Potential Protective Effect of *Zingiber officinale* in Comparison to Rosuvastatin on High-fat diet-induced Non-alcoholic Fatty Liver Disease in Rats

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Abstract

BACKGROUND: Non-alcoholic fatty liver disease (NAFLD) is a common liver disease affecting nearly 25% of adults worldwide with related risk factors including obesity, metabolic, and inflammatory diseases. Many therapeutic remedies of natural or synthetic properties were used.

AIM: This study aimed to investigate and compare the effects of ginger/rosuvastatin (ROSU) on the liver of rats with induced NAFLD.

MATERIALS AND METHODS: Forty adult male albino rats were used in this study and divided into four equal subgroups, Group I, control received the standard rat chow diet and given normal saline (1 ml/kg/day), Group II, high-fat diet (HFD) group, Group III, received HFD+ ROSU (15 mg/kg/day), and Group IV, HFD+ *Zingiber officinale* (10% W/V) for 6 weeks. At the end of our experiment, the rats were sacrificed then blood samples were collected for biochemical analysis of lipid profiles and liver enzymes, liver specimen was prepared for light and electron microscopic examination, and measurement of tissue level of malondialdehyde.

RESULTS: NAFLD caused degenerative changes and lipid deposition in liver cells as evidenced by microscopic results and laboratory tests. Treatment with ginger/ROSU alleviated those changes.

CONCLUSION: Ginger and ROSU could ameliorate liver functions in NAFLD and ginger effect is superior to ROSU.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a diverse hepatic state induced by irregular fat deposition in hepatocytes with or without inflammation. It causes many liver diseases; including steatosis, steatohepatitis, liver fibrosis, cirrhosis, and liver failure [1].

The guidelines of the American Association for the Study of liver diseases categorize the NAFLD histologically into two main categories; fatty liver and steatohepatitis.

The first one is considered a benign disorder, whereas the development of such condition in later stages has severe complications including hepatocellular injury, liver inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma [2].

High dietary intake of carbohydrates is a risk factor in the pathogenesis of NAFLD [3]. Many clinical trials were done to treat NAFLD but it remains a challenge for the scientific community without licensed therapies till date [4].

Diet and modifications of lifestyle have been shown to be important tools for the prevention and

control of NAFLD but still there is no effective clinical management of this disease [5].

Many therapeutic methods were investigated to modulate pathogenesis of NAFLD. The use of nutritional therapy and natural products for many health problems has become the interest of many researchers [6].

Ginger (_Zingiber officinale_ Roscoe), belongs to the Zingiberaceae family, is widely used in many countries for its anti-inflammatory, antihepatotoxic, antidiabetic, and antioxidant effects [7]. Rosuvastatin (ROSU) is a member of statin family, which is comprised the anti-hyperlipidemic agents. ROSU inhibits 3-hydroxy-3-methyglutaryl coenzyme reductase. Independent of its lipid-lowering effect, ROSU has also anti-inflammatory and antioxidant properties [8].

This study was performed to assess and compare the effectiveness of _Zingiber officinale_ extract and ROSU, on lipid profile, liver structure, and functions in rats with induced NAFLD.

Materials and Methods

Drugs and chemicals

ROSU was obtained from AstraZeneca Company (Giza, Egypt). Ginger was obtained in powder form and dissolved in distilled water 125 g/L for 12 h at room temperature then filtered [9]. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs), and malondialdehyde (MDA) kits were purchased from Biodiagnostics (Egypt). Anti-caspase-3 antibody was purchased from Dako Corporation (Life Trade, Egypt).

Preparation of high-fat diet

A high-fat diet (HFD) model was followed for the induction of NAFLD in rats. It implies feeding rats with high cholesterol (atherogenic) diet (79% standard diet and 21% ghee fat) for 6 weeks [10].

Animals

Animal handling was approved by the Medical Ethical Committee, Damietta Faculty of Medicine, Al-Azhar University (IRB 0001267-21-07-005).

