

MELATONIN AMELIORATES HIGH-FAT DIET-INDUCED LIVER STEATOSIS THROUGH MODULATING ANTIOXIDANT AND APOPTOTIC SIGNALS

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ABSTRACT

Aims: The continuous prevalence of obesity and its complications continues on being a noteworthy worldwide medical problem. The key pathophysiological markers for liver steatosis includes; oxidative stress and lipid peroxidation. The main goal of the present study was to demonstrate whether melatonin ameliorates hepatic steatosis induced by a high-fat diet in rats and explore the underlying mechanisms. **Methods:** Male albino rats (3-4 month-old) were randomly divided into four groups: (1) control (fed a normal diet; n=10); (2) high-fat diet (HFD; n=10); (3) high-fat diet plus melatonin (10mg / kg.b.wt; HFD + Mel, n=10); (4) vehicle (0.5% ethanolic phosphate-buffered saline, n=10). Several biochemical, histological and real time PCR analyses were used to investigate antioxidant system, morphological changes and gene expression fluctuations; respectively. **Results:** Current data showed that feeding rats on high-fat diet induced oxidative stress, extensive liver steatosis and fibrotic changes. Also, high-fat diet markedly decreased SOD activity, Nrf2 and DJ-1 mRNA expressions and induced a significantly increased the expression of Bax and p53 genes. In addition, melatonin effectively reduced hepatic steatosis and fibrogenesis. Moreover, melatonin increased SOD activity and Nrf2 and DJ-1 mRNA expressions and reduced MDA level as well as Bax, and p53 gene expressions. **Conclusion:** Altogether, this research showed that melatonin exerts its protective action against fatty liver in rats induced by high-fat diet, possibly through modulating antioxidant and anti-apoptotic signaling.

KEY WORDS

High-fat diet; Liver; Melatonin; DJ-1; Nrf2; Apoptosis

1. INTRODUCTION

Obesity has turned into a noteworthy general medical problem, and its rate has expanded strongly during the recent years. Obesity is one of the most critical metabolic disorders that is characterized by the simultaneous existence of hyperinsulinemia, hyperglycemia, dyslipidemia and hypertension. Obesity is a major risk factor for several diseases such as type 2 diabetes, coronary heart disease and some types of cancer [1]. High-fat (HF) consumption is considered the

main factor causing obesity. Moreover, obesity is a multisystem disorder correlated with abnormal neuroendocrine response to chronic excess of calorie. Accordingly, obesity occurs by either or both increasing adipocytes numbers (hyperplasia) or/and enlarging their sizes (hypertrophy) [2].

Obesity is a critical risk factor leading the progression of non-alcoholic fatty liver disease (NAFLD) [3]. It is stated that, NAFLD is portrayed by accumulation of fats in hepatocytes and precedes hepatic steatosis without alcohol consumption [4]. In this respect, NAFLD is a

developing general health concern worldwide due to its high morbidity and its relationship with hyperlipidemia, diabetes, and obesity [4,5]. In the course of NAFLD, simple steatosis takes place early and develops to nonalcoholic steatohepatitis (NASH) and finally to liver cirrhosis [6].

Melatonin (N-acetyl-5-methoxytryptamine) is the main hormone secreted by pineal gland. Melatonin is secreted in a cyclic rhythm that shows diurnal variation which increases in the dark phase of the day and decreases in the light phase of the day [7]. Melatonin is an important key regulator in several cellular, physiological and biochemical functions such as circadian rhythm [8], cardiovascular [9], neuroendocrine [10] and neuroimmunological [11], oncostatic actions [12]. Melatonin functions also as a powerful antioxidant; scavenging reactive oxygen and reactive nitrogen species [13]. Also, it was reported that melatonin works against aging, liver injury, cancer and atherosclerosis through its antioxidant actions [14,15]. In addition, it was found also that melatonin has the ability to regulate lipid metabolism [16], increase insulin sensitivity [17], regulate glucose metabolism [18], and reduce body weight [19]. It has been observed that melatonin can improve HFD-induced NAFLD and attenuated oxidative stress [20,21]. However, the underlying molecular mechanisms of action of melatonin are not yet fully understood. Through this study, the ameliorative effect of melatonin on HFD-inducing liver changes is investigated using several molecular, histological and biochemical investigations.

