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Prenatal melatonin reprograms liver injury in male pups caused by maternal exposure to a high-fat diet and microplastics

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Abstract

Prenatal exposure to a high-fat diet (HFD) or microplastics can impact liver fat accumulation in offspring. This study investigates the protective effects of prenatal melatonin on liver injury in male pups resulting from maternal exposure to a HFD and microplastics. Pregnant Sprague-Dawley rats were fed either an HFD or a normal chow diet, with some groups exposed to microplastics alone or in combination with melatonin. Male pups were evaluated on postnatal day 7. Results indicated that pups in the HFD-microplastics group (HFD-Mi) exhibited increased liver lipid accumulation (observed in histological staining), apoptosis (elevated cleaved caspase 3, phospho-AKT, and TUNEL staining), inflammation (higher IL- 6 and TNF-α), and oxidative stress (elevated malondialdehyde). Conversely, melatonin treatment (HFD-Mi +M) significantly reduced these effects, including lipid accumulation, apoptosis, and inflammation, while enhancing antioxidant enzyme glutathione peroxidase activity and improving lipid metabolism (reduced SREBP- 1 expression). These findings suggest that prenatal melatonin mitigates liver injury caused by maternal HFD and microplastics through its anti-inflammatory, antioxidative, and lipid-regulating properties, underscoring its potential hepatoprotective role.

Keywords High-fat diet · Microplastics · Apoptosis · Oxidative stress · Melatonin

Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatocytes with more than 5% steatosis, reflecting excessive fat accumulation in the liver [1, 2]. A high-fat diet (HFD) can contribute to obesity, which is associated with chronic inflammation and the release of various proinflammatory cytokines, thereby promoting the development of NAFLD [3]. The global prevalence of NAFLD is currently 25% and continues to rise. The severity of NAFLD is associated with various components of metabolic syndrome, including steatosis, inflammation, nonalcoholic steatohepatitis, advanced liver fibrosis, and overall mortality [4]. The "multiple-hit pathogenesis" hypothesis suggests that the onset of NAFLD results from a combination of genetic, epigenetic, metabolic, and environmental factors. An imbalance in energy metabolism in the liver leads to excessive energy storage, primarily as carbohydrates and fat, resulting in a net accumulation of fat in the live [5, 6]. This fat accumulation in hepatocytes triggers inflammation, cell death, and fibrosis [7]. Additionally, the development of NAFLD is further stimulated by mitochondrial dysfunction and the overproduction of reactive oxygen species (ROS) [8, 9].

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The rising global demand for industrial products has led to the widespread production of plastic items, contributing to plastic pollution [10]. Over time, these plastic materials degrade, forming microplastics (particle size <5 mm) and nanoplastics (particle size <100 nm) in the environment. Animals subsequently ingest these plastics, leading to their accumulation in various organs [11–13]. Polystyrene microplastics have been shown to damage hepatocytes. Candidate mechanisms underlying microplastic-induced hepatotoxicity include cytotoxicity, alterations in key molecular markers, ATP production, lipid metabolism disruption in liver organoids, oxidative stress, and inflammatory responses [14]. Notably, microplastics can harm not only the liver but also other systems, such as the immune [15], nervous [16], and skeletal muscles [17].

Melatonin, also known as N-acetyl- 5-methoxytryptamine, is synthesized by the pineal gland in both rats and humans [18, 19], and it is also present in dietary sources like sour cherries, walnuts, and orange juice [20]. Melatonin provides various benefits, including anticancer and anti-osteoarthritic activities, strong antioxidant properties, free radical scavenging, anti-inflammatory actions, and proapoptotic effects [21, 22]. Numerous studies have emphasized its role in preventing hepatic steatosis and the progression of NAFLD [23, 24]. Melatonin preserves the liver architecture and function in the HFD offspring [24, 25]. While one study investigated the interaction between microplastics and melatonin, it did not explore how this combined effect is influenced by the presence of an HFD in the experimental group [26].

