



Bacoside – A improves Non-Alcoholic Fatty Liver Disease in rats fed on a high fat diet

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ABSTRACT:

The chronic illness known as nonalcoholic fatty liver disease (NAFLD) is linked to morbidity in the metabolic syndrome. NAFLD is a global issue that is primarily responsible for liver damage, which can result in the loss of liver cells. We looked into how rosuvastatin (RSV; 10 mg/kg) and Bacoside - A (Bac-A; 10 mg/kg) affected hepatic steatosis brought on by a high-fat diet (HFD). Male Sprague-Dawley rats were given Bac-A or RSV for four weeks after 16 weeks of HFD. We looked at the liver's apoptotic cell death, reactive oxygen species production, lipid content, metabolic parameters, and histological changes. In addition to the expression of the following significant molecules, we investigated the liver's metabolic parameters, function, fat content, histological changes, production of reactive oxygen species, and apoptotic cell death: transient receptor potential cation channel subfamily V member 1 (TRPV1) phosphorylation of sterol regulatory element binding protein (pSREBP-1c/SREBP-1 c), total and membrane glucose transporter 2 (GLUT2), 4-hydroxynonenal (4-HNE), and cleaved caspase-3. Significantly higher levels of morphological disarray, damage indicators, oxidative stress, lipid peroxidation, and apoptosis were linked to HFD-induced hepatic steatosis. However, RSV and Bac-A treatment decreased metabolic dysfunction and hepatic damage. Bac-A reduced oxidative stress and apoptotic cell death, enhanced insulin resistance, and controlled lipid accumulation. Consequently, Bac-A is a therapy strategy that shows promise for treating metabolic abnormalities in NAFLD patients.



Introduction

Hepatocellular lipotoxicity can result from liver steatosis, also referred to as non-alcoholic fatty liver disease (NAFLD), which is defined by a gradual pattern of intrahepatic fat buildup.[1] Because NAFLD is linked to metabolic disorders, obesity-related diseases, insulin resistance (IR), dyslipidemia, oxidative stress (OxS), and systemic inflammation that can result in nonalcoholic steatohepatitis (NASH), it is becoming a major problem for communities globally.[4] Hepatic lipotoxicity linked to cellular malfunction and cell death can be exacerbated by circulating free fatty acids that are converted into triglycerides through hepatic de novo lipogenesis and fatty acid oxidation via SREBP activation.[5–6]

In addition, circulating FFAs can upregulate the production of reactive oxygen species (ROS), [4] which can interact with the phospholipid bilayer inducing lipid peroxidation within the cell, resulting in 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) formation. [7,8] Oxidative stress is observed in cases of NAFLD, providing evidence of hepatocyte apoptosis. [9,12] Therefore, agents that attenuate hepatic lipid accumulation and OxS are beneficial for the treatment of NAFLD.

Plant varieties have long been utilized to treat a range of cardiovascular conditions. Numerous plants possessing strong medicinal elements such as fibers, flavanoids, polyphenols, phytosterols, and saponins have been studied for their potential to reduce hyperlipidemia, act as antioxidants, and prevent atherosclerosis [9].

It has been demonstrated that the plant possesses strong antioxidant and free radical scavenging abilities [13]. It also has anti-inflammatory [16], anti-ulcer [19], anti-addictive [20], mast cell stabilizing [18], cardio-protective [14], vasodilatory [15], anti-inflammatory [16], and calcium antagonistic [17] qualities. Alkaloids, flavonoids, and triterpenoid saponins have all been shown to be present in *B. monniera* [21, 22].

The assessment of Bac-A's hepatoprotective mechanism is especially relevant given the rise in metabolic syndrome cases worldwide. Thus, in rats given a high-fat diet (HFD), our goal was to examine how Bac-A affected the accumulation of fat,

insulin resistance, lipid profiles, pSREBP-1 c expression, and OxS parameters in the liver in addition to the incidence of apoptotic proteins. The development of Bac-A as a natural medication to treat HFD-induced NAFLD may benefit from the study's findings.

2. Materials and methods

2.1. Animal experiments and diets

150–180 g male Sprague–

Dawley rats were purchased from the University Animal House.

The rats were kept in a temperature-controlled environment ($25 \pm 1^\circ\text{C}$) with a 12 hour light/dark cycle.

The University's Institutional Animal Care and Use Committee approved all experimental protocols in accordance with National Institutes of Health (NIH) regulations.

The rats were randomized into two groups: the high fat diet (HFD) and the normal chow diet (NCD) after a week of acclimatization.

Standard rat chow was given to the control group; fat accounted for 19.79% of the total calories.

The NCD, NCD plus 10 mg Bac-A per kilogram body weight (BW) (N + Bac-A), and NCD plus 10 mg rosuvastatin per kilogram BW (N + RSV) groups were further split into six members each. For 20 weeks, the HFD rats were fed a diet high in fat (65.26% of total calories). Three groups ($n = 6$ per group) of HFD, HFD plus Bac-A (H Bac-A), and HFD plus rosuvastatin (H + RSV) were created from the HFD rats. Following 16 weeks, for the last 4 weeks of the trial, either Bac-A or RSV was administered to the H Bac-A and H + RSV groups. The Bac-A groups' animals received oral administration of 10 mg/kg/day of Bac-A. For four weeks, rosuvastatin (Sandoz, Princeton, NJ, USA) was given by gastric gavage at a dose of 10 mg/kg/day after being freshly dissolved in 0.5% carboxymethyl cellulose (Sigma—Aldrich, St. Louis, MO, USA).²¹ The identical vehicle was used for the rats in the NCD and HFD control groups as it was for the treatment groups. The RSV dose was chosen based on earlier studies in rats that showed its potential to enhance metabolic parameters, while the Bac-A dose was determined by looking at the findings of a preliminary investigation. [23, 24] Every week, the weight of the rats and their consumption of food and water were noted. Both before and after the treatments,



metabolic parameters and the oral glucose tolerance test (OM) were conducted. The rats were euthanized at the conclusion of the trial, and samples of liver tissue and blood were taken for further research.

2.2. Oral glucose tolerance test

The rats were given an oral gavage of a glucose solution (1 g/kg BW) following a 12-hour fast. [24] Tail vein blood samples were obtained, and a commercial kit (Erba Mannheim, Mannheim, Germany) was used to assess the blood's glucose levels. Before glucose loading, right away (0 min), and 30, 60, 90, and 120 min after glucose loading were all sampled. The function of the pancreatic β -cell was evaluated using the area under the curve (AUC) for glucose.

2.3. Determination of serum insulin levels and homeostasis model assessment of insulin resistance (HOMA-IR) index

The concentrations of serum insulin were measured with a commercial ELISA kit (Millipore, Billerica, MA, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) index, which was computed as follows, was used to measure insulin resistance. $\text{HOMA-IR} = \text{fasting glucose (mg/dL)} \times \text{fasting insulin (}\mu\text{U/mL)} / 22.5$. [25]

2.4. Metabolic blood parameter analysis

Serum samples were extracted from the rats' blood samples by centrifuging them for 10 minutes at 4 °C at 1600 xg. Using commercial kits (Erba Mannheim, Mannheim, Germany) with absorbance at 600 nm, the following parameters were measured: albumin, alkaline phosphatase (ALP), total cholesterol (TC), albumin-triglyceride (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

2.5. Hepatic triglyceride and total cholesterol concentrations

The Bligh and Dyer method was used to extract lipids from liver tissues. Isopropylene was used to homogenize [26] liver tissues. Following dispersion, the entire mixture was shaken three times for one minute at room temperature. The homogenates were then centrifuged for five minutes at 8000 rpm. After collecting the supernatants, commercial kits (Erba

Mannheim, Mannheim, Germany) with absorbance at 505 nm were used to quantify the amounts of TG and TC.