2. MATERIALS AND METHODS

2.1. Animals and housing conditions

Fourty young (3-4 months) adult male albino rats (*Rattus norvegicus*), were obtained from the Ministry of Health (Helwan-Egypt). The animals were weighed; housed (three animals in each cage) and kept under normal laboratory conditions. Rats were then acclimatized for two weeks prior starting experiments. All procedures were performed according to institutional guidelines and follow the Guide for Care and Use of Laboratory Animals.

2.2. Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) was bought (Sigma, St. Louis, Mo, USA). Melatonin was prepared in ethanol due to its instability in non-sterile solutions. Briefly, melatonin was dissolved in 0.5 ml of 100% ethanol and diluted with phosphate-buffered saline (PBS) to a final concentration of 10 mg melatonin/ 1ml of 0.5% ethanolic PBS [22]. The bottles of melatonin solution were covered with aluminum foil; and kept in a refrigerator; fresh solutions were prepared every two days.

2.2. Experimental design

After acclimatization of rats for two weeks, four groups of young adult male rats were used in this experiment. The first group of rats was designated as normal control (N. adult Group; n=10) and received normal laboratory diet throughout the study.

The second group of rats [high-fat diet (HFD) Group; n=10] was reared on a high-fat diet as previously described [23] over a period of 12 weeks.

The third group of rats [high-fat diet + melatonin (HFD + Mel) Group; n=10] was subjected to the same experimental manipulation as the second group (HFD Group) but they received 10 mg / kg. b.wt melatonin five times a week by oral intubation in the late afternoon (4.00-6.00 pm).

The fourth group of rats was used as vehicle-controls for the HFD + Mel animals (n=10); they were fed on a high-fat diet and received 0.5% ethanolic phosphate-buffered saline in the late afternoon for 12 weeks.

2.3. Blood and tissue sampling

After decapitation of rats under deep ether anesthesia, blood samples were then collected. Samples were centrifuged at 4000 rpm for 30 minutes. Serum aliquots were then stored at -08°C in microcentrifuge tubes until use. Liver tissues were immediately removed after taking blood samples from each animal. Tissue samples were then fixed in 10% neutral formalin solution. Other fresh liver tissue samples were used for RNA extraction for gene expression analyses.

2.4. Biochemical analysis

Serum superoxide dismutase (SOD) and malondialdehyde (MDA) activities were investigated in the blood serum using standard assay kit (Bio-diagnostic, Egypt). The data were expressed as a unit per milliliter for SOD and nanomol per milliliter for MDA.

2.5. Gene expression analyses

The effect of high-fat diet and its co-treatment with melatonin on the expression levels of the indicated genes regulating antioxidant system and apoptosis induction in liver tissues of rats were investigated using real-time PCR. The procedure of analyses was performed as previously described [24]. Briefly, Qiazol (Qiagen, Valencia, CA, USA) was used for extracting total RNA according to the manufacturer's instructions. From each sample 5 µg RNA was then reverse transcribed into cDNA using the first strand cDNA synthesis kit (Applied Biosystems, CA, USA). The sequence of the primers were obtained by the primer Express 1.5 software (Applied Biosystems) as follows: GAPDH forward, 5'-ATCTTCTGTGCAGTGCCAGC-3' and GAPDH reverse, 5'-GAAGGCAGCCCTGGTAACC-3' and Bax forward, 5'-TCATGAAGACAGGGGCCCTTT-3' and Bax reverse, 5'-CTGCAGCTCCATGTTGTTGT-3' and NRF2 forward, 5'-TGTCAGTACTCCAGGTTG-3' and NRF2 reverse, 5'-ATCAGGGGTGGTGAAGACTG-3' and DJ-1 forward, 5'-GGAGCAGAGGAGATGGAGAC-3' and DJ-1 reverse, 5'-TCACCAAAGCCGACTCAGAT-3' and P53 forward, 5'-CTCCTCTCCCCAGCAAGA-3' and P53 reverse, 5'-GTAGACTGGCCCTTCTTGGT-3'. QPCR was carried out using 7500 fast (Applied Biosystems, USA). All PCR reactions were then performed in triplicates in a 96-well plate. The cycling conditions were as follows: 10 minutes at 95 °C, and 40 cycles at 95 °C for 15 seconds followed by one minute at 60 °C. Calculations of real time PCR results were performed by determining the values of Δ cycle threshold (Δ Ct) using the endogenous control (GAPDH) for normalization. Then for each treatment, $2^{-\Delta\Delta Ct}$ was calculated and statistical differences were investigated and calculated as previously shown [25].