Adult-onset chronic non-communicable diseases can originate in early life through developmental programming [27]. Regarding prenatal programming, evidence has shown that the deleterious effects of a prenatal HFD can impair the liver of rat offspring, even into adulthood [28]. Environmental factors, including drinking water–induced microplastic exposure [29], which are critical for children's growth and development, have been associated with an increased risk of adult NAFLD [30–32]. Maternal exposure to polystyrene microplastics has also been linked to metabolic disorders in offspring [33]. Several lines of evidence indicate an association between suboptimal fetal and neonatal environments and the development of adult diseases later in life [34]. The combined effect of exposure to polystyrene microspheres and a HFD-induced metabolic dysfunction–associated steatotic liver disease has been reported [35]. However, few studies have examined the combined effects of microplastics and chemical contaminants on liver toxicity in offspring [36]. Our previous study demonstrated that prenatal exposure to a HFD combined with microplastics induces liver injury via oxidative stress in male pups [5].

Reprogramming strategies refer to interventions aimed at reversing maladaptive developmental programming and restoring normal development [27]. This study aimed to simulate a more realistic scenario by combining maternal microplastic and HFD exposure to evaluate whether melatonin can reprogram these conditions and to investigate the underlying mechanisms involved.

Materials and methods

Animals

The study was conducted at the Kaohsiung Chang Gung Memorial Hospital Animal Experimental Center in Taiwan and was approved by the Institutional Animal Care and Use Committee of the hospital (No. 2021083001). Seven- to eight-week-old Sprague Dawley rats were used for the study. The rats were housed in an animal facility under a 12-hour light/dark cycle, with lights turned on at 7:00 a.m. Litters were inspected daily at 10:00 a.m. The rats were divided into two groups: one group of females fed a high-fat diet (HFD) and another group fed a normal chow diet (NCD) for a minimum of seven weeks before mating. The HFD consisted of 23 g/100 g protein, 35.5 g/100 g carbohydrate, and 35.8 g/100 g saturated fat (58% kcal from fat), with fat sources derived from soybean oil and coconut oil. In contrast, the NCD consisted of 19.2 g/100 g protein, 67.3 g/100 g carbohydrate, and 4.3 g/100 g saturated fat. Both diets were purchased from the Research Diet Company (D12331i, Research Diet, New Brunswick, NJ, USA). Male rats consumed the NCD, except during mating. Female rats were given 24 h to mate with male rats while consuming either the HFD or NCD. After mating, the rats were separated and individually housed in standard plastic cages. Pregnant females were then randomly assigned to one of two groups: maternal HFD (N= 12) or NCD (N= 12) exposure paradigms until delivery. Some pregnant females in both paradigms were exposed to microplastics (Bangs Laboratories; Fishers, IN, USA). These dams were given drinking water containing PS-MPs (5 µm in size) at a concentration of 1000 µg/L (N= 4), or were co-treated with both microplastics and melatonin (40 mg/L) (N= 4) from conception onward. Each litter consisted of one male and two female rats, resulting in a total of twelve male and twenty-four female rats. The mating period lasted seven days to improve the pregnancy rate, achieving a 100% pregnancy rate and a live birth rate 94.9%, with a male-to-female ratio of 1:1.04. The average litter size was 13.9 pups. The experimental groups were as follows: HFD-Mi: HFD +microplastics (5 µm, 1000 µg/L), HFD-Mi +M: HFD +microplastics (5 µm, 1000 µg/L) +melatonin (40 mg/L), NCD-Mi: NCD +microplastics (5

μm, 1000 μg/L), and NCD-Mi + M: NCD + microplastics (5 μm, 1000 μg/L) + melatonin (40 mg/L). Comparisons were made between the NCD group and the NCD-Mi or NCD-Mi + M groups, as well as between the HFD group and the HFD-Mi or HFD-Mi + M groups. On postnatal day 7 (PD7), male pups were sacrificed for subsequent analysis (Fig. 1).