2.6. Hematoxylin and eosin staining

The liver tissues of the rats were immediately removed upon their euthanasia, preserved for 24 hours in 4% paraformaldehyde, and then embedded in paraffin. Five o'clock thick pieces were prepared using a microtome. Hematoxylin and eosin (H&E) staining was applied to the slices for histological examination [27], and they were viewed using a light microscope.

2.7. Oil Red O staining

Liver tissues were quickly extracted, preserved in 4% paraformaldehyde for twenty-four hours, embedded in Tissue-Tek® OCT solution (Sakura Finetek USA, Inc. Torrance, CA, USA), and sliced into five-hour thick sections. Under a light microscope, the cryosections were inspected and stained with Oil Red solution to see how the tissue stored lipids.

2.8. 2', 7'-dichlorofluorescein diacetate (H2DCF-DA) assay

The liver samples were homogenized in 10 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4) with 1 mM EDTA and 140 mM KCl, to which a protease inhibitor was added, in order to identify the formation of ROS. The homogenate was centrifuged for 10 minutes at 4 °C at 8000 rpm. After removing the supernatant, 100 μ M of 2',7'-dichlorofluorescein diacetate (H2DCF-DA) was added, and the mixture was incubated for an additional 30 minutes at 37 °C. With excitation at 485 nm and emission at 538 nm, a microplate reader was used to measure the DCF fluorescence level.

2.9. TUNEL assay

The liver slices were examined using a TUNEL assay kit in compliance with the manufacturer's instructions in order to identify apoptotic cell death (Roche Diagnostics, Indianapolis, IN, USA). Under a light microscope, both total cells and TUNEL-positive cells were examined. Eight high-power fields (x 200) were chosen at random, and each field's quantity of apoptotic cells was counted. This is how the apoptotic index (AI) was determined: AI is equal to the total number of positive cells.



2.10. Tissue preparation and Western blot analysis

Using a modified version of the differential centrifugation method reported by Thallas-Bonke et al. [28], the liver tissues were homogenized in lysis buffer (1.5 mmol/L MgCl₂, 10 mmol/L KCl, 20 mmol/L HEPES, 1 mmol/L EGTA, 1 mmol/L EDTA, 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulphonyl fluoride, 1 mmol/L dithiothreitol, and proteinase inhibitor cocktail; pH 7.0) in order to prepare a cellular component. Each fraction's proteins were extracted and measured using the Bradford test, which used bovine serum albumin (BSA) as the reference.

Proteins of equal quantities were separated using 12% SDS poly-acrylamide gel electrophoresis (SDS-PAGE) and then transferred for 35 minutes at 400 mA to a PVDF membrane (Immobilon-P, Millipore, MA, USA). The membrane was blocked for two hours at 4 °C with 5% skim milk. Thereafter, it was incubated for an overnight period at 4 °C with the primary antibodies: anti-VR1 (Abcam, Cambridge, UK), anti-SREBP-1 c (Abcam, Cambridge, UK), anti-pSREBP-1 c (Millipore, Darmstadt, Germany), anti-GLUT2 (Abcam, Cambridge, UK), anti-4HNE (Abcam, Cambridge, UK), anti-Na⁺/K⁺ ATPase (Millipore, Darmstadt, Germany), and anti-caspase-3 (Millipore, Darmstadt, Germany). After that, the membrane was probed for one hour at room temperature using secondary antibodies (Millipore, Darmstadt, Germany) coupled to horseradish peroxidase. The enhanced chemiluminescence (ECL) substrate solution was incubated on the blots for a duration of 5 minutes. Blue X-ray films

allowed for the detection of chemiluminescent bands. Image-W was used to analyze densitometry.

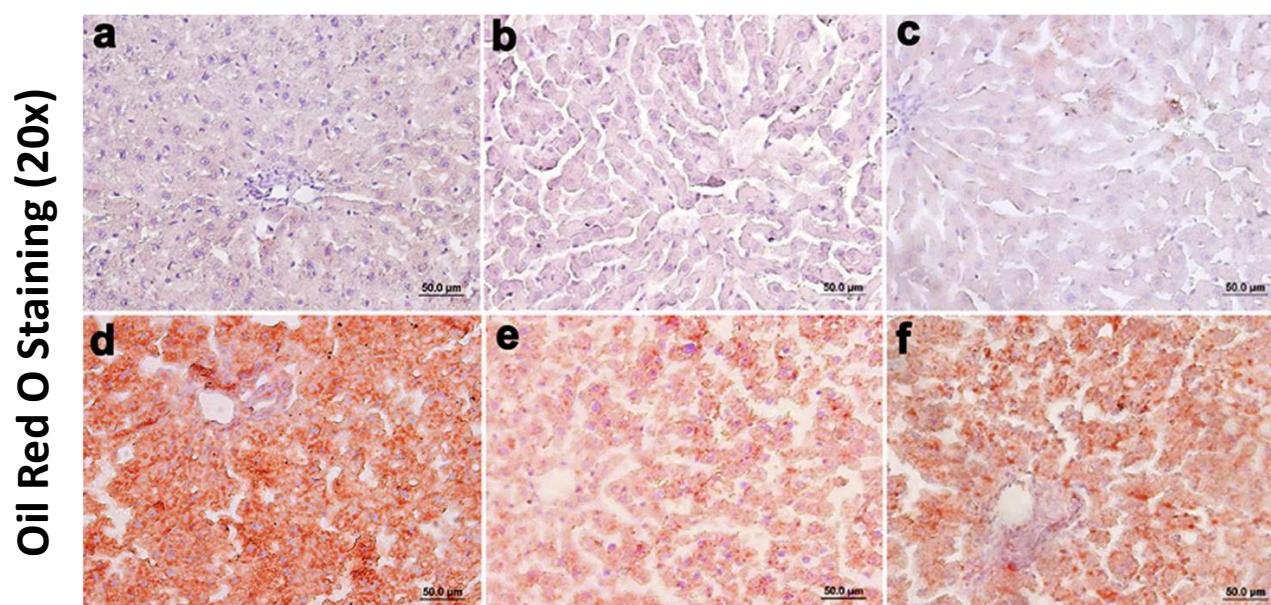
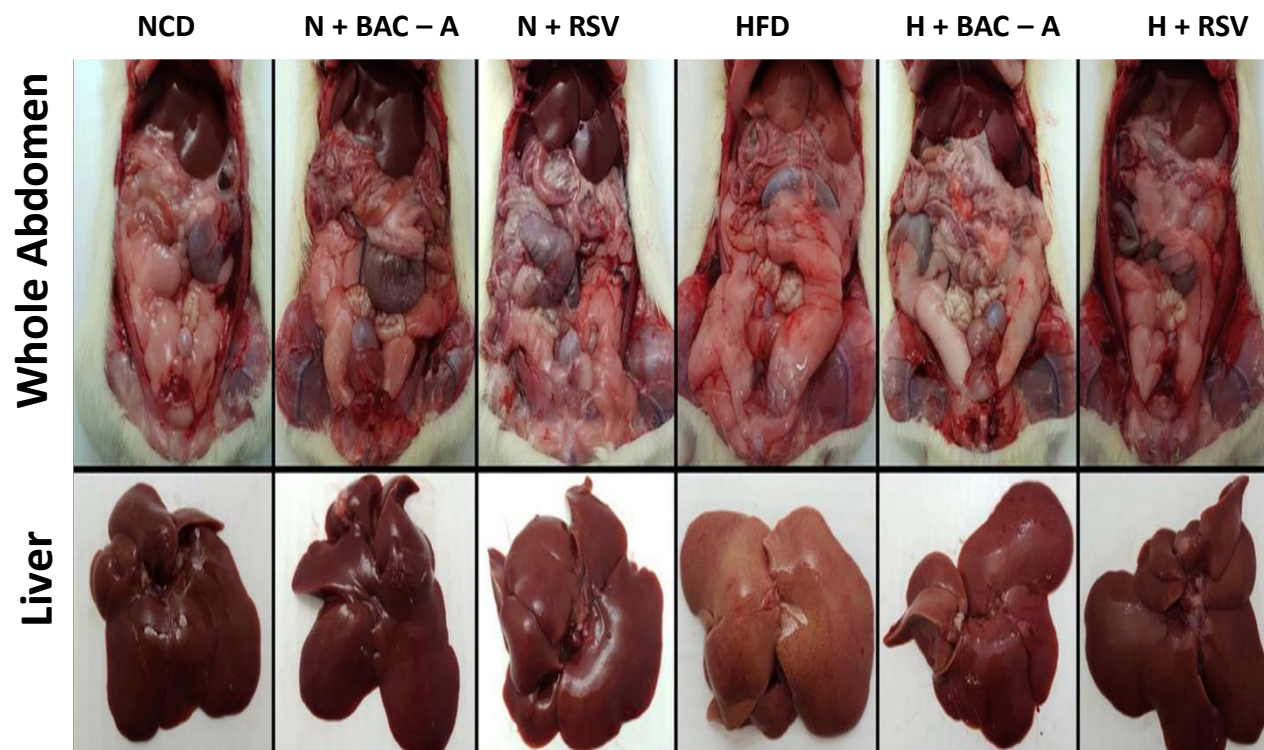
2.11. Statistical analysis