2.6. Histopathological Studies

After fixing liver tissues in formalin, samples were dehydrated in a series of ethyl alcohol and were then embedded in Paraplast (Sigma, m.p. 56-58). Tissue samples were then cut at 5 µm thickness. Sections were then stained with hematoxylin and eosin (H&E) for general histological investigations. Masson's trichrome stain was also used to observe collagen fibers and to highlight the existence and distribution of reactive fibrosis.

2.7. Statistical analysis

The data were presented as mean $\pm$ standard error. Statistical investigations were performed using ANOVA (analysis of variance) followed by Post-Hoc test for multiple comparisons. P -value <0.05 was considered statistically significant (using the SPSS program, version 16.0).

3. RESULTS

3.1. Effect of high-fat diet on SOD and MDA activities

The effect of high fat diet on the oxidative/antioxidant system was investigated. In the present study, feeding rats on high fat diet for a period of 12 weeks (HFD group) induced a significant ($P < 0.05$) reduction in the serum activity of SOD (Fig. 1A) with concurrent elevation of MDA activity (Fig. 1B) when compared to normal adult rats (N. adult group).

3.2. Effect of melatonin against high-fat diet-inducing changes in SOD and MDA activities

Next, the ameliorative effect of melatonin against high-fat diet-inducing oxidative stress was assessed. The current findings showed that, co-administration of melatonin with high-fat diet (HFD+Mel group) caused a significant increase ($P < 0.05$) in the serum SOD activity (Fig. 1A), along with marked inhibition ($P < 0.05$) in the MDA level (Fig. 1B) compared to obese rats (HFD group). Therefore, it was apparent that co-administration of high-fat diet with melatonin restored the SOD activity and MDA level in the serum close to that measured in the normal adult animals (Fig. 1).

3.3. Effect of high-fat diet on DJ-1, Nrf2, p53 and Bax mRNA expressions

Feeding rats with high-fat diet for 12 weeks (HFD group) showed marked inhibition ($P < 0.05$) in the levels of Nrf2 and DJ-1 mRNA (Figs 2A & 2B) and a significant increase ($P < 0.05$). The expression of p53 and Bax mRNA levels (Figs. 3A&3B) in the liver tissues when compared to those of normal adult group.

3.4. The modulatory effects of melatonin against high-fat diet-inducing DJ-1, Nrf2, p53 and Bax mRNA expression changes

The effect of high-fat diet on the anti-oxidant regulators (DJ-1 and Nrf2) and apoptosis-related genes (P53 and Bax) was significantly reversed by melatonin administration (Figs. 2 & 3). In this respect, our data showed that, co-treatment of rats with high-fat diet and melatonin for 12 weeks (HFD+Mel group) significantly

up-regulated ($P < 0.05$) both Nrf2 and DJ-1 mRNA expression levels and markedly repressed ($P < 0.05$) both p53 and Bax mRNA levels in liver tissues (Figs. 2A, 2B, 3A & 3B).

3.5. Histological investigations

a- Haematoxylin and Eosin (H&E) staining

In the present study, histological investigations on the effect of high-fat diet and melatonin co-treatment on the liver tissues of rats was performed using H&E staining (Fig. 4). In this respect, feeding rats with high-fat diet resulted in microvesicular and macrovesicular steatosis (fatty changes) accompanied by cytological ballooning (Fig. 4). Microvesicular steatosis was characterized by the existence of various small vesicles of fat that did not displace the nucleus to the edge of the cell (Fig. 4C). But, in macrovesicular steatosis the cytoplasm was replaced by a large vacuole of fat that displaces the nucleus to the edge of the cell (Fig. 4C). In addition, many hepatocytes appeared with pyknotic nuclei. We found also that, central and portal veins were

dilated and congested with haemolized blood (Fig. 4D). Also, perisinusoidal fibrosis was not observed.

Melatonin treatment along with HFD abrogated the HFD-induced changes in liver tissues (Figs. 4E & F). Liver steatosis was apparently attenuated in all liver sections of this group compared to that observed in the liver tissues of HFD group (Figs. 4E & F). Dilated central and portal veins were significantly decreased compared to that seen in the liver of HFD-fed rats.

b- Masson's trichrome staining

The liver sections of normal adult rats (N. adult group) showed minimal collagen deposition around central vein and portal traid (Fig. 5A). Collagen fibers were distinctly accumulated around the central vein as well as at portal traid in liver tissues of HFD- fed rats (Fig. 5B). On the other hand, an apparent decrease in the amount of collagen fibers around the central vein and portal traid was noticed in animals received high-fat diet plus melatonin (HFD+Mel group) compared to that of HFD-fed rats (Fig. 5C).