Measurement of plasma biochemistry parameters

Blood samples were collected by cardiac puncture when the rats were sacrificed to alleviate their suffering [37]. The plasma levels of total cholesterol, aspartate transaminase (AST), and alanine aminotransferase (ALT) were determined by a standard auto-analyzer (Hitachi model 7450, Tokyo, Japan).

Tissue preparation

On PD7, the rats were anesthetized with 25 mg/kg Zoletil and 23 mg/kg Xylazine. Continuous perfusion with normal saline was performed using a peristaltic pump. The liver was promptly extracted and placed on ice. Liver tissue designated for immunohistochemistry was sectioned and embedded in paraffin, while the remaining liver samples were stored at −80 °C for future analysis. Tissue preparation for Oil Red O staining differs significantly from that for H&E staining, as it requires a separate fresh liver tissue sample. Due to the limited amount of liver tissue available from 7-day-old rat pups, we were unable to collect a sufficient sample for Oil Red O staining at that time.

Hematoxylin and eosin staining

The liver was excised and fixed in 4% paraformaldehyde at 4 °C overnight. They were then dehydrated through a gradient of ethanol concentrations (70%, 75%, 85%, 90%, 95%, and 100%), followed by clearing in xylene and embedding in paraffin wax at 55 °C. Sections of the liver 3 μm thick, were cut and stained using an H&E staining kit (ScyTek Laboratories, West Logan, WV, USA). Histological lesions were observed using a Leica DMI- 3000 microscope equipped with a digital camera. ImageJ (Fiji version 1.8.0) software was used to quantify liver lipid accumulation. The image was first converted to an 8-bit grayscale image and then inverted to black and white so that the lipid droplets appeared black. A threshold of 20 out of 255 on the grayscale was applied to remove inter-hepatocyte structures that did not indicate lipid droplet features, followed by particle analysis. All particles with the circularity between 0.5 and 1.0 and a diameter between 0.1 μm and 50 μm were counted. The area fraction (%) occupied by the counted particles was then determined. The semi-quantification of lipid droplets was calculated using approximately 500 liver cells.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay was conducted on fixed tissues embedded in paraffin, sectioned at 3 μm thickness, and mounted on slides. An apoptosis detection kit (Roche, 11684817910, Mannheim, Germany) was used to assess cellular apoptosis according to the manufacturer's instructions. The rates of TUNEL-positive cells were determined by counting the positively stained cells in the liver of each rat, based on observations from 10 randomly selected fields.

Western blot

Livers were dissected and subsequently frozen in liquid nitrogen. The tissue from each liver was homogenized in lysis buffer (17081; iNtRON Biotechnology, Seongnam, Korea) and centrifuged at 14,000×g. The supernatant proteins were quantified with a protein assay dye (5000002, Bio-Rad, Hercules, CA, USA). A total of 65 μg of protein