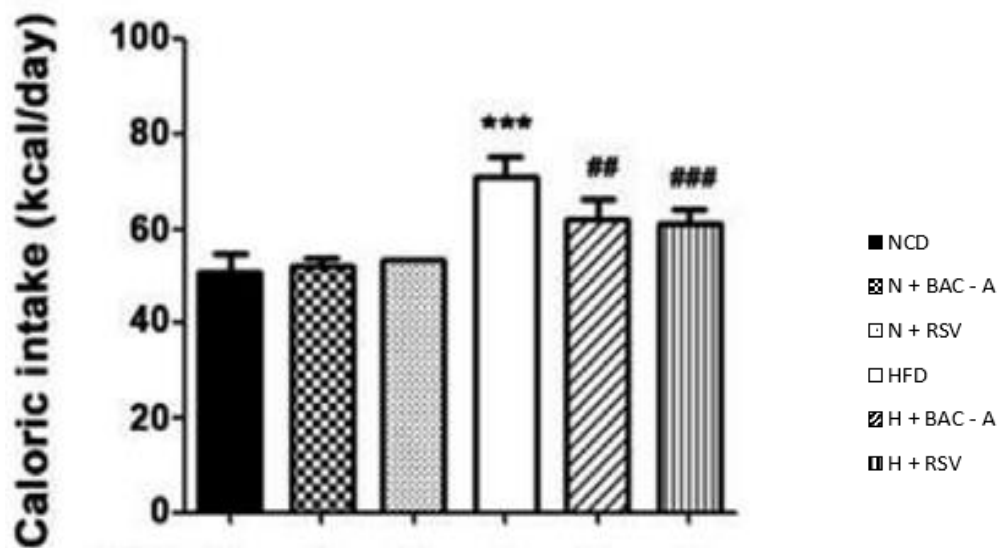
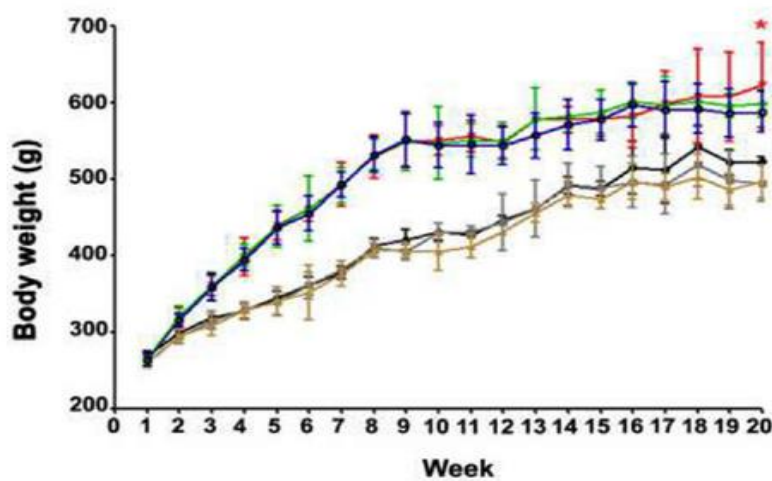
Every data point is displayed as mean $\pm$ SD. One-way ANOVA was used to ascertain the statistical differences between the groups, and Dunnett's post-hoc test was then used to assess the significance between the individual groups. When the p-value was less than 0.05, the difference was deemed significant.

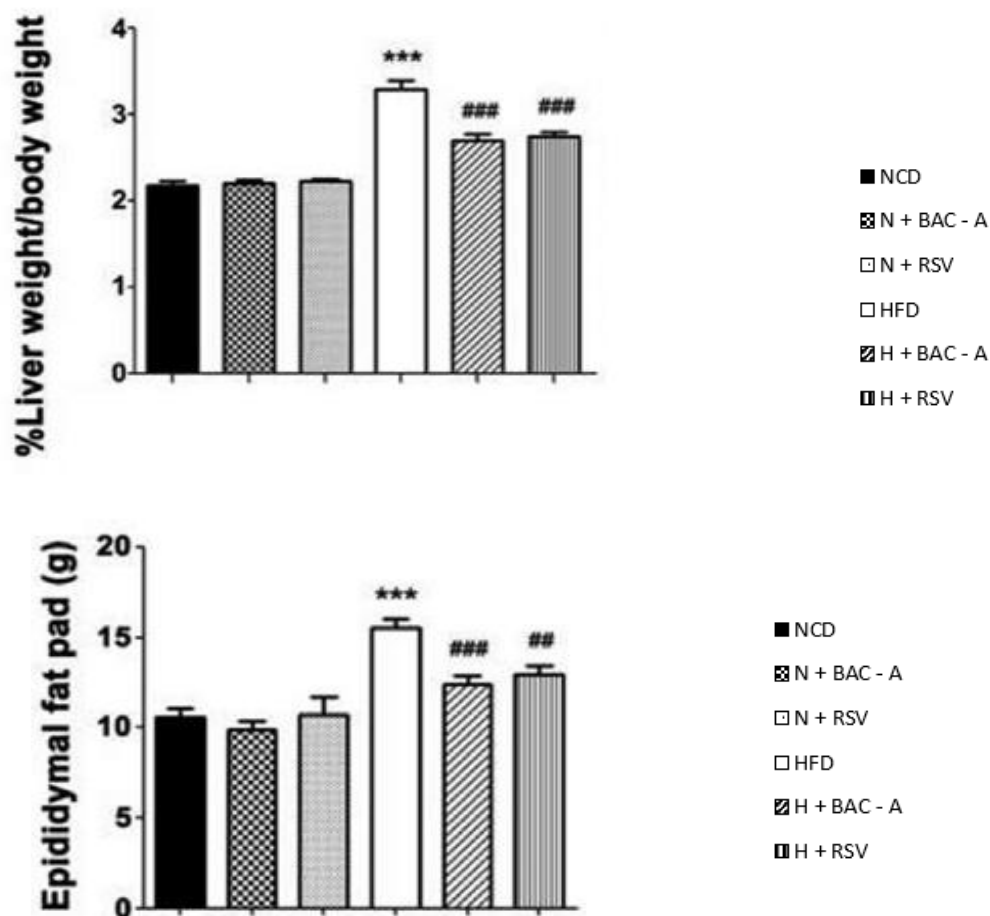
3. Results

3.1. Effects of Bac-A and rosuvastatin on body weight, external features of the liver, liver/body weight ratio, and epididymal fat pad weight

Compared to rats fed a regular diet, rats fed an HFD showed a progressive rise in body weight gain, calorie intake, liver/body weight ratio, and weight gain in the epididymal fat pad (Fig. 1C—F). After four weeks of treatment, HFD-fed rats given Bac-A exhibited a decrease in body weight increase, liver/body weight ratio, and epididymal fat pad mass in comparison to rats that did not receive Bac-A. Nonetheless, rats fed a high-fat diet and administered with RSV or Bac-A had a considerably greater liver/body weight ratio than rats with non-communicable disease ($p < 0.001$). animals in the HFD group had dull, yellowish brown livers on the outside, while animals treated with RSV and Bac-A had livers that resembled those of control rats (NCD rats; Fig 1A).







