Aswan University Medical Journal, volume 4 / No.2/ September 2024 (67-79) Online ISSN: **2735-3117**

ORIGINAL ARTICL

Ameliorative Effect Of Rosuvastatin On High Fat Diet Induced Nonalcoholic Fatty liver Disease In Adult Male Albino Rats

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ABSTRACT

Keyword: NAFLD, rosuvastatin, HFD.

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Background: Nonalcoholic fatty liver disease (NAFLD) is emerging as one of the major causes of chronic liver damage that includes a wide spectrum of liver injury ranging from steatosis to steatohepatitis, evolving to fibrosis. **Aim of the work:** The purpose of our present study is to investigate the role of rosuvastatin in ameliorating the histological changes observed in an animal model of HFD-induced NAFLD. **Materials and Methods**: forty adult male albino rats were divided into 2 groups: the control group, the HFD group, which was divided into 3 groups: HFD-control, crestor group, and withdrawal group. **Results:** Our results showed that rosuvastatin significantly ameliorated the histopathological lesions induced by HFD. Furthermore, HFD induced a significant elevation in blood glucose level, lipid profile, and liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This was significantly attenuated by rosuvastatin treatment and withdrawal of HFD. **Conclusion:** These findings indicate that rosuvastatin and withdrawal of HFD possess a marked role in modulation of NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinical pathological syndrome characterized by excessive fat deposition in liver cells in the absence of excessive alcohol consumption and other specific liver-damaging factors **(1).** NAFLD comprises a spectrum of pictures ranging from benign (simple hepatic steatosis) to non-alcoholic steatohepatitis (NASH), in which the intra-hepatocytic accumulation of triglycerides is further accompanied by an intense inammatory activity, till overt liver cirrhosis and all its allied complications (e.g., liver failure, portal hypertension, and hepatocellular carcinoma (HCC))**(2).** As a hepatic manifestation of the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) is linked to abnormalities in glucose and lipid metabolism as well as insulin resistance (IR) **(3).** NAFLD is now considered to be the most common cause of chronic liver disease, with an estimated prevalence of 20–30% in the adult population and peaks of 70–90% among obese and diabetics. This is due in large part to the high frequency of blood tests and ultrasound examinations performed on asymptomatic individuals **(2).** The medical and economic NAFLD burden is because of progressive liver disease and an increased risk of chronic kidney disease, cardiovascular disease (CVD), and some kinds of extrahepatic cancer, such as colorectal cancer or others **(4).** The multiparallel hypothesis for NAFD proposed that it results from numerous conditions acting in parallel, including genetic

predisposition, abnormal lipid metabolism, oxidative stress, lipotoxicity, endoplasmic reticulum stress (ER stress), mitochondrial dysfunction, altered production of cytokines and adipokines, and gut dysbiosis [(5)].

Statins are lipid-lowering agents acting as inhibitors of the 3-hydroxy-3 methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol. Besides their lipid-lowering effect, statins have lots of wonderful positive pleiotropic effects, including anti-inflammatory, anti-proliferative, antithrombotic, improving endothelial dysfunction, and neuroprotective effects [(6)]. Statins are classified under three categories on the basis of their chemical structure, origin, and solubility (e.g., **rosuvastatin**, pravastatin, simvastatin, fluvastatin, and lovastatin) [(6)]. Rosuvastatin has exhibited a more potent affinity for the active site of HMG-CoA reductase than other statins. In addition, the hepatic uptake of rosuvastatin in rats has been found to be more selective and efficient than that with other drugs [(7)].

MATERIALS AND METHODS

Ethical considerations

Adult male albino rats (8 weeks old) were purchased from the Animal House of Assiut University. Rats were housed in the animal house of Assiut University under strict care and hygiene. The animals were kept in a room with a light/dark cycle of 12 hours and a controlled temperature between 22 and 24 °C, with free access to food and water. Animals were fed a normal chow diet (NCD) for 1 week to allow them to acclimate to the environment before experiments began. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Assiut University.

Drugs, chemicals, and kits:

The following drugs, chemicals, and kits were used and purchased from sources indicated.

1. Crestor: trade name of rosuvastatin purchased from A pharmacy in Assuit.
2. High-fat diet: containing 58% fat; classified as follows (20% cholesterol from ramtail; 20% hydrogenated oil; 18% proteins; and 24% carbohydrates).
3. Immunohistochemical kits:

Primary antibodies:

Anti-caspase-3 antibody and NFKB were purchased from Thermo Fisher Scientific Company (Waltham, MA, USA), and anti-iNOS antibody was purchased from Bioss Antibodies Company (Boston, MA, USA).

Detection System for antibodies:

Ultra Tek horseradish peroxidase (HHRP) anti-polyvalent (DAB) staining system (Scy Tek laboratories, West Logan, west UT, USA, AMF080).