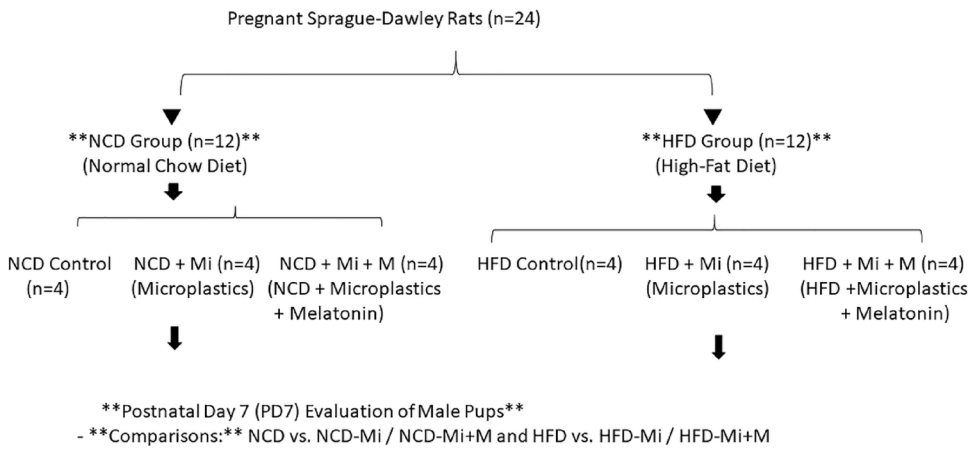

Fig. 1 A scheme for the experimental groups and the treatments

from each sample was separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBST buffer containing 10% non-fat milk for 1 h at room temperature. Immunoblotting was performed by incubating the blocked membranes overnight at 4 °C with the following primary antibodies at the indicated dilutions: cleaved caspase- 3 (#9661, Cell Signaling, Denver, MA, USA) at 1:1000, caspase- 3 (#9662, Cell Signaling, Denver, MA, USA) at 1:1000, IL- 6 (ab6672, Abcam, Cambridge, MA, USA) at 1:1000, AKT (#9272, Cell Signaling) at 1:1000, phospho-AKT (#4060, Cell Signaling) at 1:1000, SREBP- 1 (PA1 -335, Thermo Fisher Scientific, Waltham, MA, USA) at 1:4000, ECSH- 1 (11305 -1-AP, Proteintech, Rosemont, IL, USA) at 1:1000, MCAD (sc- 365108, Santa Cruz, Dallas, TX, USA) at 1:1000, and GAPDH (ab181602, Abcam) at 1:5000. The membranes were then incubated with secondary HRP-conjugated anti-rabbit (1:5000; Jackson Immuno Research, West Grove, PA USA) or anti-mouse antibody (1:10,000; Jackson Immuno Research) for 1 h at room temperature. Western blots were visualized using an ECL kit (PerkinElmer, NEL 105001EA, Boston, MA, USA). Western blot quantification was conducted using Quantity One software (version 4.52, Bio-Rad), which measured the background-subtracted band densities. All results were normalized to GAPDH expression [5].

Estimation of lipid peroxidation

Lipid peroxidation in liver tissue was determined using a thiobarbituric acid reactive substances (TBARS) assay kit (10009055, Cayman, MI, USA) according to the manufacturer's instructions. Briefly, 25 mg of liver tissue from each sample was homogenized in 250 µl of lysis buffer (17081; iNtRON Biotechnology, Seongnam, Korea). Then, 100 µl of the liver tissue homogenate was mixed with 900 µl of color reagent containing TBA, concentrated acetic acid, and NaOH. The mixture was heated at 95 °C for one hour and then centrifuged at 1,600 × g. The absorbance of the clear supernatant was measured using an ELISA plate reader against a reference blank at 532 nm.

Glutathione peroxidase activity assay l

Each supernatant sample from the homogenized liver tissue was used to measure the activities of hepatic antioxidant enzyme Glutathione peroxidase 1 (GPX1, Cayman, 703102, MI, USA) level by using commercially available kits according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using SPSS (Version 24, Chicago, IL, USA). Biochemical parameters, enzyme activities, and Western blot results were evaluated using a two-way analysis of variance with Tukey's post hoc tests. For comparisons between two groups, the Mann–Whitney U test was used. Results are presented as the mean ±standard error of the mean, with a P value of <0.05 indicating statistical significance.