Forty male adult albino rats were left in the experimental room for 1 week before the study for acclimatization, then, animals were randomly divided into four groups (10 rats in each group – 5/cage): Group I, control group received the standard chow diet and given normal saline (1 ml/kg/day) by gastric intubation for 6 weeks, Group II: HFD group (received HFD orally for 6 weeks), Group III: HFD (received HFD orally)+ ROSU (15 mg/kg/day, and Group IV, HFD+ _Zingiber officinale_ (125 mg ginger powder dissolved in 1 L of distilled water). The ginger powder (10% W/V), ROSU tablet in the form of watery solution in a dose of 15 mg/kg was freshly prepared and given daily by oral gavage tube.

Blood sampling, serum, and tissue preparations

At the end of our experiment (on day 42), blood samples were obtained from the retro-orbital plexus, then, we separated serum by centrifugation at 1200 g for 15 min, collected, and kept at 20 °C for further laboratory analyses. Moreover, liver specimen was rapidly excised after scarification of rats and washed with normal saline solutions (0.9% NaCl in distilled water) to be prepared for histological evaluation.

Serum biochemical analyses

The serum concentrations of lipid profile (TG, TC, LDL, and HDL) and liver enzymes (ALT and AST) were examined by Beckman Coulter chemistry analyzer.

Assay of oxidative stress marker

The liver specimen was perfused with ice-cold 50 mmol/L sodium phosphate-buffered saline (100 mmol/L Na_2HPO_4/NaH_2PO_4, pH 7.4) containing 0.1 mmol/L EDTA to wash away the RBCs and clots. Tissue samples were homogenized in 5–10 mL ice-cold buffer/1 g tissue. Then, centrifugation of the homogenate was done for 30 min at 3000 g. The obtained supernatant was stored at −80 °C for MDA analysis.

The concentrations of MDA in plasma and in hepatic tissue were measured indirectly with thiobarbituric acid [11]. The hepatic tissue MDA concentration was normalized against the protein concentration.

Hematoxylin and Eosin staining and immunohistochemistry

The liver specimens were fixed in 10% formalin for at least 24 h. The collected liver tissues were processed and sectioned at a thickness of 5 μm and stained with hematoxylin and eosin and immunostain for caspase 3. The prepared slides were examined under light microscopy by the histologists and pathologists. The images were photographed and the percentage area density of caspase 3 was measured using a Raywild E5 microscope with a Raywild M-300

digital camera with image analyzing system (Mvi-mage program v12).

For electron microscope examinations

Samples of liver tissue were cut into 1 mm³ thickness, fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C. Routine processing for E.M was carried out in the unit of electron microscope. Semi-thin sections (0.5–1 um thickness) stained with toluidine blue for structural assessments using L.M. Ultrathin sections, 60 nm thick, were also prepared and stained by lead citrate 2% and uranyl acetate [12]. Photographing was done by E.M (JEM-100 Cx11, JEOL, Egypt) at 80 kV.

Statistical analysis

Data were presented as the mean ± standard deviation and analyzed by the two-tailed Student's test and one-way analysis of variance test using the statistical software package SPSS for Windows OS (Version 21.0; SPSS Inc., Chicago, IL, USA) followed by the Duncan's *post hoc* test for multiple group comparison. Statistical significance was considered at $p < 0.05$.

Results

Effects on lipid profile

There was a significant increase in the level of cholesterol; TGs and LDL in animals treated with HFD for 6 weeks in comparison to the control group, while a significant reduction in level of HDL were observed in comparison to other groups. Daily administration of 2 ml ginger (10% W/V)/ROSU (15 mg/kg) for 6 consecutive weeks induced a significant reduction in serum LDL, TG, and significant increase in HDL level in normal rats. Compared to HFD rats model, 2 ml ginger 10% (W/V)/ROSU produced significant reduction in serum cholesterol, TG, serum LDL, and serum HDL. Furthermore, the use of ginger showed significant changes in comparison to ROSU (Table 1).

Effects on serum levels of liver enzymes and MDA

Serum AST and ALT of rats on HFD had significantly increased when they compared with normal rats. No significant change in ALT and AST was observed in HFD rats treated with ginger/ROSU as compared with rats received HFD alone. Tissue level of MDA in the liver of the rats treated with HFD showed significant increase when compared to the control group, while daily administration of 2 ml ginger 10% (W/V)/ROSU significantly reduced MDA when compared to rats of HFD. Furthermore, the use of ginger showed significant changes in comparison to ROSU (Table 2).