Experimental design and animal treatment

A total of forty adult male albino rats were used in this study and divided into two groups:

Group I: control group (n = 10); they were fed a NCD during the whole experiment and served as an untreated control group.

Group II: (n = 30), they were fed a 60% HFD for 10 weeks to induce NAFLD and NASH changes. Afterwards (starting from the 11th week to the 15th week), group II further subdivided into three groups:

Group IIa: HFD group (n = 10); they were fed a 60% HFD and served as a HFD control group.

Group IIb: HFD-RSV group (n = 10); they were fed a 60% HFD plus rosuvastatin suspension dissolved in distilled water with a concentration of 10 mg/mL and will be administered orally using gastric gavage [8]. For 5 weeks and served as the treated group.

Group IIc: HFD-NCD group (n = 10); they will be fed a NCD and served as a withdrawal group.

Animals were weighed weekly until the end of the experiment for comparison.

Biochemical Analysis:

Fasting blood glucose was determined also, and the following biochemical parameters were measured: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC), HDL, LDL, and VLDL.

Histological examination

Immediately after the animals were sacrificed, small specimens were taken from the liver of all groups and prepared for light microscopic examination.

Hepatic tissue samples were fixed in 10% formalin for 24 h, then they were dehydrated by putting them in ascending concentrations of ethanol (50%, 70%, 90%, and 100%), followed by a clearing agent (xylol). After that, the tissues were embedded in melted paraffin wax, then cooled down to form paraffin blocks. The paraffin blocks were sectioned at 4-5 m thickness with a slicing machine (the microtome) [9]. Sections separated from the paraffin blocks were put on glass slides. The slides were warmed on a hotplate to straighten the sections and then dried in an incubator, ready for staining with:

1. **Hematoxylin and eosin (H&E) stain** for general histological examination.
2. **Masson's trichrome stain** to assess collagen fibers.

These stains were used according to **Bancroft and Gamble [10].**

Immunohistochemistry (IHC):

The following primary antibodies were used: Anti-caspase-3 antibody: for detection of apoptotic cells, **Anti-nuclear factor kappa B (NFKB) antibody**: as an inflammatory marker; **Anti-inducible nitric oxide synsthase (iNOS) antibody**: as an oxidative stress marker.

Statistical analysis:

Data were expressed as the mean ± standard deviation (SD). All analyses were performed with the IBM Statistical Package for the Social Sciences (SPSS) 20.0 software. A one-way ANOVA followed by a post hoc test will be used for comparisons among groups. $P < 0.05$ was considered statistically significant.

RESULTS

A-Histological Results

H&E-stained liver sections of the control group showed the normal architecture of hepatic lobules, with the central vein in the center and the portal areas at the periphery. The liver cells (hepatocytes) radiate from the central vein in the form of plates of two cell thickness and separated from each other by blood sinusoids. The liver cell is polyhedral with pale acidophilic cytoplasm and rounded, vesicular nucleus with prominent nucleolus **(Figs. 1a&b).**

Group IIa (HFD-control) showed accumulation of lipid droplets (steatosis) and hepatocellular ballooning; many apoptotic cells with dark stained nuclei and highly eosinophilic cytoplasm were observed, and inflammatory cellular infiltration and congested blood vessels were detected **(Figs. 1c&d).**

Group IIb (crestor) showed marked improvement in liver architecture with resolution of steatosis, ballooning, and inflammation. Most liver cells appeared with vesicular nuclei and pale acidophilic cytoplasm. Only a few apoptotic cells appeared with dense nuclei and deeply stained acidophilic cytoplasm **(Figs. 1e&f).**

Group IIc (withdrawal of high-fat diet) There was improved hepatic architecture with resolution of steatosis, ballooning, and cellular infiltration. Some hepatocytes appeared with vesicular nuclei and pale staining cytoplasm, while others appeared apoptotic with dense nuclei and deeply stained cytoplasm **(Figs. 1g&h).**