Results

After female rats gave birth and were sacrificed, their body weight, liver weight, and biochemical parameters were measured. Maternal body weight and liver weight increased in both the HFD and HFD-Mi groups compared to the NCD group ($p< 0.05$) and decreased after melatonin administration (Table 1). There were no significant differences in GPT and total cholesterol levels among the groups. However, GOT levels increased following microplastic and HFD exposure compared to the NCD group. Triglyceride levels

Table 1 Body and liver weights of mothers and pups, and biochemical data of mothers

Maternal	NCD	NCD-Mi	NCD-Mi +M	HFD	HFD-Mi	HFD-Mi +M
Body weight (g)	$314.50 \pm 19.19^{+\$}$	$333.50 \pm 11.02^{+\$}$	$329.75 \pm 1.43^{+\$}$	$364.50 \pm 44.50^{*\#\&※}$	$383.00 \pm 22.05^{*\#\&※}$	$317.50 \pm 18.37^{+\$}$
Liver weight (g)	$13.57 \pm 0.36^{+\$}$	$14.065 \pm 1.56^{+\$}$	$12.065 \pm 0.50^{+\$}$	$18.73 \pm 2.55^{*\#\&※}$	$18.00 \pm 2.65^{*\#\&※}$	$14.08 \pm 0.59^{+\$}$
GOT	$61.67 \pm 9.54^{\#+\$}$	$98.67 \pm 16.78^{*}$	94.00 ± 11.28	$106.40 \pm 4.70^{*}$	$106.33 \pm 3.11^{*}$	85.00 ± 14.06
GPT	53.00 ± 1.02	59.33 ± 11.50	55.50 ± 2.88	59.00 ± 4.10	61.67 ± 2.49	57.67 ± 2.65
T-cholesterol	67.50 ± 5.76	70.20 ± 6.89	64.67 ± 5.71	67.50 ± 7.98	72.33 ± 5.87	63.33 ± 8.10
Triglyceride	$30.80 \pm 2.32^{+\$※}$	$32.67 \pm 2.94^{+\$※}$	$28.00 \pm 1.55^{+\$※}$	$52.50 \pm 9.80^{*\#\&}$	$66.33 \pm 10.12^{*\#\&}$	$50.33 \pm 2.15^{*\#\&}$
Male pups						
Body weight (g)	$8.58 \pm 0.25^{\#\&+\$※}$	$13.54 \pm 0.29^{*\$}$	$13.14 \pm 0.55^{*\$}$	$13.61 \pm 0.85^{*}$	$14.86 \pm 0.31^{*\#\&}$	$13.61 \pm 0.29^{*}$
Liver weight (g)	$0.34 \pm 0.01^{\#\&+\$※}$	$0.40 \pm 0.02^{*+\$※}$	$0.39 \pm 0.01^{*+\$※}$	$0.49 \pm 0.02^{*\#\&\$}$	$0.54 \pm 0.02^{*\#\&+※}$	$0.46 \pm 0.02^{*\#\&\$}$

GOT: Glutamic Oxaloacetic Transaminase, GPT: Glutamic Pyruvic Transaminase, T-cholesterol: total cholesterol

*p<0.05 compared to NCD, # p<0.05 compared to NCD-Mi,

&p<0.05 compared to NCD-Mi+M, + p<0.05 compared to HFD,

$p<0.05 compared to HFD-Mi, ※ p<0.05 compared to HFD-Mi+M

increased in the HFD, HFD-Mi, and HFD-Mi +M groups compared to the NCD group.

The body weight of pups in the NCD-Mi, NCD-Mi +M, HFD, HFD-Mi, and HFD-Mi +M groups increased compared to that of pups in the NCD group (Table 1). The HFD-Mi group showed an increase in liver weight compared to the HFD and NCD groups, but liver weight decreased in the HFD-Mi +M group (Table 1).

Lipid accumulation

Reactive lipid accumulation in the offspring liver was assessed using H&E staining (Fig. 2a). As expected, an HFD significantly increased reactive lipid accumulation compared with an NCD. In this study, microplastic exposure further promoted lipid accumulation in both the NCD-Mi and HFD-Mi groups. However, melatonin administration led to a more pronounced reduction in lipid accumulation in the HFD-Mi +M group compared to the NCD-Mi +M group (Fig. 2b).