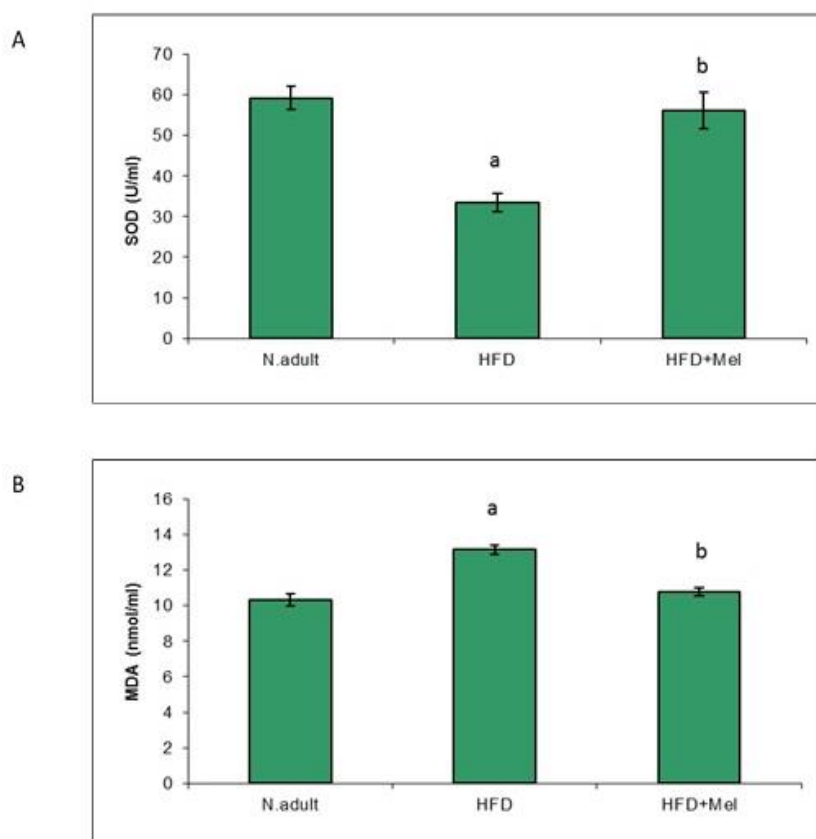


Fig. 1: (A) Serum activity of superoxide dismutase (SOD; U/mL); (B) Serum levels of malondialdehyde (MDA; nmol/mL) in normal adult rats, HFD-fed rats and HFD-fed rats treated with melatonin for 12 weeks. Data represent means $\pm$ SEM. N = normal; HFD= high fat diet; Mel= melatonin. ^a= $P < 0.05$ vs. normal adult animals, ^b= $P < 0.05$ vs. high fat diet-fed rats.