3.2 Bacoside-A and rosuvastatin treatment attenuates insulin resistance in rats fed a high fat diet

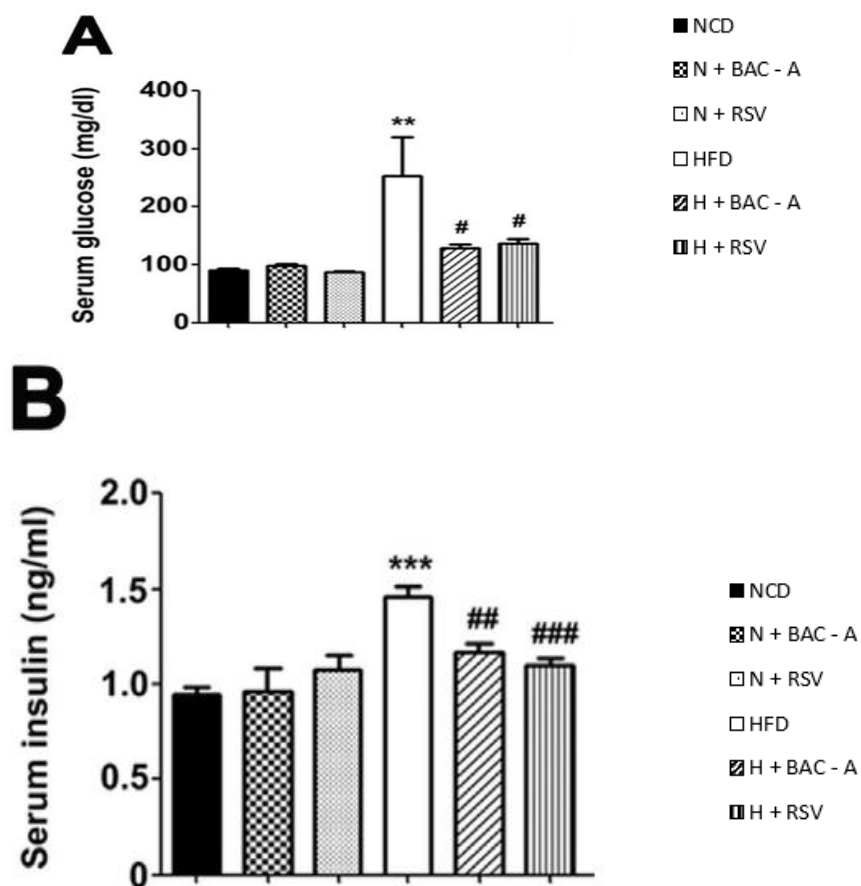
In Fig. 2D, the effects of Bac-A on the OGTT results are shown as the area under the curve (AUC). The HFD group's glucose tolerance was compromised, while it improved in the RSV and Bac-A-treated animals. Fig. 2A and B shows a significant decrease in serum glucose and insulin levels when compared to groups that were only administered Bac-A or RSV treatments.

Rats who were fed an HFD had reduced insulin sensitivity; rats that got Bac-A or RSV had notable improvements in this area. A reduction in the homeostasis model assessment of

insulin resistance (HOMA-IR index) to almost control levels, which was significantly different from that of HFD rats ($p < 0.001$), indicated improved insulin sensitivity. Additionally, compared to HFD rats, there was a substantial decrease in HFD-fed rats treated with Bac-A ($p < 0.01$) or RSV ($p < 0.001$) (Fig. 2C). Furthermore, the ratio of membrane GLUT2 to total GLUT2 was assessed (Fig. 2E). There was no difference in the liver's membrane/total GLUT2 ratio between the treatment groups. The membrane/total GLUT2 ratio tended to be lower in the HFD group, but the Bac-A and RSV-treated animals exhibited an increasing trend. In the membrane fraction, GLUT2 expression was shown to be

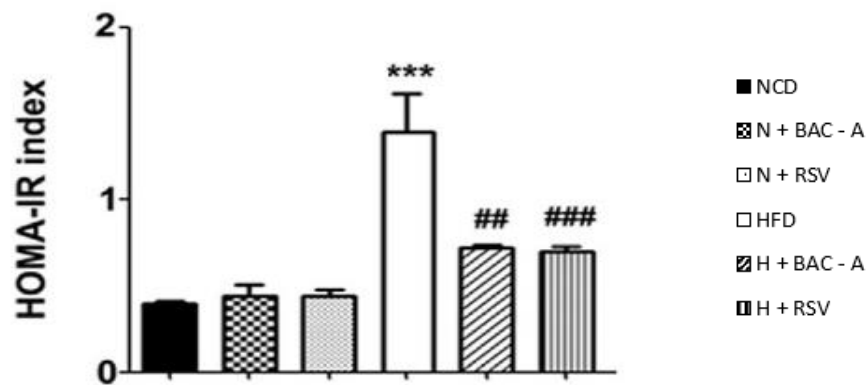


considerably lower in the HFD group and significantly higher in the Bac-A or RSV-treated groups.