Apoptosis

Phospho-AKT overexpression was identified as a key factor in triggering apoptotic cell death. Microplastic exposure led to a significant increase in phospho-AKT expression in both the NCD-Mi (NCD +microplastics) and HFD-Mi (HFD +microplastics) groups. However, following melatonin administration, phospho-AKT expression significantly decreased in both groups (Fig. 3a and b). Apoptosis, indicated by cleaved caspase- 3, serves as a marker of liver cellular damage and apoptotic pathways activation. Microplastics increased cleaved caspase- 3 expression, but this apoptotic effect was mitigated by melatonin (Fig. 3c). The results of TUNEL staining indicated late- stage apoptosis in the offspring liver cells, suggesting that liver cellular DNA fragmentation was more advanced at this stage following microplastic exposure in both the NCD-Mi and HFD-Mi groups. However, melatonin alleviated microplastics-induced stress, leading to a reduction in apoptosis in both groups (Fig. 4a. 4b).

Inflammation

To assess the inflammatory responses, we examined the expression of IL- 6 (Fig. 5a). Exposure to microplastics led to a significant increase in IL- 6 expression in the HFD-Mi group, whereas melatonin treatment reduced its expression (Fig. 5b). Consequently, melatonin appears to play a role in reducing the inflammatory response.

Oxidative stress

MDA serves as an indicator of oxidative stress in the offspring liver. MDA levels were significantly decreased in the HFD-Mi +M group compared to the HFD and HFD-Mi groups (Fig. 6). The intrinsic anti-free radical and antioxidant capabilities of GPX1 in cells were assessed, with the results presented in Fig. 7. GPX1 expression was decreased in both the HFD and HFD-Mi groups, indicating a reduced anti-free radical and antioxidative stress response due to the HFD and microplastics in these 7-day-old pups. Melatonin enhanced the anti-free radical effect in the HFD-Mi +M group (Fig. 7).

Lipid metabolism

SREBP- 1, ECSH1, and MCAD serve as indicators of lipid metabolism (Fig. 8a). Exposure to microplastics significantly increased the expression of SREBP- 1 in both the NCD-Mi and HFD-Mi groups, but this increase was markedly alleviated following melatonin treatment (Fig. 8b). Conversely, no significant changes in ECSH1 or MCAD expression were observed, even after exposure to microplastics and melatonin (Fig. 8c and d).

Discussion

Our study findings revealed that prenatal exposure to HFD and microplastics increases apoptosis, oxidative stress, inflammatory response, and lipid accumulation. However, prenatal melatonin administration effectively mitigated these adverse effects. Therefore, we propose minimizing prenatal exposure to HFD and microplastics and considering prenatal melatonin supplementation to prevent potential liver cell damage in offspring.

Evidence supports the concept of “developmental programming” in livestock. This suggests that a stimulus or insult occurring during critical periods of pre- or postnatal growth and development may result in permanent, programmed alterations in the health and wellbeing of the offspring [38]. Several factors, such as maternal nutritional perturbations and inflammation, have been recognised as prominent causes of developmental programming [39] Perturbations to the emerging immune system might have long-term consequences for the physiology and disease risk of the offspring due to programming effects [24] However, the critical developmental stages during which the immune system is vulnerable to environmental programming have not yet been elucidated and likely vary between different species [24]. In our study, we also found that the programming