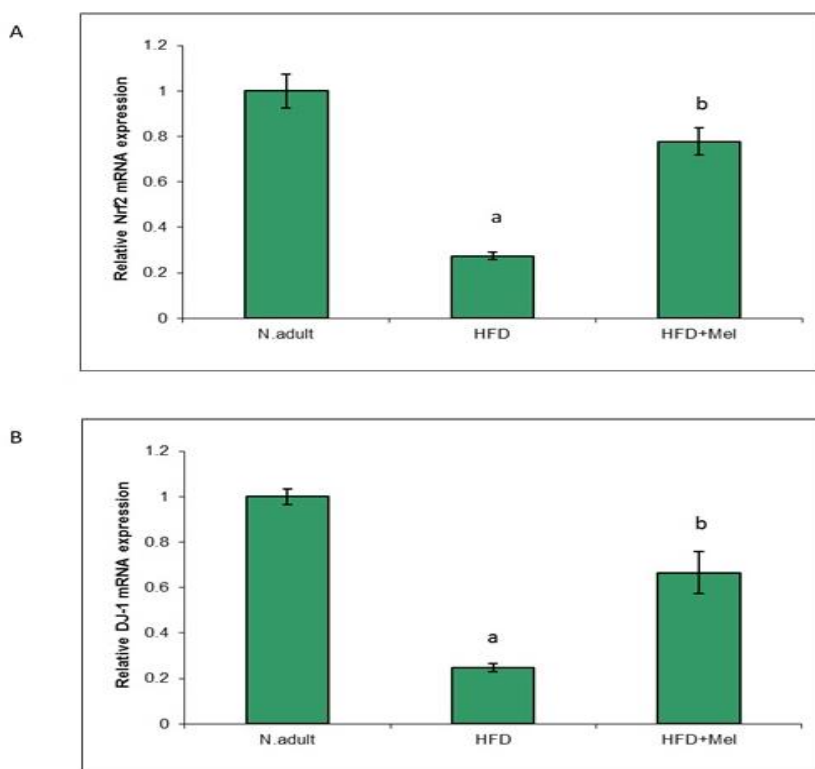


Fig. 2: Changes in mRNA expression of Nrf2 (A) and DJ-1 (B) in liver tissue. N = normal; HFD= high fat diet; Mel= melatonin. ^a= $P < 0.05$ vs. normal adult animals, ^b= $P < 0.05$ vs. high fat diet-fed rats.

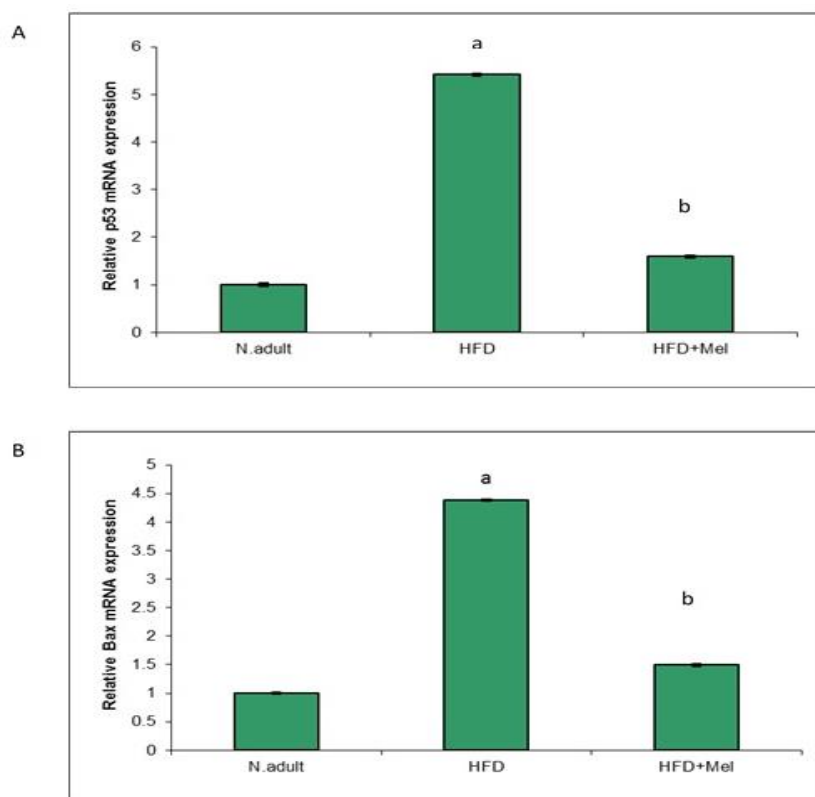


Fig. 3: Changes in mRNA expressions of p53 (A) and Bax (B) in liver tissue. N = normal; HFD= high fat diet; Mel= melatonin. ^a= $P < 0.05$ vs. normal adult animals, ^b= $P < 0.05$ vs. high fat diet-fed rats.