Table 2: Assay of serum levels of liver enzymes, malondialdehyde, and caspase 3

Parameters (mean ± SD)	Control group	HFD	HFD+ROSU	HFD+Ginger
ALT (iu)	30.8 ± 7.2	70.4 ± 6.5	82.1 ± 19.3	63.3 ± 17.8
p	< 0.005			
AST (iu)	151.3 ± 4.43	197.3 ± 14.1	266 ± 23.2	165.2 ± 15.5
p	< 0.005		< 0.005	
MDA (mg/dl)	12.5 ± 1.29	23.60 ± 2.43	7.30 ± 1.25	5.5 ± 1.2
Caspase 3 (%)	0.728 ± 0.847	6.47 ± 1.0250	2.65 ± 0.924	1.34 ± 1.048
p	< 0.005			

MDA: Malondialdehyde, HFD: High-fat diet, ROSU: Rosuvastatin, SD: Standard deviation, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase.

Light microscopic findings

Hematoxylin and eosin stained sections results

The liver from control rats group showed normal structure of hepatic lobules. The liver cells appeared polyhedral in shape exhibiting acidophilic cytoplasm and central round vesicular nuclei. Hepatocytes were arranged in cords radiating from the central vein and separated by blood sinusoids. Portal area was normal in shape, with no infiltration of inflammatory cells. In contrast, the liver structure of rats of HFD showed that minimal dilation of blood sinusoids, many Kupffer cells, and vacuolar fatty degeneration were detected in hepatocytes near the central vein. The nuclei of liver cells were deformed and darkly stained, dilated portal vessels surrounded by cell infiltrates. Liver sections from HFD rats treated with ROSU showed slight dilatation of the sinusoids with prominent Kupffer cells. Hepatocytes show homogeneous cytoplasm, with no lipid deposits; they have normal central vesicular nuclei. The portal tract area appeared with normal vessels and nearly normal hepatocytes. The liver of HFD rats treated with ginger showed hepatocytes, central vein, and portal areas appear more or less similar to control (Figure 1).

Ultrastructural results

The control liver showed the hepatocytes with normal eukaryotic nucleus, mitochondria, RER, and

Table 1: Assay of lipid profile in different groups

Parameter	Control Group (1)	HFD Group (2)	p	HFD+ROSU (3)	HFD+Ginger (4)	p
Cholesterol (mg/dl) (mean ± SD)	55.74 ± 6.36	98.35 ± 4.69	< 0.005	71.98 ± 8.9	72 ± 5.4	p2–3 and 2–4 < 0.005
TG (mg/dl) (mean ± SD)	47 ± 8.48	102.56 ± 9.13	< 0.005	38.72 ± 11. 8	36.6 ± 7. 2	p2–3 and 2–4 and 3–4 < 0.005
LDL (mg/dl) (mean ± SD)	20 ± 5.92	55 ± 7.83	< 0.005	12 ± 2.7	15.40 ± 1.96	p2–3 and 2–4 < 0.005
HDL (mg/dl) (mean ± SD)	24 ± 2.16	17.60 ± 1.029	> 0.005	15.90 ± 7.5	15.50 ± 1.2	p2–3 and 2–4 < 0.005