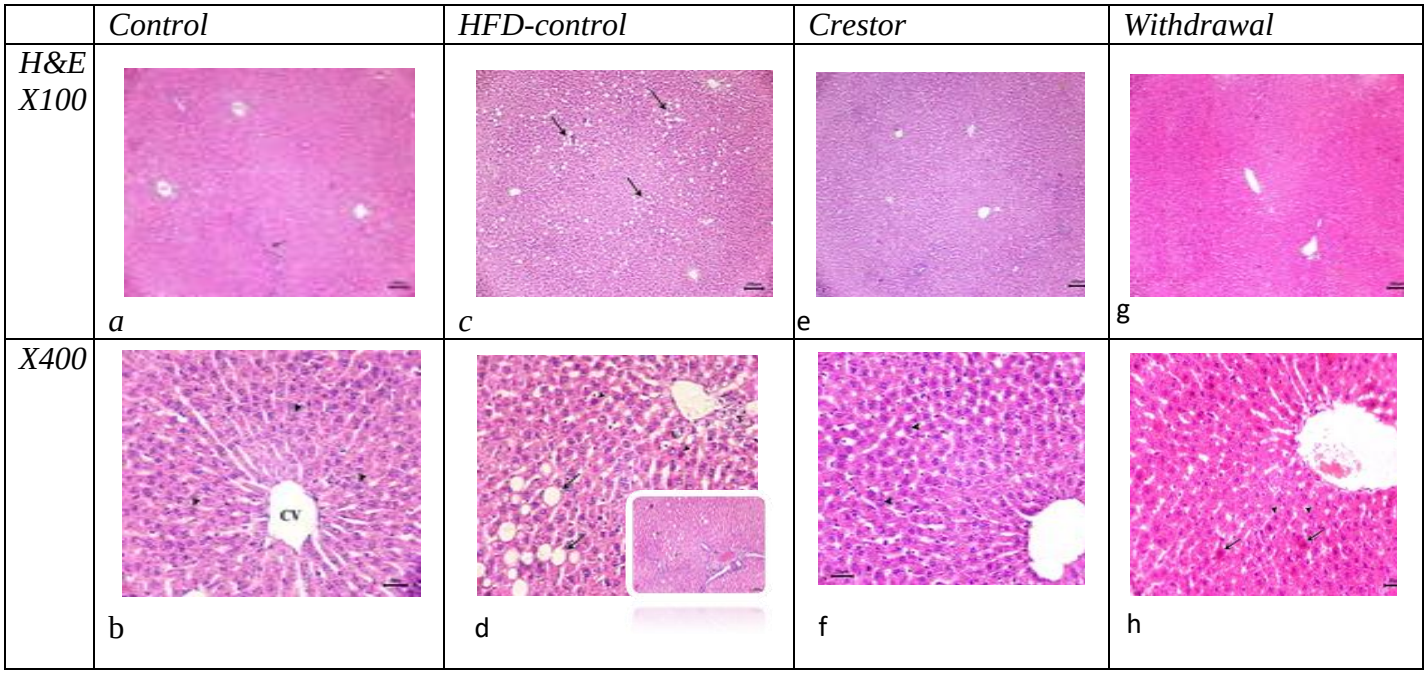


Fig.1. H&E stained liver sections of experimental groups showed normal classic liver architecture of the control group (a&b), accumulation of lipid droplets (steatosis), hepatocellular ballooning (aarrows), many apoptotic cells observed(▲) and inflammatory cellular infiltration (*), and congested blood vessels were detected *in the HFD-control group (c&d); obvious improvement of the liver structure and normalization of the hepatocytes in the crestor group (e&f); and moderate improvement in the withdrawal group (g&h). CV central vein; V vaculation;* inflammatory infiltration.*

Masson`s tricome*-**stained liver sections in the control group There were delicate collagen fibers surrounding the central vein and in between the liver cells**.** Minimal collagen fibers around the portal area ***(Figs. 2a&b).

Group IIa (HFD-control) there was an increase in collagen fiber deposition around the central vein and massive collagen deposition in the periportal area **(Figs. 2c&d).**

Group IIb (crestor) Minimal collagen fiber deposition around the central vein, in between liver cells, and around the portal area **(Figs. 2e&f).**

Group IIc (withdrawal of high-fat diet) Mild collagen fiber deposition was observed around the central vein and in between hepatocytes, while there was moderate collagen fiber deposition around the portal area **(Figs. 2g&h).**

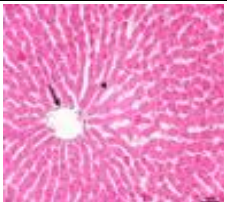
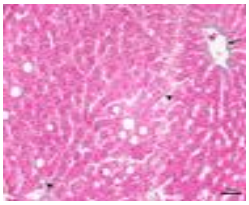
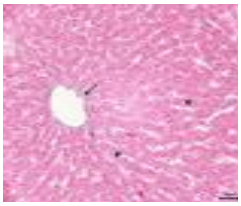
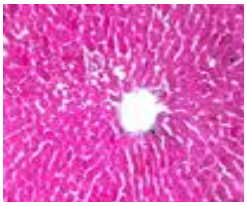
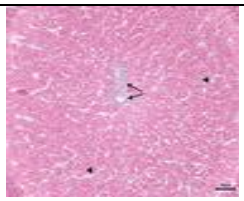
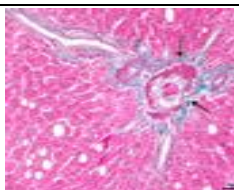
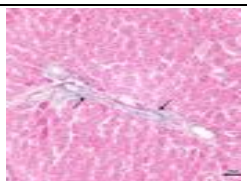
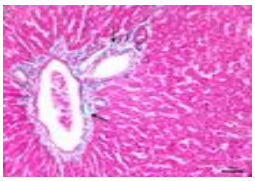
	Control	*HFD-control*	*Crestor*	*Withdrawal*
Masson`s tricome staining X100	 a	 *c*	 *e*	 *g*
X400	 *b*	 *d*	 *f*	 h