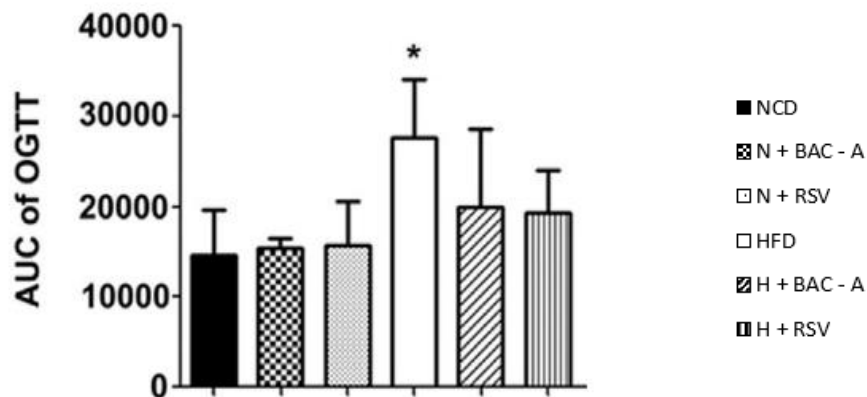


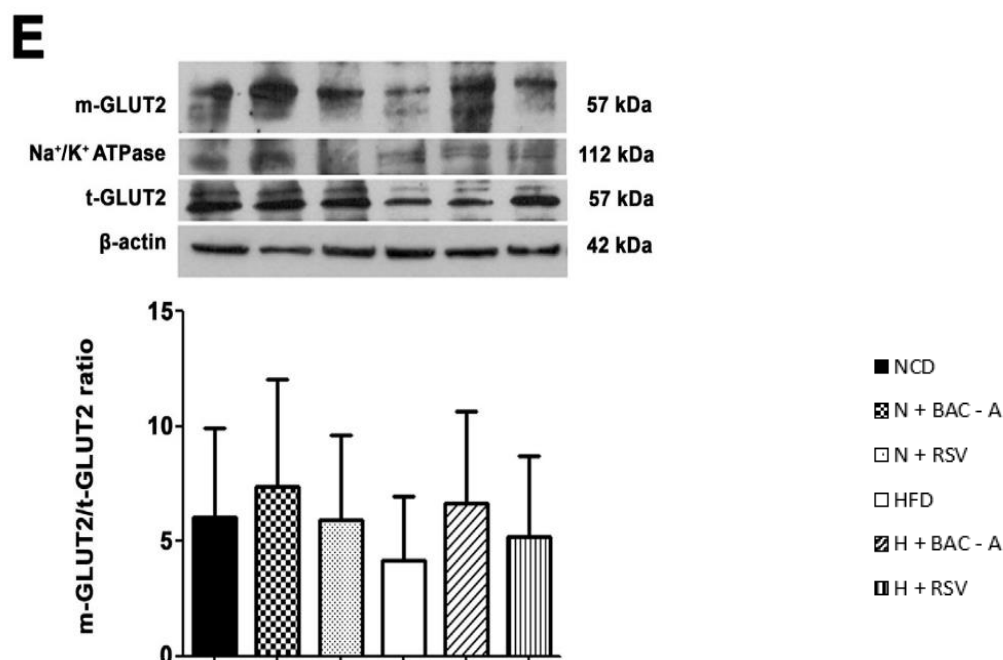


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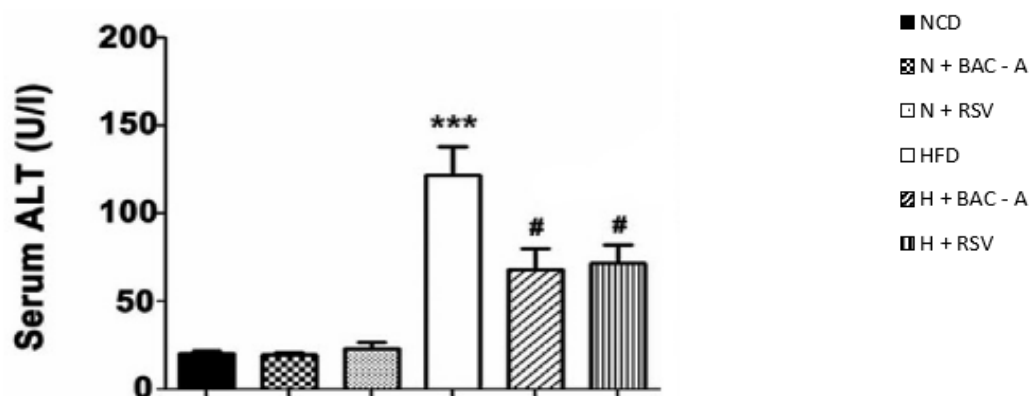


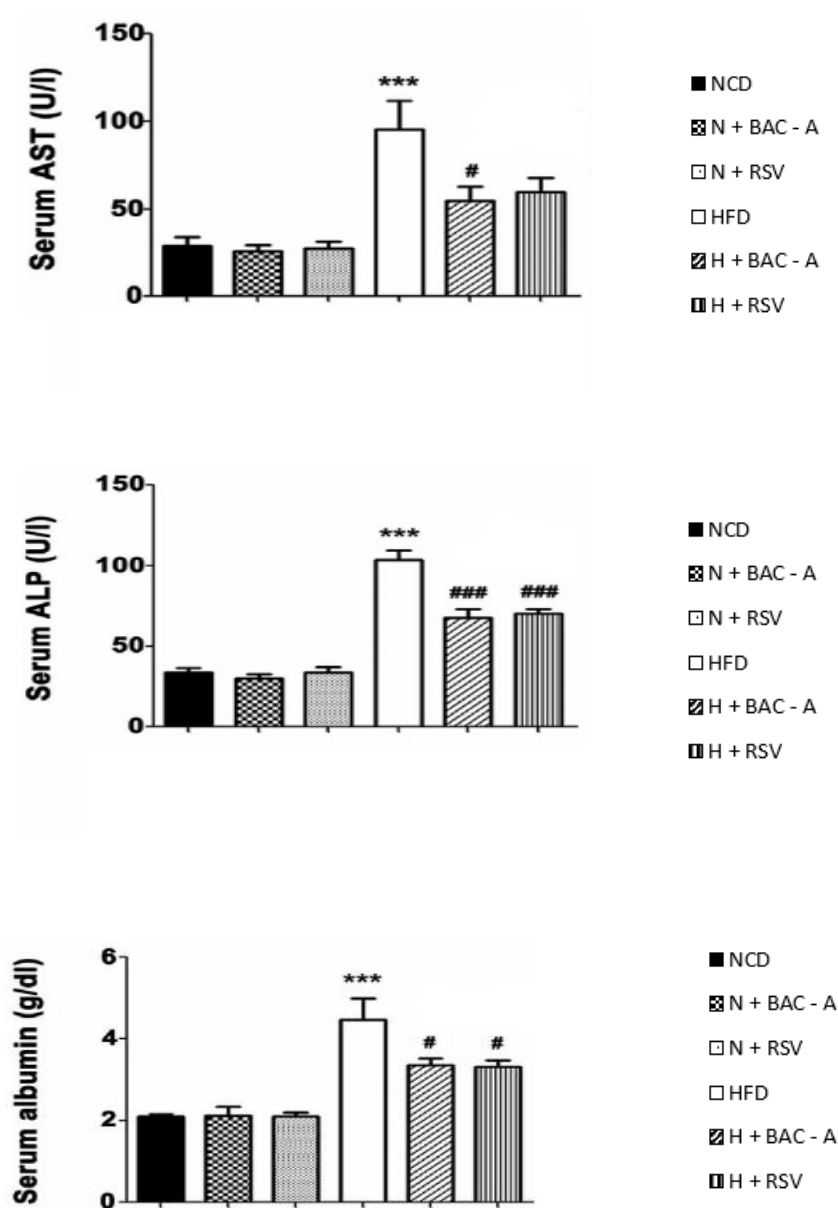


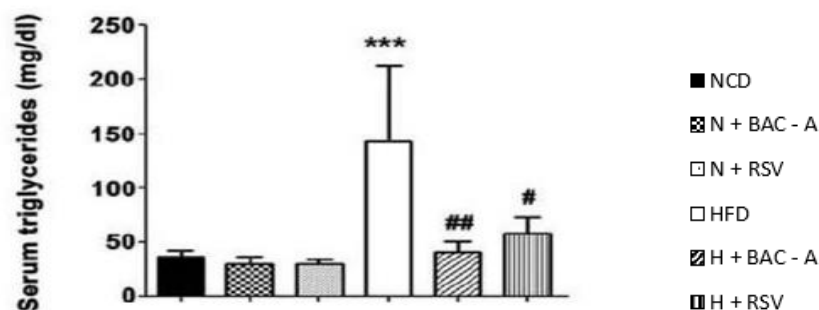
3.3. Effects of Bac-A and rosuvastatin on hepatic injury in rats fed a high fat diet

When compared to the NCD group, the HFD rats' serum levels of albumin, alkaline phosphatase (ALP), aspartate

aminotransferase (AST), and alanine aminotransferase (ALT) were significantly higher. Compared to normal rats, HFD-fed animals treated with Bac-A and RSV exhibited considerably lower levels of liver damage markers (Fig. 3C—F).