HFD: High-fat diet, ROSU: Rosuvastatin, SD: Standard deviation, TG: Triglyceride, LDL: Low-density lipoprotein, HDL: High-density lipoprotein.
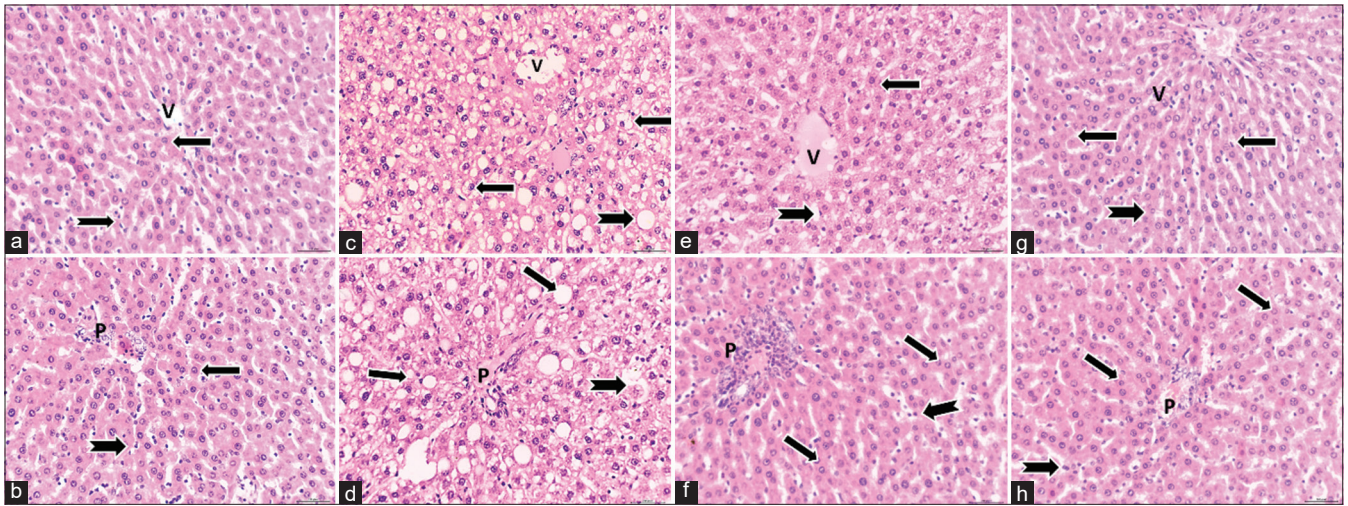
Figure 1: Section of liver from rats of Group I showing classic histological liver structure. (a) (Central vein region) the hepatic cords were radiating from the central veins (V), hepatocytes were polygonal and had vesicular nuclei (arrow), and the blood sinusoids and their von Kupffer cells (notched arrow) were seen among the cords. (b) (Portal area region) shows normal portal vein and normal bile ducts (P). Nearly, hepatocytes show normal cytoplasm and nuclei (arrow). (c) Section of the liver (central vein area) from high-fat diet rat showing slightly dilated and congested central vein (V). The hepatocytes show vacuolar degeneration, their nuclei were deformed and darkly stained (black arrows). Some of sinusoids are disorganized and dilated (notched arrow). (d) Liver from high-fat diet group (portal area) showing dilated portal vessels (P), with ballooning of hepatocytes (arrows). (e) Section in rat liver of high-fat diet with rosuvastatin group (central vein region) showing dilated and congested central vein (V) with restoration of normal hepatocyte cell cord (arrow) with slightly congested sinusoids (notched arrow). (f) Section in rat liver of high-fat diet with rosuvastatin group (portal area, region) showed normal portal vein and bile ducts (P) with marked lymphocytic infiltrations (notched arrow). The hepatocytes showed normal cytoplasm and nuclei (arrows). (g) Liver from high-fat diet and ginger group (central vein region) showed normal liver cells radiating from the central vein (V) with no lipid deposition, having vesicular round central nuclei (arrows) and normal blood sinusoids in between (notched arrow). (h): Liver from high-fat diet and ginger group (portal area) showing nearly normal portal area (P), normal hepatocytes (arrows), normal blood sinusoids in between (notched arrow) (hematoxylin and eosin, ×400), scale bar = 200 µm

glycogen granules with few fat globules. While that of HFD showed, marked vacuolation in hepatocytes due to accumulation of lipid droplets, increased swollen mitochondria with loosen cristae and distorted r.ER. The liver of ROSU/ginger groups showed improvement of hepatocyte structure with less fatty vacuolation and less swelling of mitochondria. The ginger group showed better results than that of ROSU group (Figure 2).