Fig. 2. Masson`s tricome-stained sections show delicate collagen fibers surrounding the central vein and in between the liver cells and minimal collagen fibers around the portal area (a&b*),* massive collagen deposition around the central vein and in the periportal area in the HFD group (c&d)**,** mild collagen deposition in the crestor group (e&f), and a moderate amount in the withdrawal group (g&h*).*

B-Immunohistochemical results

In the control group, there was a weak immunopositive reaction for caspase 3 antibody in a few liver cells**.** A weak reaction for NFKB antibody was observed in the cytoplasm of liver cells, and a few liver cells showed a weak reaction for iNOS antibody **(Fig. 3.a, b & c).**

The HFD-control group showed a strong positive reaction for caspase-3 antibody in many liver cells around the central vein**.** There was increased immunoreactivity for nuclear factor KB in the cytoplasm of most hepatocytes, and there was a positive reaction for iNOS in many liver cells around the central vein **(Fig .3.a, b &c). .** The number of caspase-3, NFKB, and iNOS-positive cells was highly significantly increased in the HFD group compared to the control group (P = 0.001) **(Fig. 4).**

Crestor group there were few positive reactions for the caspase-3 antibody and few weak reactions for the nuclear factor KB antibody**.** A weak immunopositive reaction for iNOS was observed in a few hepatocytes **(Fig.3.a, b& c)**. There was a highly significant decrease in caspase-3 NFKB and iNOS-positive cells in crestor groups compared to the HFD-control group (P = 0.001) **(fig.4).**

The withdrawal group showed a positive reaction for caspase 3 in a few liver cells**.** Some hepatocytes showed a positive reaction for nuclear factor KB and Positive reactions for the iNOS antibody were detected in some liver cells **(Fig.3.a, b& c).** There was a highly significant decrease in caspase-3 and iNOS positive cells (P = 0.001) and a non-significant decrease in NFKB positive cells (P = 0.103) in withdrawal groups compared to the HFD-control group **(fig.4)**

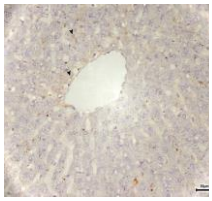
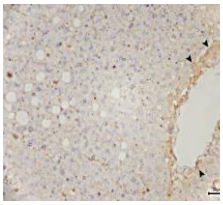
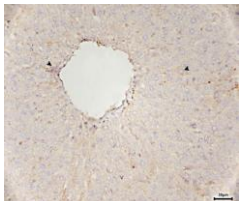
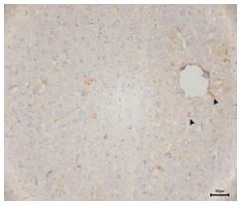
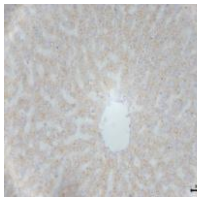
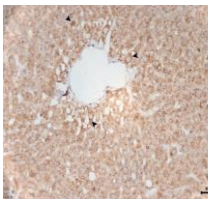
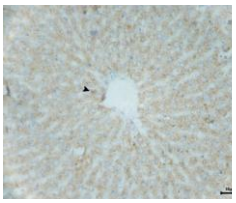
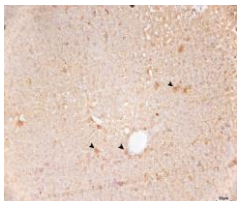
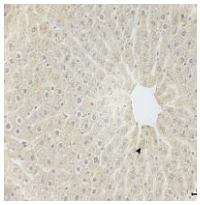
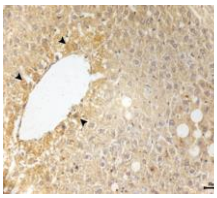
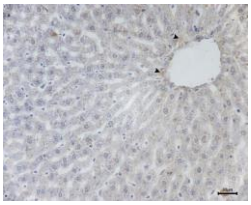
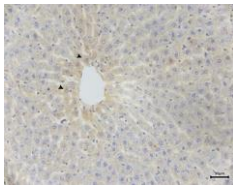
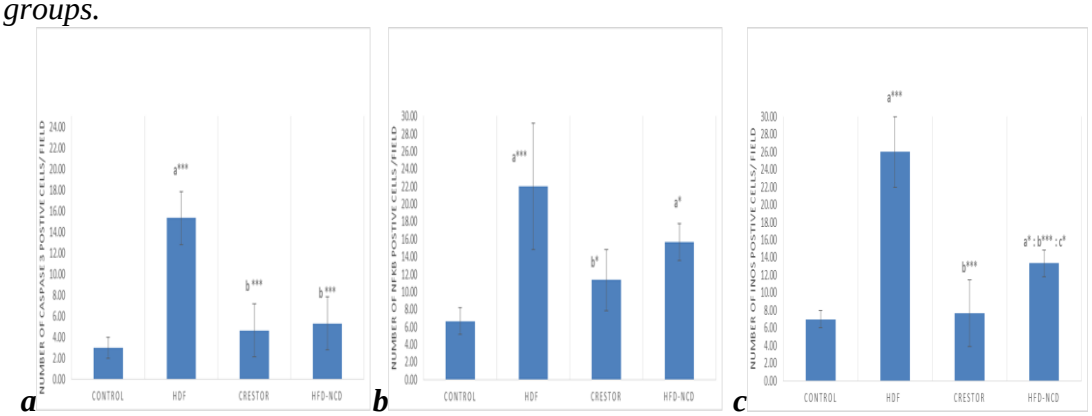
	Control	HFD-control	Crestor	Withdrawal
(a) Caspase 3 X400				
(b) NFKB X400				
(c) iNOS X400				