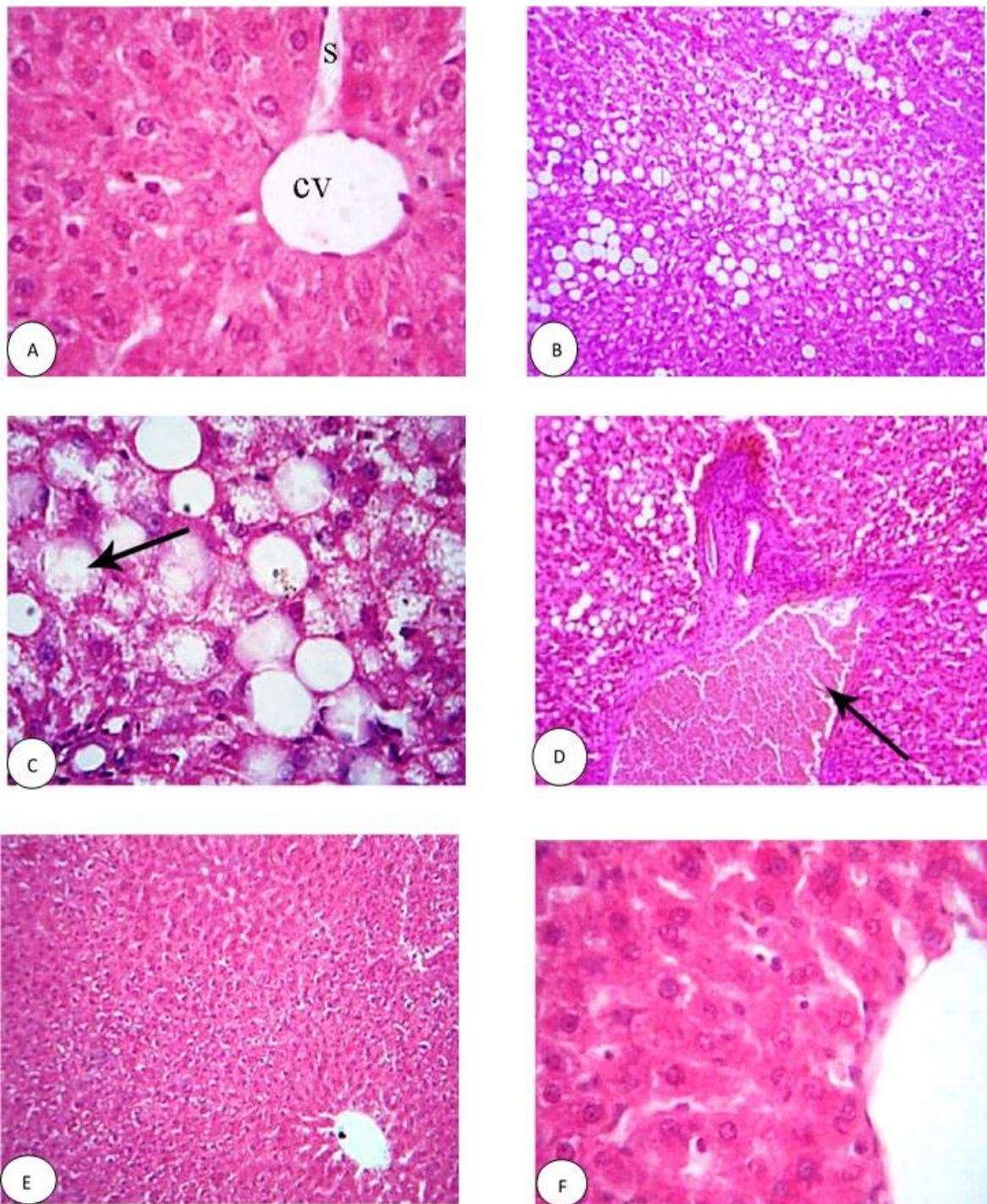


Fig. 4: A photomicrograph of liver sections. (A) Normal adult rats showing normal central vein (cv) surrounded by strand of hepatocytes and sinusoids (s). (B-D) HFD-fed rats for 12 weeks showing microvesicular and macrovesicular steatosis (fatty changes) accompanied by cytoplasmic ballooning; note large vacuole of fat in the cytoplasm of hepatocytes with displacement of the nucleus in macro-steatosis (arrow); note also dilated and congested of portal area (arrow). (E&F) HFD-fed rats cotreated with melatonin for 12 weeks showing amelioration of fatty changes and normal structure of hepatocytes. (B, D, E, H&E, x100; A, C, F, x400).