Immunohistochemical assessment of caspase 3

Liver sections for caspase-3 showed weakest expression of caspase-3 in the control group, moderate expression of caspase-3 were found in treated groups with ROSU/ginger groups while the marked expression of caspase-3 was in HFD group as proved statistically (Figure 3 and Table 2).

Discussion

Human studies on NAFLD were very limited due to ethical considerations in administration of drugs, use of liver tissue biopsy, and the long period of disease development and progression, so, the animal models could give more information not only in identifying the etiology of NAFLD but also in investigating therapeutic effects of various compounds [4].
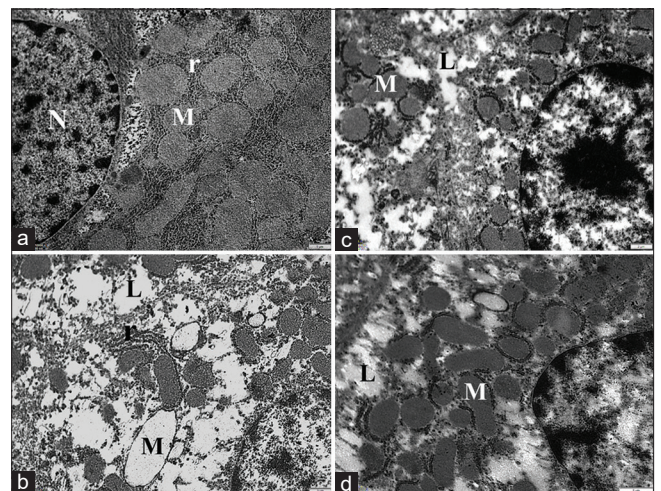

Figure 2: Electron micrographs from control liver (a) showed the hepatocytes with normal eukaryotic nucleus, mitochondria (M), and RER (r), with few fat globules. While that of high-fat diet-induced liver disease (b) showed multiple vacuolations in hepatocyte due to many lipid droplets (L), swollen mitochondria (M) with loosen cristae and distorted RER (r). (c) The rosuvastatin group showed hepatocytes with less fatty vacuolation (L), less mitochondrial swelling (M) than that of the fatty liver. (d) The ginger group showed that hepatocytes have ultrastructure feature nearly similar to the control group with restoration of mitochondria (M) and less lipid droplets (L) than that of the rosuvastatin group (lead acetate and uranyl citrate ×10,000, scale bar = 1 um

The present study investigated the potential therapeutic effect of *Zingiber officinale* (ginger) in comparison with ROSU on diet-induced nonalcoholic fatty liver disease.
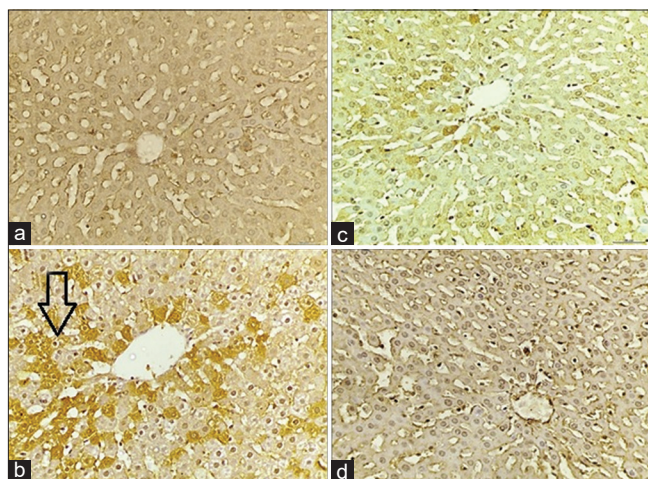
Figure 3: Photomicrographs of immunostained liver sections for caspase-3 show no expression of caspase-3 in the control group (a); marked caspase-3 expression in high-fat diet group (b); weak expression (arrow) of caspase-3 in treated groups with rosuvastatin (C), and the less expression with ginger group (d). A control group; B: High-fat diet rat group; C: NAFL-ROSU group; and D: NAFL-ZO group. The positive staining of caspase-3 is presented as brown hepatocytes (anti-caspase 3 immunostaining ×400, scale bar = 200 pixel)