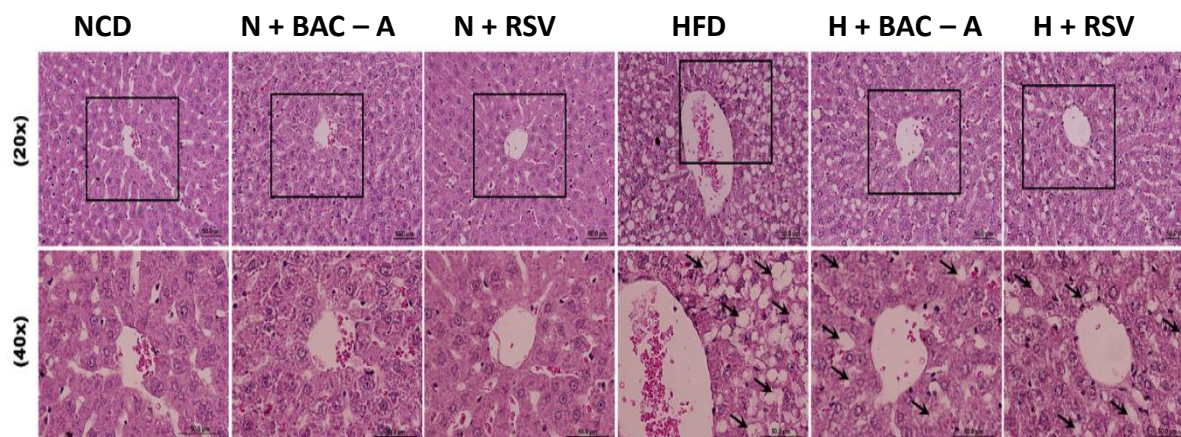


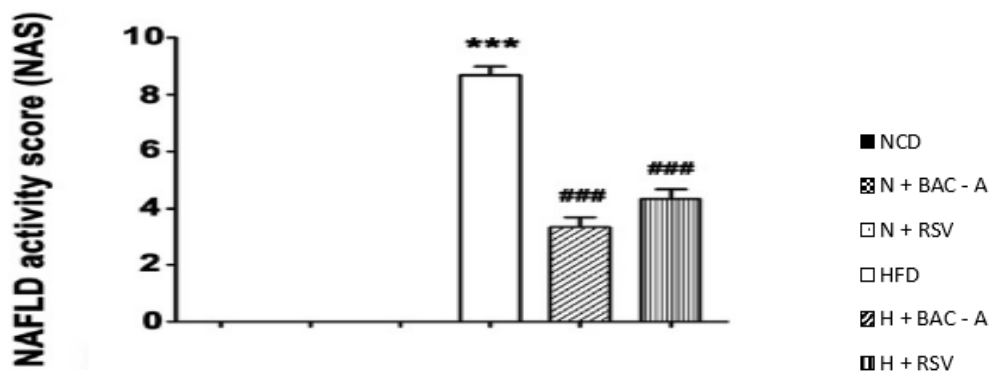


Hepatic lobules arranged in a thick plate, commonly known as the hepatic cord, with distinct cell borders devoid of lipid vacuoles were visible in sections stained with hematoxylin and eosin. Rats in the usual diet group had spherical and vesicular nuclei in the core of their liver cells. In contrast, the HFD group's liver cells had pyknotic nuclei along with disordered hepatic lobules that included vacuoles of different sizes. Nonetheless, rats treated with Bac-A showed observable reductions in the levels of fatty degeneration, unclear cell boundaries, inflated hepatocytes, and liver cell damage compared to the HFD

group. Rats treated with Bac-A had observably fewer irregular cell boundaries than the HFD group.

In rats treated with RSV, these results were comparable (Fig. 3A). Steatosis, lobular inflammation, and hepatocyte ballooning were assessed as part of the Brunt et al! approach, which was used to construct the NAFLD activity score (NAS) (Fig. 3B). Rats on HFD and NCD showed a significant difference in their NAS, although the NAS was significantly lower in HFD-fed rats who received RSV or Bac-A treatment ($p < 0.001$).



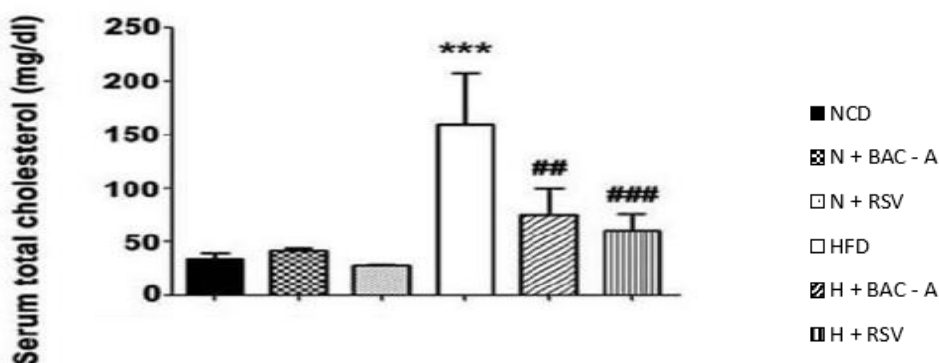


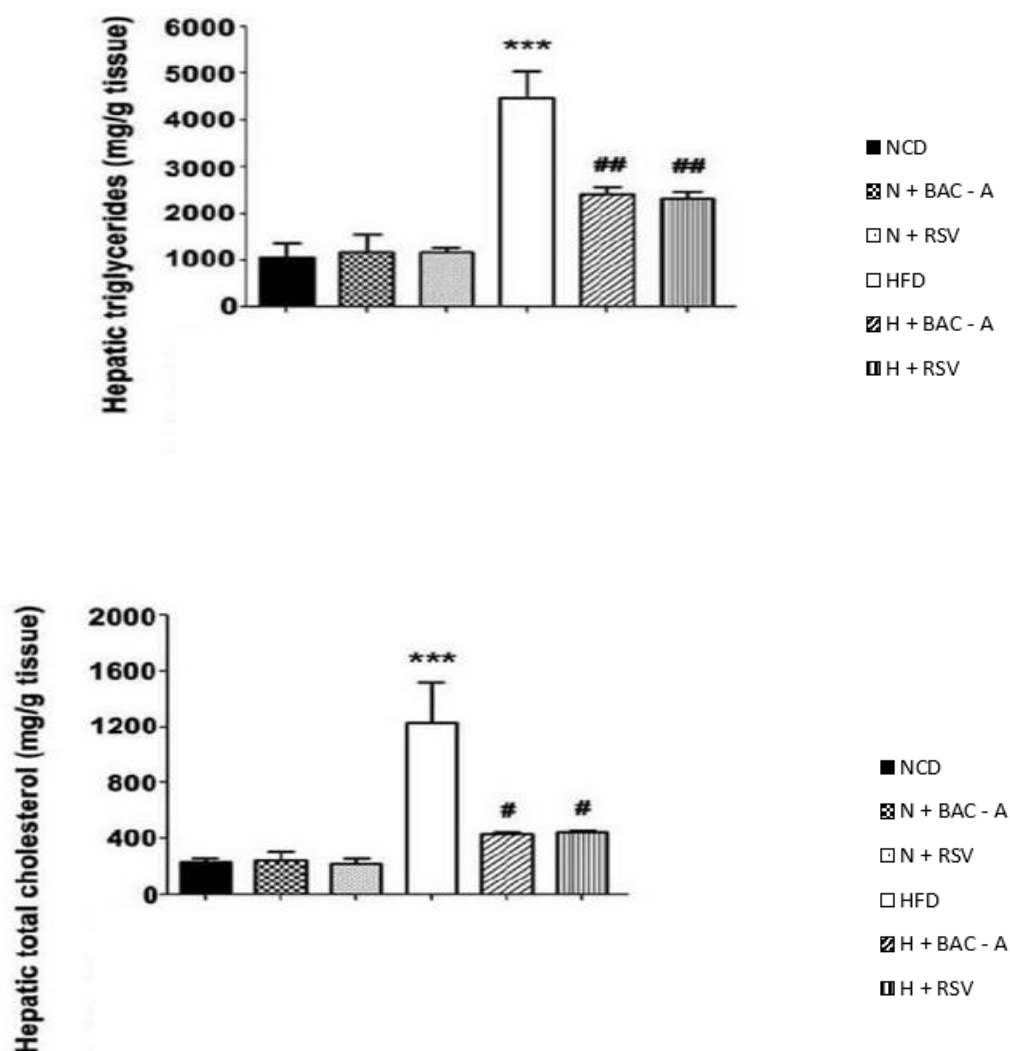
3.4. Effects of Bac-A and rosuvastatin on hepatic lipid accumulation and TRPV1 expression

The HFD animals showed a marked increase in fat deposition as seen by Oil Red O staining, while the treatment of Bac-A or RSV resulted in a decrease in hepatic fat accumulation (Fig. 1B).

