammation and fat accumulation in the hepatocytes [11,17,20,23]. Our histological analysis with ORO staining showed a reduction in fat droplet content (~10%) in NAFLD animals treated with 10 and 15 mg/kg/day AT and NASH animals treated with 10 mg/kg/day.

Concerning liver damage, HFD induced about a 60% increase in ALT activity, which was reduced in animals administered 5 and 10 mg/kg/day AT treatment, but not in the AT15 group. It should be pointed out that the high AT dose (15 mg/kg/day) induced an increase of approximately 20% in the blood determination of this enzyme, suggesting potential liver and/or muscle toxicity as previously described [16]. These results indicate that using uncalculated high doses of AT in mice can lead to liver or muscle damage. In WD animals, perhaps because of the liver damage already present in this model [11,17], the optimal dose (10 mg/kg/day) of AT could not change it. Use the "Insert Citation" button to add citations to this document.

The present study further expanded on whether a calculated equivalent human dose [13] can modulate the ERS in NAFLD and NASH models. The use of high statins doses and a very extensive treatment time (both not calculated) altered the expression of ERS-related proteins [24-27]. Additionally, high statin doses can lead to apoptosis [28], new-onset diabetes [29,30], and muscle damage [31]. It is well-established that under unstressed conditions, the three main molecules (PERK, IRE1α and ATF6) are inactivated upon binding to GRP78/BiP. Under stress conditions, this protein dissociates from the ER stress sensors and activates the unfolded protein response [6,32]. Following a cascade of reactions, other proteins become involved, leading to an adaptative or apoptotic process. Despite decreasing fat droplet accumulation and liver damage, we found no statistical difference in the expression of ERS-related proteins when administering AT at 10 mg/kg/day.

The present study showed that AT at a human equivalent dose could reduce liver fat content in both NAFLD and NASH models but only attenuated liver damage in the NAFLD model. However, AT did not affect ERS-related protein expression. Finally, our data show the importance of paying attention to the dose and treatment time and always making correlations with clinical applicability.

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Conflict of Interest

All authors declare no conflicts of interest.

Author contributions

SLM performed experiments, analyzed and interpreted the data, wrote the manuscript with input from all authors. FNC performed experiments, analyzed and interpreted the data. AGA performed experiments, analyzed and interpreted the data. JPC analyzed and interpreted the data. CROC analyzed and interpreted the data. All authors have read and agreed to the published version of the manuscript.

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