In this study, induction of non-alcoholic fatty liver disease was done by feeding rats with high cholesterol diets (79% standard diet and 21% ghee fat) for 6 weeks, confirmed by significant increase in lipid profile (i.e., hyperlipedemia) as the serum cholesterol, TGs, HDL, and LDL of hyperlipidemic rats were increased due to the intake of atherogenic diet when compared to the control group. This was in agreement to Dizaye and Mohammed in 2019 [10] who used the atherogenic diet containing (30% vegetable oil and 3% pure cholesterol) given to 36 adults male rats. Other published study [13] has obtained similar results to our study and hyperlipidemia has been induced in Wistar rats using atherogenic diet which contains 50% carbohydrate (fructose) and 24% fat (beef tallow) with 25% fructose in drinking water.

The mechanism of the induction of NAFL by high-fat diet may be due to increased level of cholesterol biosynthesis precursor (acetyl CoA) which increases the activity of HMGCoA reductase, leading to downregulation of LDL receptor either by changing the activity of hepatic LDL receptor or by changing the production rate of LDL or by both [14]. Moreover, several researches revealed that the increase in serum LDL, cholesterol, and TGs and reduced HDL were involved in etiology of many diseases such as the fatty liver disease [15].

The histopathological and ultrastructural changes observed in the hepatic tissue in the form of dilation of blood sinusoids and vacuolar fatty degeneration in hepatocytes near to central vein. Hepatocyte nuclei were deformed and darkly stained nuclei (sign of cell apoptosis). Portal vessels are dilated and surrounded by cell infiltrates. Moreover that, mitochondrial dysfunction manifested by mitochondrial swelling and loss of cristae. Similar to our findings, these structural and ultrastructural changes of hepatocytes have been observed in animal models and patients of NAFLD [16], [17].

Together with the laboratory findings, these abnormalities proved the successful induction of NAFLD which is in agreement with former reports in animal models [18], [19]. The histological examination of liver structure in rat-induced NAFL was also accompanied by changes in lipid profile and liver enzyme. This coincides with the results of recent studies [4], [13], [20].

In this study, the outcome of the induction of NAFL in rat influences the liver functions by a significant increase in liver enzymes (ALT and ASAT) in the induced NAFL group compared to control group. This indicates destruction of liver tissue in the group with HFD. This in agreement with the finding of a recent study [20] which recorded that liver enzymes were significantly increased after induction of NAFL in rats. This indicates hepatic destruction in the rat-induced NAFL group. The increase in ALT and AST enzymes is due to their leakage into blood serum from the different tissues in case of muscular and hepatic damage. ALT is known as specific marker of liver damage which leaks into the blood serum from liver cell cytoplasm through cell membrane [21], [22].

Aerobic metabolism of the liver induces peroxidant (e.g., reactive oxygen species) at a continuous rate, which is balanced with continuous production of antioxidants. Peroxidant/antioxidant imbalance for peroxidant substitution (peroxidation) suggests the induction of oxidative stress which causes pathological changes in the liver. Reactive oxygen species with toxic effects lead to membrane lipid peroxidation [23].

Various studies [4], [24] have revealed that the most significant theory in the pathogenesis of NAFLD suggested the oxidative damage, which leads to inflammation and progression of the disease.

In the present study, the serum melondialdehyde (MDA) level showed significant increase in NAFLD compared to the control group. This was in agreement to a previous study in 2020 [3] which recorded a significant increase in the liver tissue level of MDA in NAFLD rats in comparison to the control group. Furthermore, lipid dysregulation in high-fat diet-fed rats was connected to induction of oxidative stress in related hepatocytes which favors a disease progression. Hypothesis for NAFLD progressive cycle suggests the involvement of metabolic disease, ROS formation, and inflammation. ROS-induced oxidative stress produces MDA as a byproduct [1], [25].

The elevated tissue levels of positive caspase 3 in the HFD group compared to the control group indicate hepatocyte apoptosis in rat with induced NAFLD. This coincides with the results of a recent study [26] who found a significant increase in the expression of

caspase-3 in HFD-exposed liver in comparison to the control group. Hepatocyte apoptosis is a key mediator in the initiation of NAFLD. Apoptosis is a high conserved genetically controlled pathway of cell death which leads to the induction of liver injury and fibrosis, and the development of several hepatic diseases [27].