Fig.3. The control group showed a negative or weak reaction for caspase 3, NFKB, and iNOS, while a strong reaction was seen in the HFD-Control group and a mild reaction in the crestor and withdrawal groups.



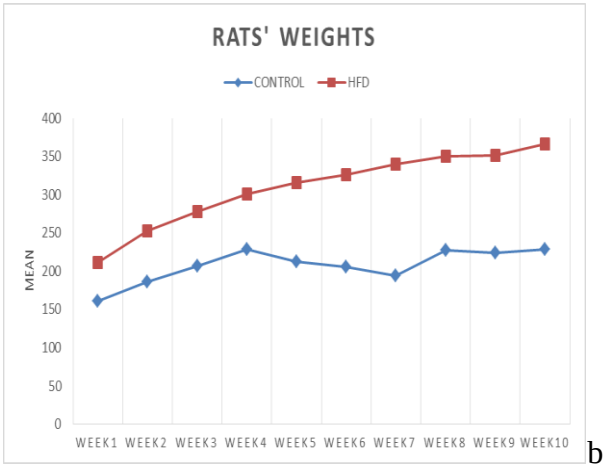
a ***b*** ***c***

Fig. 4. Quantitative estimation of the mean count of (a) caspase-3, (b) NFKB, and (c) iNOS positive cells. All of the values are expressed as mean ± SD; statistically significant differences are shown as *p < 0.05, **p < 0.01, and ***p < 0.001. 'a' vs. control, 'b' vs. HFD-control, 'c' vs.crestor. HFD=high fat diet,crestor=trade name of rosuvastatin, HFD-NCD=Withdrawal Group

Statistical analysis

Rats body weight

Results showed that feeding rats with HFD was accompanied by a significant increase in body weight gain as compared to the normal control group (p = 0.001) (Fig. 5a**).**There was a significant decrease in rats body weights in the crestor & withdrawal groups compared to HFD-control groups (p = 0.001). However, the achieved values of these parameters were still significantly higher than those of the normal control group **(Fig. 5b).**

a 

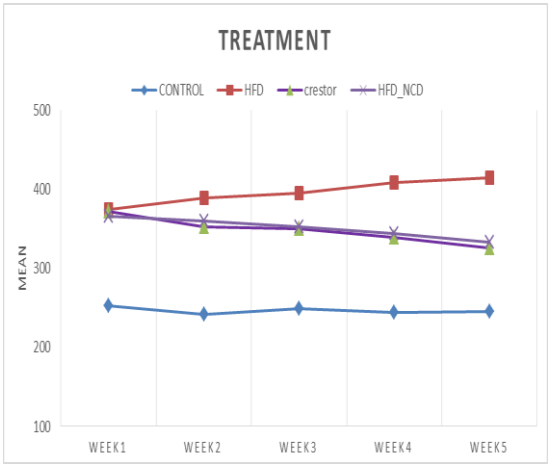
b 

Fig.5. (a) Comparison between control and HFD-fed rats body weights in the first 10 weeks; (b) Effects of rosuvastatin and withdrawal of a high-fat diet on body weight gain of different rat groups. One-way ANOVA test, Statistically significant difference (p<0.05), ** Highly statistically significant difference (p<0.01).*

Blood glucose level

There was a highly statistically significant increase in plasma glucose in the HFD group compared to the control group (P = 0.001). There was a highly significant decrease in blood glucose observed in the crestor group and withdrawal group compared to the HFD-control group (P < 0.001) **(fig.6).**However, the values of BGL were still significantly higher than those of the normal control group **(Fig. 6).**