Rats in the HFD group were found to have extraordinarily high lipid profiles when compared to rats fed NCD. The

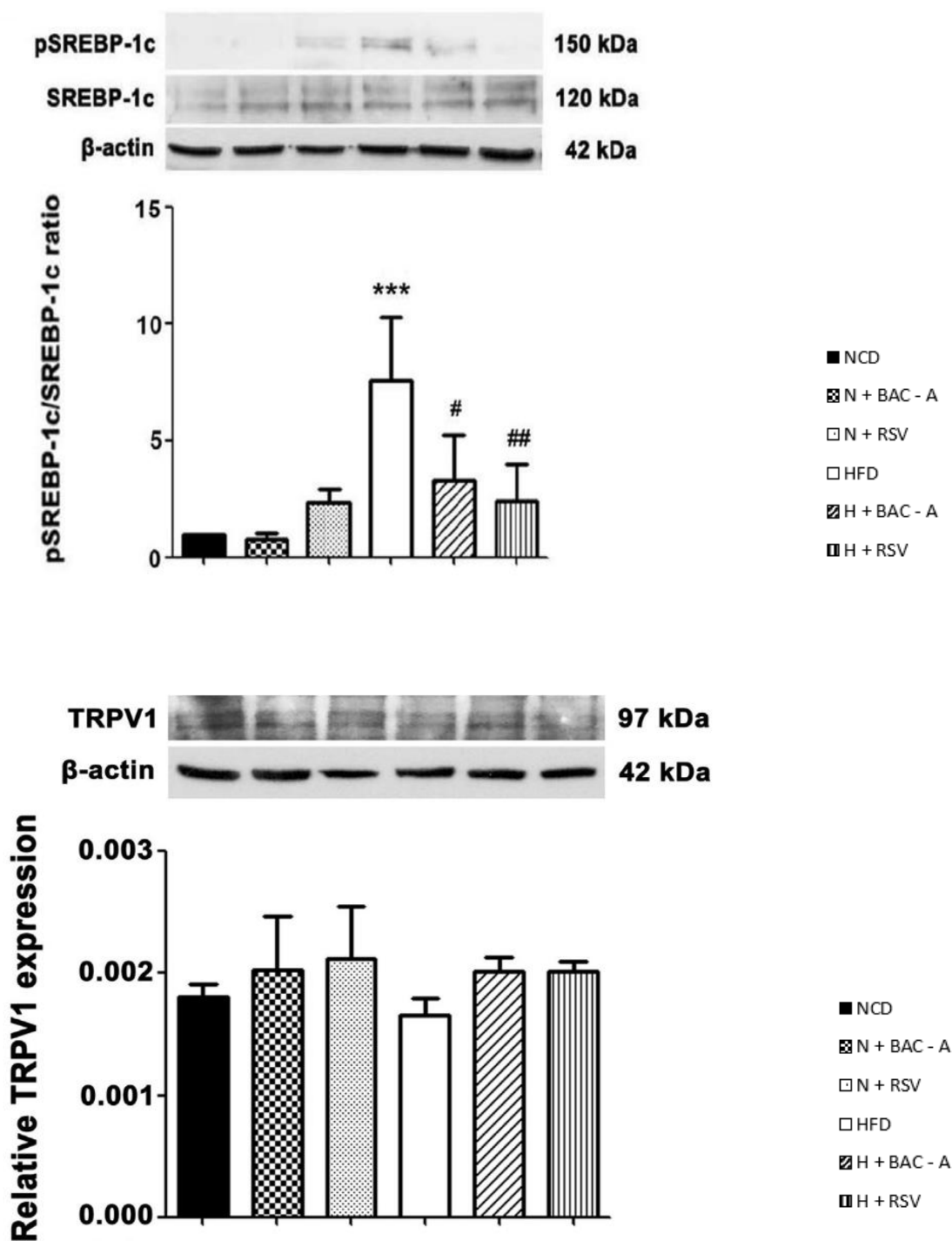
HFD group had considerably higher serum levels of total cholesterol (TC) and serum triglycerides (TG), as seen in Figs. 4A and B. When compared to the HFD group, we found that the injection of Bac-A or RSV considerably reduced the amounts of TG and TC in the serum. In a similar vein, the HFD group's levels of TG and TC were noticeably greater than those of the control group. Rats in the NCD group and those treated with Bac-A or RSV exhibited similar liver TG levels (Fig. 4C and D).





Rats given either Bac-A or RSV did not, however, exhibit a statistically significant difference in serum TC levels from the NCD group. The HFD group exhibited a considerable increase in the ratio of phosphorylation of the sterol regulatory element binding protein (pSREBP)-1 ϕ /SREBP-1c expression as compared to the normal group. When compared to HFD rats, the Bac-A and RSV

treatment significantly reduced the pSREBP-1 ϕ /SREBP-1c expression ratio ($p < 0.01$; Fig. 4E). There was no discernible difference in the groups' transient receptor potential cation channel subfamily V member 1 (TRPV1) expression. These findings suggest that Bac-A may have additional mechanisms by which to alleviate hepatic steatosis (Fig. 5).

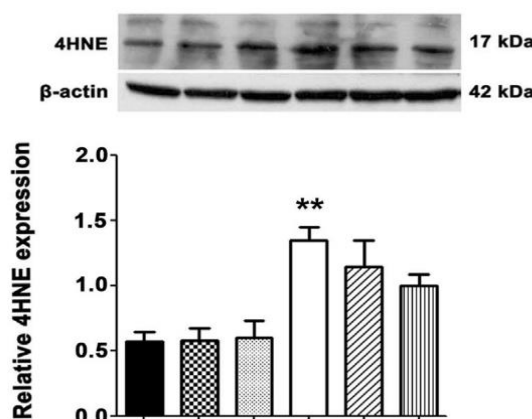
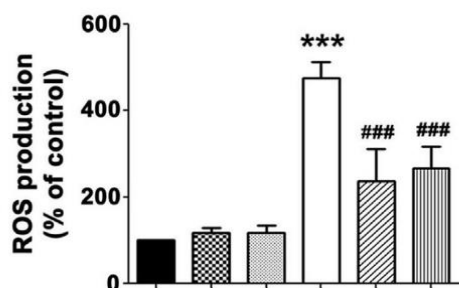




3.5. Effects of Bac-A and rosuvastatin on hepatic oxidative stress and lipid peroxidation

Hepatocellular deterioration and mortality may result from oxidative stress. ROS can cause lipid peroxidation, which in turn can cause the onset and progression of liver injury. This is why the formation of ROS in the liver and the expression of 4-HNE were investigated (Fig. 6A and B, respectively). Compared to the usual control group, the HFD group had a considerably higher ROS level. Comparing the HFD-fed