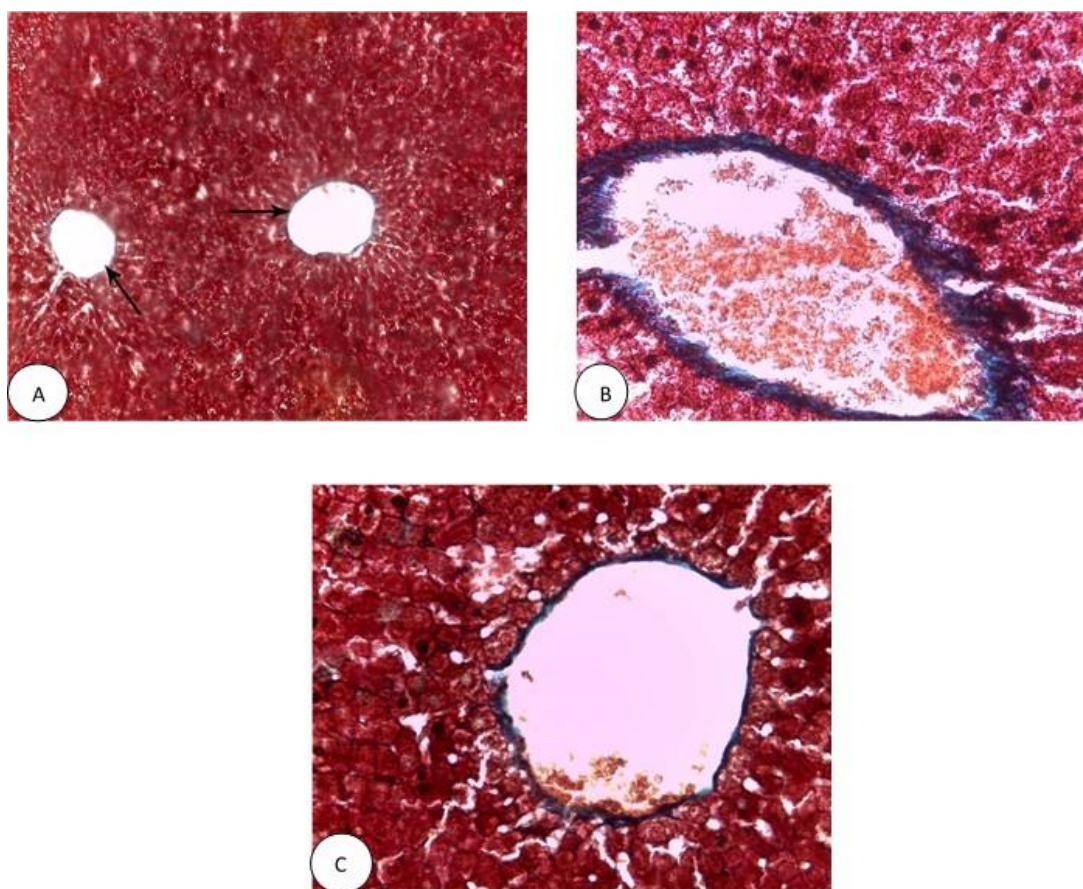


Fig. 5: A photomicrograph of liver sections. (A) Normal adult rats showing minimal collagen deposition around central veins (arrows). (B) HFD-fed rats showing accumulation of collagen fibers around central vein. (C) Rats received HFD plus melatonin for 12 weeks showing decrease in the amount of collagen fibers around the central vein. (A, Masson's trichrome, x100; B, C x400).

4. DISCUSSION

The increasing incidence of obesity is currently recognized as a major health issue. High fat-rich food is considered as a main factor causing obesity and obesity related diseases [26]. In consequence, changes in lifestyle and food habits increased the prevalence of liver steatosis, which may be accompanied by oxidative stress and lipid peroxidation. The current results indicate that melatonin administration can greatly improve liver changes associated with high-fat diet through its antioxidant and anti-apoptotic actions.

In the present study, feeding of rats with a high-fat diet for 12 weeks induced a marked decrease in the activity of SOD and a significant increase in the level of MDA in comparison to normal adult animals. The data presented herein are supported by earlier reports which demonstrated that feeding a high-fat diet acts as an inducer of oxidative stress, since it significantly attenuates the antioxidant enzymes and increases the

levels of lipid peroxidation products in the liver [27,28] and plasma of adult rats [29]. Taken together after feeding a high-fat diet an imbalance could occur between oxidative stress generation and antioxidants formation.

Nrf2 is a key regulator of anti-oxidative responses [30] and acts as a Potential Therapeutic Target against Oxidative Stress [31]. Also, the transcription factor Nrf2 is reported to be a master regulator for tissue damage [32]. It is being activated on oxidative damage and increased antioxidant enzyme activities through binding to its antioxidant responsive element [33]. It was believed that this the mechanism by which Nrf2 protect cells against oxidation damage. In this respect, it was suggesting that Nrf2 ameliorated HFD-induced lipid peroxidation in liver tissue [34]. Consistently with our results, feeding rats for 12 weeks with HFD markedly reduced Nrf2 expression in liver tissues.