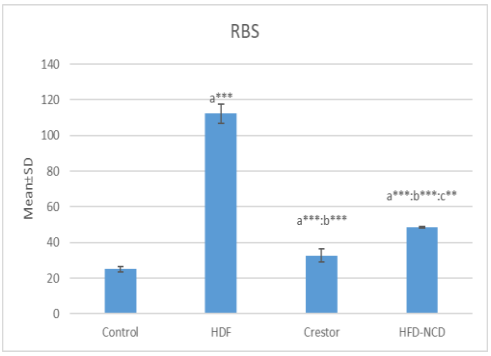


Fig.6. Effects of HFD, crestor and withdrawal of high fat diet on RBS of different rat groups. Data represent the mean ±S.E. of 8-10 observations. a: significant difference compared to the control group,b: significant difference compared to the HDF group,C: significant difference compared to the Rosuvastatin group at p<0.05. RBS=random blood sugar

Lipid Profile:

There was a significant increase in the HFD group in serum levels of total cholesterol, triglyceride, LDL-C, and VLDL-C. However, the serum level of HDL-C was highly significantly reduced as compared to the normal control group **(p = 0.001) (Fig. 6).**

There was a highly significant decrease in TC, TG, LDL, and VLDL values, plus a significant increase in HDL values in both the crestor group and withdrawal group compared to the HFD-control group **(p = 0.001)**. However, the levels of TC & LDL were still significantly higher than those of the normal control group, plus the level of HDL was still higher than the normal control group. **(fig.6).**

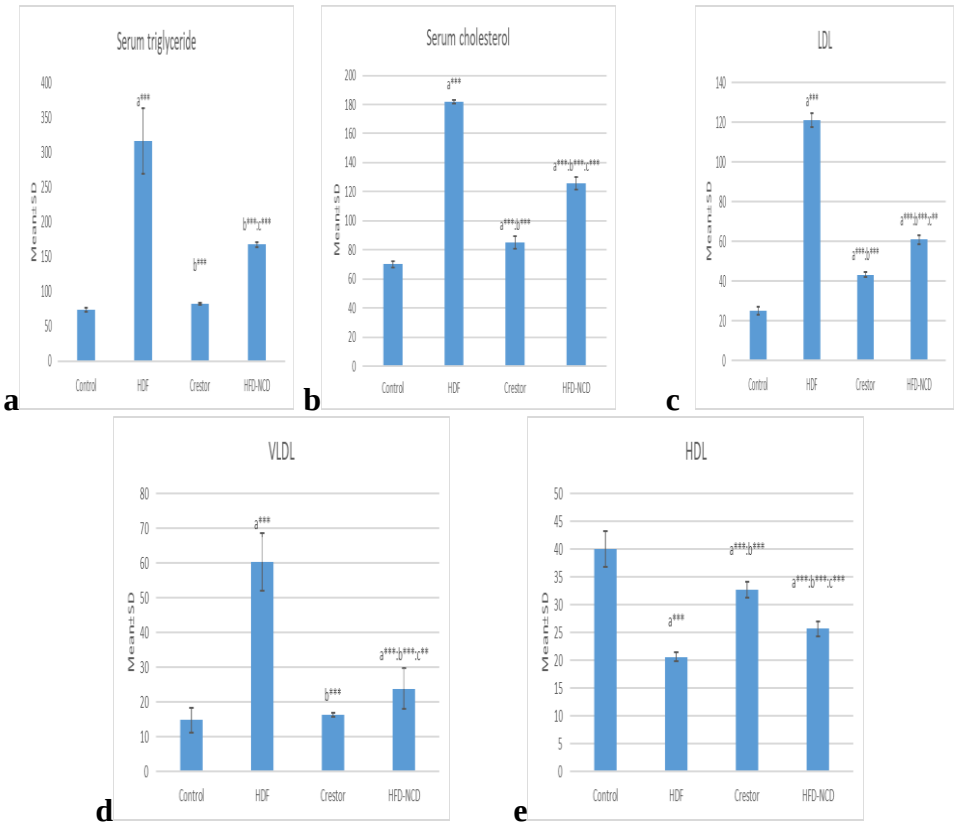


Fig.7. Effects of HFD, Crestor, and withdrawal of high-fat diet on lipid profiles of different rat groups. Data represent the mean ±S.E. of 8-10 observations. a: significant difference compared to the control group, b: significant difference compared to the HDF group, C: significant difference compared to the rosuvastatin group at p<0.05.LDL-C=low density lipoprotein cholesterol, HFD-C=high density lipoprotein cholesterol, crestor=trade name of rosuvastatin.

Alanine transaminase and aspartate transaminase:

There was a highly significant increase in both ALT & AST levels in the HFD group compared to the normal control group (p=0.001) **(Fig.8)**.There was a significant decrease in ALT& AST levels in both the crestor and withdrawal groups compared to the HFD-control group (p = 0.001). However, the achieved values of these parameters were still significantly higher than those of the normal control group (Fig. 8).