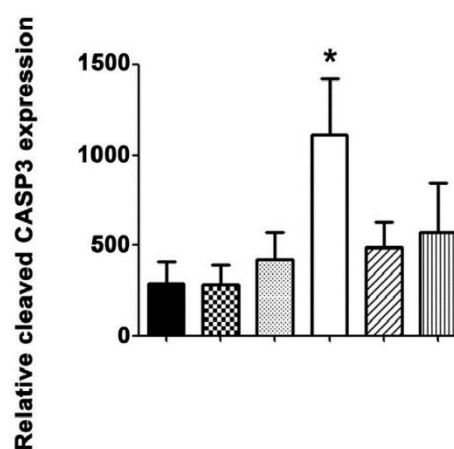
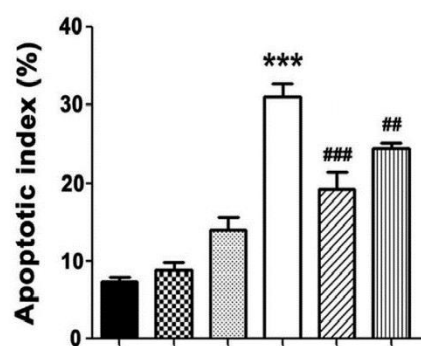
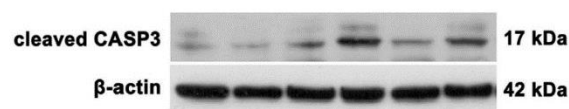
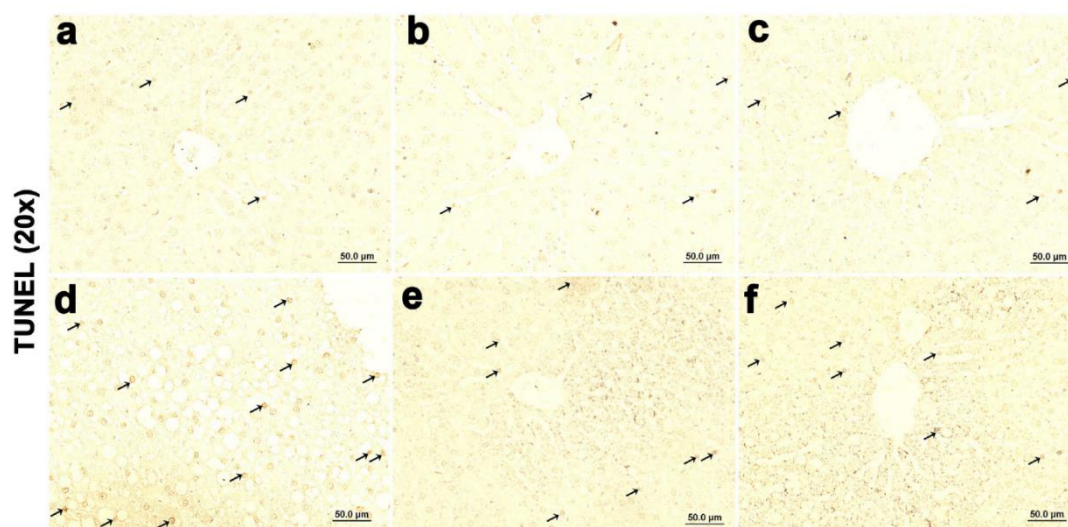
group to the normal control group, there was a substantial increase in 4-HNE expression. These findings suggest that the HFD rats had higher OxS levels. Significantly, treatment with RSV or Bac-A decreased the amount of ROS and the relative expression of 4-HNE. This suggests that giving Bac-A to HFD-fed rats improves hepatic Oxygen Stress and lowers lipid peroxidation.



3.6. Effects of Bac-A and rosuvastatin on apoptotic cell death.

A TUNEL assay was run to find out how many cells were apoptotic. The brown staining of these cells suggests that the nucleus contains fragments of DNA (Fig. 7A). Rats treated

with Bac-A or RSV showed a significant decrease in AI when compared to the HFD group (Fig. 7B). The HFD-fed rats had significantly higher levels of cleaved caspase-3 expression, but the Bac-A-treated HFD-fed rats showed lower levels of this expression (Fig. 7C).





4. Discussion

Rats given an HFD are used in this study's animal model of non-alcoholic fatty liver disease (NAFLD). In the HFD-fed rats, conditions indicative of non-alcoholic fatty liver disease (NAFLD) included insulin resistance, hepatic steatosis, liver damage, hyperlipidemia, and oxygen stress. [28-33] In these rats, the administration of Bac-A was observed to decrease hepatic damage, lipid accumulation in the liver, and NAFLD. The primary discovery of our research indicates that using Bac-A as a therapy is a potential protocol for hepatic injury prevention or therapy.

Our investigation showed that HFD-induced NAFLD was linked to a number of metabolic hepatic pathologies brought on by excess fat accumulation, including hepatic dysfunction, "ROS production," and insulin resistance. These findings are consistent with those of prior research. In addition to expanding hepatocellular lesions, the disarray of hepatic lobules in steatosis is typified by lipid vacuoles of different sizes as a result of fatty cell degeneration.[37] Furthermore, since excess TGs are deposited in the liver where they build up as lipid droplets, the TG level is another important factor linked to NAFLD. Fasting lipid profiles are typically utilized because they offer up-to-date metabolic information and show alterations in vital organs including the liver and heart. Furthermore, elevated free cholesterol levels have cytotoxic effects that primarily lead to the accumulation of triglycerides, which accelerates the advancement of non-alcoholic fatty liver disease (NAFLD).[38,39]

Similar to the effects of RSV treatment, Bac-A was found to have a positive effect on fat deposition in this investigation. RSV was shown to alleviate hepatic steatosis brought on by diets heavy in fat and cholesterol by inhibiting the expression of SREBP-1c, according to a prior study [40]. An essential transcription factor for the production of fatty acids is SREBP-1c. Critical mechanisms, such as elevating the expression of LDL receptors in liver cells and encouraging the esterification of FFA, may be impacted by the overexpression of SREBP-1c. Insulin increases SREBP-1c regardless of blood glucose levels. [41] Furthermore, hepatic TG deposition is encouraged by SREBP-1c transcription, which results in NAFLD.

Bac-A therapy decreased the ratio of pSREBP-1 α /SREBP-1c expression, which in turn decreased the fat content of the liver. Therefore, through the relationship between FFA influx and insulin resistance, increased fat deposition in the liver may play a role in liver damage and the advancement of liver disease. IR is a common reaction to hyperinsulinemia and is linked to chronic low-grade inflammation. [42] Numerous mediators are released by immune cells and adipocytes as a result of insulin resistance. Hepatocytes contain the facilitative glucose transporter GLUT2, which moves to the cell membrane when the insulin signaling pathway is activated.

Unstable byproducts of intracellular metabolism, reactive oxygen species interact with a variety of substances, including DNA, fatty acids, and proteins. Production of ROS can result in an imbalance between oxidant products and pro-oxidants, which can harm liver cells by causing lipid peroxidation. The protein that has been changed with 4-hydroxy-2-nonenal (4-HNE) has a longer half-life than ROS. [43] 4-HNE can enter cells freely and cause cell death and the disintegration of the phospholipid cell membrane. OxS is the primary cause of NAFLD and a key marker of developing NASH, as shown by a marked elevation in 4-HNE level. OxS is essential for NAFLD and liver damage. According to several researchers, therapy with Bac-A and RSV improves OxS. Therefore, it is possible that RSV and Bac-A have antioxidant activity and a protective impact against OxS, which may aid to lessen NAFLD.

Excess TG accumulation is a major factor in the development of NAFLD-related apoptosis, since lipids can passively diffuse over the plasma membrane into cells and cause lipotoxicity, or they can enter liver cells by fatty acid translocase.* The expression of caspase-3, cleaved caspase-3 in its active form, is the cause of DNA fragmentation. Positive TUNEL labeling indicated hepatocyte death because hepatic apoptosis is a common characteristic of NASH. [44] According to our findings, Bac-A therapy decreased the expression of cleaved caspase-3 and prevented apoptosis in NAFLD cases, which is associated with a favorable prognosis for liver damage in rats fed a high-fat diet.

Lastly, in rats with metabolic syndrome brought on by a high-fat diet, Bac-A decreased liver damage. Along with



lower OxS and apoptotic cell death, the reduction in dysfunction is probably caused by changes in the management of lipid accumulation and insulin resistance. Bac-A should therefore be given priority in next research as a possible pharmaceutical or functional food therapy to guard against OxS and metabolic dysfunction linked to NAFLD.

In conclusion, an HFD causes rats to produce ROS, which is linked to the buildup of fat and the death of apoptotic cells in the liver. The results of this investigation show that Bac-A medication improves NAFLD by lowering insulin resistance, dyslipidaemia, hyperglycaemia, glucose intolerance, and fat accumulation. [45] The improvement in liver disease linked to Bac-A may be due to a molecular process that involves the stimulation of the insulin signaling system, which in turn causes GLUT2 translocation to the membrane. Moreover, it was shown that Bac-A therapy inhibited the apoptotic pathway, which decreased the generation of ROS (Fig. 8). Consequently, Bac-A may offer a viable substitute therapy to slow the advancement of chronic NAFLD.

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