DJ-1 is an oncogene working upstream and regulating and stabilizing the antioxidant master regulator Nrf2 [35]. DJ-1 has wide biological and physiological effects [36]. Current data showed that feeding rats with high-fat diet for 12 weeks induced a significant decrease in the level of DJ-1 expression in liver tissues in comparison to those of normal adult animals. Simultaneous administration of melatonin with high-fat diet induced apparent increase in the activity of SOD and a marked decrease in the level of MDA. This provides support for the protective effect of melatonin against oxidative stress induced by high-fat diet. These results are consistent with previous reports that melatonin reduced high-fat diet-induced oxidative stress in rats [20,37]. In addition, administration of melatonin improved the antioxidant capacity compromised by high-fat diet via activation of the Nrf2 and DJ-1 genes. Melatonin has been shown to increase the Nrf2 and DJ-1 genes under various experimental manipulations other than high-fat diet feeding [38,39].

Moreover, the current data showed a marked increase in the gene expressions of the apoptosis-related genes; Bax and p53 in the liver tissues of rats fed a high-fat diet. The present data are supported by the observation that HFD feeding increased pro-apoptotic Bax [40]. In addition, feeding rats with high-fat diet for 12 weeks induced a marked elevation in the P53 level in peripheral and adipose tissues [41]. Also, current data showed a significant decrease in the p53 and Bax mRNA levels in the liver tissues of rats administered melatonin in combination with high-fat diet feeding relative to adult animals. These results suggest that melatonin reverse the effects of high-fat diet feeding. The antiapoptotic effects noticed for melatonin in the present study is supported by previous studies [42]. No data has been found on the effect of melatonin on apoptosis after high-fat diet feeding, however, it has been shown that melatonin downregulated the Bax and p53 expression under different experimental regimens [43].

In this study, feeding rats a high-fat diet resulted in microvesicular and macrovesicular steatosis (fatty changes) accompanied by cytological ballooning. In addition, many hepatocytes appeared with pyknotic nuclei. The data presented herein are supported by the other reports which demonstrated that feeding high-fat diet induced microvesicular and macrovesicular steatosis [21].

Hepatic steatosis occurs when the liver tissues accumulate more than 5% of its weight as fat [44], which may due to an imbalance between lipid availability and disposal [45]. It is generally believed that oxidative stress and increased production of proinflammatory cytokines combined with insulin resistance eventually leads to the fatty degeneration in liver [46].

In the present study, melatonin administration to high-fat diet-fed rats for 12 weeks resulted in a significant improvement in the histological structure of liver tissues as compared to high-fat diet group. These results are consistent with the findings that administration of melatonin (5 or 10 mg/kg) was effective in reducing hepatic steatosis induced by a high-fat diet [20,21]. It is stated that, melatonin reduced liver tissue damage mainly through its antioxidant actions, which includes scavenging of free radicals [21], and its regulation of antioxidative enzymes transcription [47].

The observed collagen fibers accumulated around the portal tract and central vein in liver tissues of HFD-fed rats in the current work, which is the picture of central and portal fibrogenesis could be attributed to generation of reactive oxygen species. Ample data have suggested the role of oxidative stress in the etiopathogenesis of liver tissue fibrosis [48,49]. In addition, liver fibrosis was induced by oxidative stress via stellate cell activation [49].

Furthermore, in this study, the histological examination of the liver sections of HFD-fed rats treated with melatonin showed reduction in the amount of collagen fibers around the portal tract as well as central vein compared to that recorded in sections of the HFD-fed rats. These results support data from other studies which demonstrated that melatonin may reduce fibroblast proliferation and collagen synthesis [50]. From the above, melatonin was suggested as a therapeutic agent against chronic liver diseases to prevent the formation and progression of liver fibrosis. Finally, melatonin administration improved the pathological abnormalities of the liver induced by high-fat diet. Also, serum MDA level was reduced while SOD activity was increased in serum of rats given high-fat diet and melatonin. We also found that the HFD supplemented with melatonin exhibited significantly increased levels of DJ-1 and Nrf2 mRNA and decreased levels of p53 and Bax mRNA in the liver tissue. Therefore, our findings suggested that melatonin exerts its

protective and ameliorative effects against high-fat diets-induced fatty liver in rats through upregulation of DJ-1/Nrf2 and downregulation of p53/Bax pathways.

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