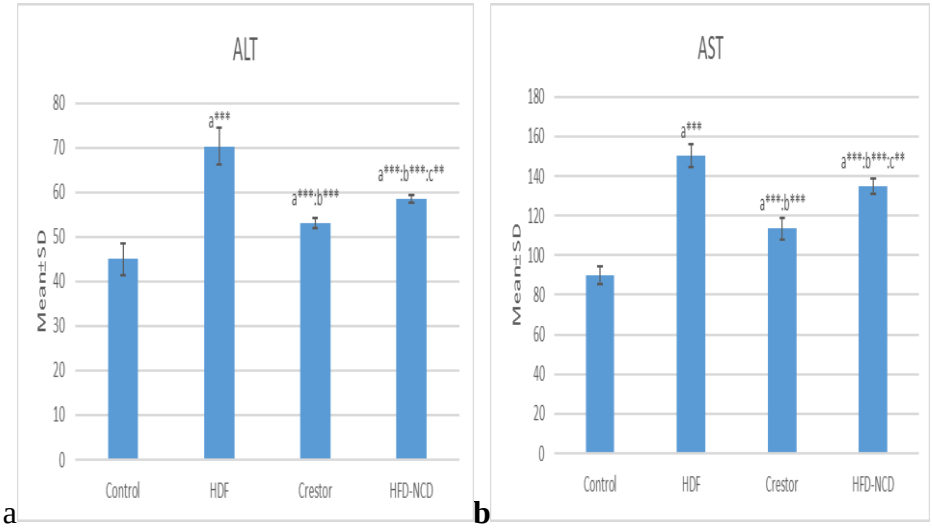


Fig.8. Effect of HFD, crestor, and withdrawal of high-fat diet on liver enzymes in different rat groups. Data represent the mean ±S.E. of 8-10 observations. a: significant difference compared to the control group, b: significant difference compared to the HDF group, C: significant difference compared to the rosuvastatin group at $p<0.05$.HFD=high fat diet, ALT= Alanine transaminase, AST= aspartate transaminase, crestor=trade name of rosuvastatin.

DISCUSSION

NASH was confirmed by histological findings of the liver tissues.liver of HFD-Control displayed steatosis, clusters of ballooned hepatocytes, and hepatic lobular inflammation accompanied by fibrosis[(11)].These findings are the necessary histological features defining human NASH and are in harmony with those previously observed in animal models of NAFLD [(12,13)].

Results of our present study indicated that treatment of the HFD group with rosuvastatin (crestor) caused attenuation of steatohepatitis. This was clearly evident in the liver pathology, which showed improvement of the severity grade of the disease near resolution and was demonstrated by a reduction in the elevated ALT &AST levels. The present findings are in agreement with previous studies confirming the beneficial role of rosuvastatin [(14–18)].

Meanwhile, in the current study, treatment of the HFD-fed rats group by withdrawal of a high-fat diet resulted in a reduction in ALT&AST levels compared to those of HFD-Control, which were still higher than the control group. The reduction in enzymes was associated with an improvement in the severity of the disease.

Consistent with our results, withdrawal of a high-fat diet induced a modest effect in inhibiting the progression of NAFLD and hyperlipidymia in HFD-fed rats [(19, 20)].

The forgoing results revealed that HFD-Control not only showed histological hepatic findings of NASH but also reproduced most of the metabolic features of the disease known to be implicated in the occurance of NAFLD in humans.For instance, HFD-Control visceral obesity as evidenced by the increased body weight gain along with dyslipidemia as reflected by elevation of serum TC,TG, and LDL--C levels accompanied with decreased HDL-C levels; in addition, these rats also exhibited increased serum levels of glucose. The same altered patterns of these metabolic indices were previously demonstrated in mice [(21)] fed with HFD and also in several clinical cases of NAFLD patients [(22, 23)].

The present data revealed that treatment of HFD-fed rats with rosuvastatin led to improvement of hyperglycemia together with reduction of the elevated lipid profile of serum TC, TG, and LDL-C and VLDL-C levels, accompanied by increased HDL-C levels. Rosuvastatin succeeded in improving obesity by decreasing body weight gain in HFD-Control compared with the control group. These results are in harmony with previous experimental studies that documented the improvement of glucose hemostasis and lipid profile [(15-17, 24)].

The present study demonstrated the ability of withdrawal of a high-fat diet to decrease body weight gain, decrease blood glucose level, and improve serum levels of TG,TC,LDL-C &VLDL-C accompanied by increased HDL-C levels. These results are in harmony with previous studies like [(19, 20, 25)].