From the above findings we suggest that fat accumulation in liver leads to membrane lipid peroxidation, oxidative stress, cellular apoptosis and consequently leakage of liver enzymes in the patients with fatty liver disease. This was in agreement to several studies [3], [4], [27], [28], [29] claimed that both inflammation and oxidative stress play a vital role in the initiation of NAFLD.

In the present study, we used ginger (*Zingiber officinale*) or ROSU to alleviate those conditions and we found that they significantly reduced the level of serum TG, LDL-C, TC, and increased serum HDL in hyperlipidemic rats when they compared with normal rats after 6 weeks of administration. Similar results were recorded after the use of ROSU in the study of Abdul-Kafy *et al*. [4], they revealed that ROSU treated group showed non-significant liver changes when compared to the control group while it showed significant decrease in comparison to NAFL group. Furthermore, Antonopoulos *et al*. [30] reported that treatment with ROSU (10 mg/day) normalized lipid profiles and transaminases in NAFLD patients with hyperlipidemia.

Similar significant results were recorded after the use of ginger in NAFLD, as it reduced HF-diet-mediated hypercholesterolemia. These results were in agreement with other experimental study [31] which carried out on 40 rats received ethanolic extract of ginger (500 mg/kg/day) for 9 weeks, showed significant reduction in serum level of TC, TGs, LDL-C, and significant increase in serum HDL. However in contrast to the present study, Prasad *et al*. [32] did not observe any significant changes in level of serum LDL and TG after the use of ginger. These differences indicated that using of ginger for 21 days is not sufficient to induce significant changes in serum TG and LDL.

Regarding the mechanism behind the anti-hyperlipidemic effect of the ginger, it comes from its ability to activate cholesterol 7α-hydroxylase, which is considered a rate-limiting enzyme in biosynthesis of bile acid. It induces conversion of cholesterol to bile acid, thus leads to enhance clearance very LDL (VLDL) and reduce TGs level [33].

Furthermore, ginger had a niacin component which may induce its anti-hyperlipidemic effect through increase in VLDL clearance, decrease of TGs level, and an increase in liver uptake of LDL. Concerning the beneficial effects of ginger on liver enzymes, our results are in agreement with the findings obtained from the previous study [24] which showed that hepatic enzymes ALT and AST were increased significantly in rats received two different doses of ginger extract (200 and 400 mg/kg) for 6 weeks showing reduction in serum AST and ALT at same time.

As observed in our results, the use of ginger in NAFLD rats produced significant reduction in MDA concentration. This agrees with the previous findings [34], [35] which recorded that ginger decreased the concentration of MDA in rats and so decreased the lipid peroxidation. Ginger also given to pregnant women in the first trimester safely [36]. In contrast to our results, Abd-Allah *et al*. [37] observed that using of ginger solution for 6 days in 40 rats showed no effect on MDA level of normal rats. The variation in the influence of ginger conforms that the plant decoction needs more than 1 week to exert its effect on MDA.

In the present study, rats treated with ginger showed that a decrease in apoptosis in the injured hepatic tissues, as the expression of protein levels of the pro-apoptotic caspase 3 decreased. Therefore, the ameliorative effect of ginger on NAFLD-induced by ROS production may be the underlying mechanism for its protective effect against apoptosis, which plays a role in the pathogenesis of NAFLD.

Conclusion

Our findings provide evidence that ginger/ROSU may be used to protect against non-alcoholic fatty liver disease and ginger effect is superior to ROSU.

The possible mechanisms of such effects involve the anti-apoptotic, anti-inflammatory, and antioxidant actions, decreased oxidative stress, additionally, lipid peroxidation. Therefore, further studies are required to establish its clinical application.

Institutional review board statement

The study was approved by the Institutional Review Board of at Damietta Faculty of Medicine, Al-Azhar University, ID number: IRB 0001267-21-07-005.

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