Since oxidative stress and inflammation, which are likely culprits in the pathogenesis of NAFLD [(26)], it's not surprising to attract considerable attention in our study.Data from the present study showed a significant increase in iNOS activity in NAFLD rats. These findings are in agreement with previous studies [(27-30)]. The interesting finding in the current study is that the effectiveness of both rosuvastatin and withdrawal of HFD showed antioxidant properties as they decreased iNOS activity in rats fed with HFD. In the case of rosuvastatin, these findings are in accordance with those of a study by Wang et al. that demonstrated that male Wistar rats were fed a high-fat diet to develop NASH and exhibited an increase in expressions of iNOS. While rats in the simvastatin

group had decreased expressions of iNOS [30], **Chong et al.** showed that fluvastatin decreased hepatic steatosis besides decreasing Il6 and iNOS [31].

One regimen that guards against oxidative stress is dietary restriction (DR). By lowering the generation of reactive oxygen species (ROS) and raising the activity of antioxidant enzymes, food restriction lowers oxidative stress in rodents and minimizes oxidative damage to macromolecule [32].These results are in agreement with previous studies [33,34].

Inflammation plays a very important role in the development of NAFLD. When lipid peroxidation occurs, it will lead to an inflammatory response and might further lead to apoptosis and cell death, which prevents the reversal of NAFLD [35]. Our results show that immunohistochemical staining of NKKB in the HFD-Control group showed a significant increase in immunoreactivity in the cytoplasm of hepatocytes. These results are in harmony with [35, 33].

Independently of its lipid-lowering capacity, rosuvastatin has been shown to decrease the expression and/or plasma levels of various proinflammatory cytokines [34]. In our study, there was a significant decrease in immunoreactivity of NFKB in liver cells in the rosuvastatin-treated group and withdrawal group in comparison with the HFD group. These results matched several studies that demonstrated that rosuvastatin suppressed inflammatory responses through inhibition of nuclear factor-kappa B in endothelial cells [16, 37].

Until today, there have been limited epidemiologic data assessing the role of diet's inflammatory potential on NAFLD. In our study, withdrawal of HFD decreased inflammation by decreasing expression of nuclear factor KB immunohistochemically. These results keep with previous studies. Yang et al.'s study shows that the alternate-day fasting (ADF) regimens inhibited the expression of nuclear factor κB protein in the liver, as well as the genes involved in the inflammatory process [38] and Park et al.'s study shows that weight loss caused by moderate caloric restriction (CR) shows a significant decrease in the expression of pIκBα (an indirect way to evaluate NFκB activation) in the high fat caloric restriction group (HFCR)[33].

Apoptosis has been identified as an important mechanism for liver damage and is strongly associated with the pathogenesis of NAFLD [39]. Anti-caspase-3 antibody was used in this study for immunohistochemical detection of apoptotic hepatocytes and revealed a prominent increase in Caspase-3 immunoexpression in HFD-Control group compared to the control one this keep with some studies like **Elalfy et al** , results of this study indicates that expression of caspase-3 as detected by immunohistochemical staining was significantly higher in NASH patients in comparison to patients with simple steatosis, proving a strong relationship between the amount of fatty liver and caspase-3 expression [40]; **Ferreira et al.** which showed a significant increase in DNA fragmentation and Caspase-3 and -2 activation in the livers of patients with severe NASH compared to those with simple steatosis ($p < 0.01$) [41] and according to studies by Thapaliya et al., WT mice given an MCD diet exhibited hepatocytes that were markedly activated by caspase 3, along with steatohepatitis, elevated hepatic triglyceride levels, hepatocyte ballooning, inflammation, and fibrosis. [42]

Surprisingly, results in our study showed that rosuvastatin had a prominent decrease in caspase-3 immunoexpression in liver cells of the HFD-Control group, as rosuvastatin suppressed activity of caspase-3 enzyme. These effects of rosuvastatin shown in some studies like [37] **s**howed that rosuvastatin has anti-inflammatory and anti-apoptotic effects by regulation of oxidative stress. Also, rosuvastatin reduced cell death through reduction of caspase-3 activity in human neuroblastoma [43].

In spite of the lack of data about the relation between caloric restriction and apoptosis induced by caspase caspase-3 pathway, in our study, diet restriction showed inhibition of the activity of caspase-3. According to one study by Gültekin et al., calorie restriction shields rats' hippocampal and dorsal root ganglion cells from apoptosis and mitochondrial oxidative stress [44].

CONCLUSION

The results of our current study indicated that treating the high-fat group of mice with either rosuvastatin or removing fat from the diet caused an attenuation of steatohepatitis. This was clearly evident in the histological examination of the liver, which showed an improvement in the degree of severity of the disease near complete recovery, and this is shown by a decrease in the high levels of ALT and AST, in addition to the improvement of hyperglycemia with a reduction in the high level of lipids in the levels of TC, TG, LDL-C, and VLDL-C accompanied by increased levels of HDL-C, and finally both decreased immunoexpression of caspase-3, NFKB, and iNOS.

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