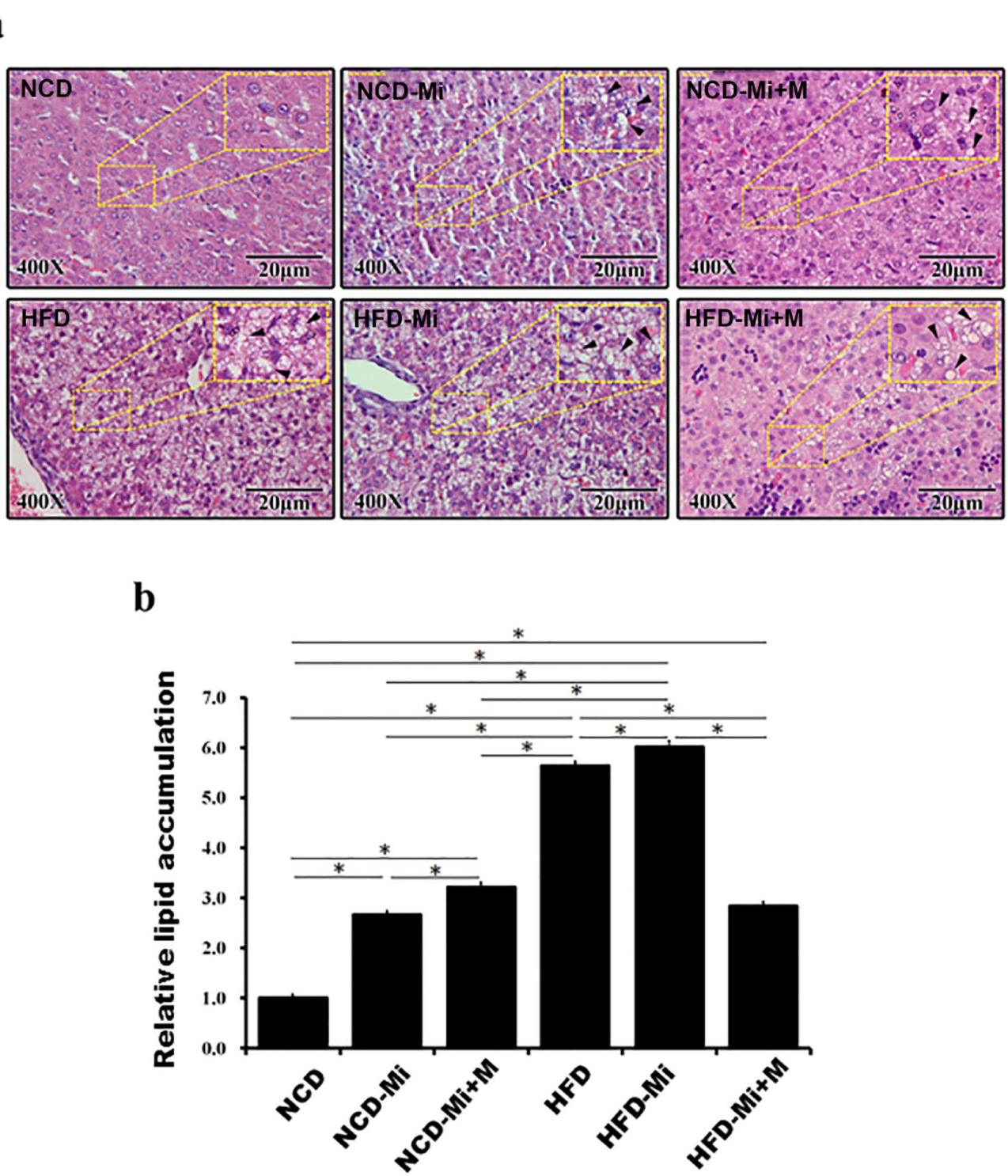

Fig. 2 Lipid accumulation analysis. H&E staining revealed a significantly lower level of lipid accumulation in the HFD-Mi +M group compared to the HFD-Mi group. (a) H&E staining results illustrating lipid accumulation. (b) Quantification of lipid accumulation. All values are expressed as mean ±standard error ($n = 36$, each group had 6 animals). *$P < 0.05$. Different letters denote the respective groups: NCD: normal chow diet group; NCD-Mi: chow diet with microplastics intervention group; NCD-Mi +M: chow diet with microplastics +melatonin intervention group; HFD: high-fat diet group; HFD-Mi: high-fat diet with microplastics intervention group; HFD-Mi +M: high-fat diet with microplastics +melatonin intervention group. (original magnification 400x; bar =20 μm)

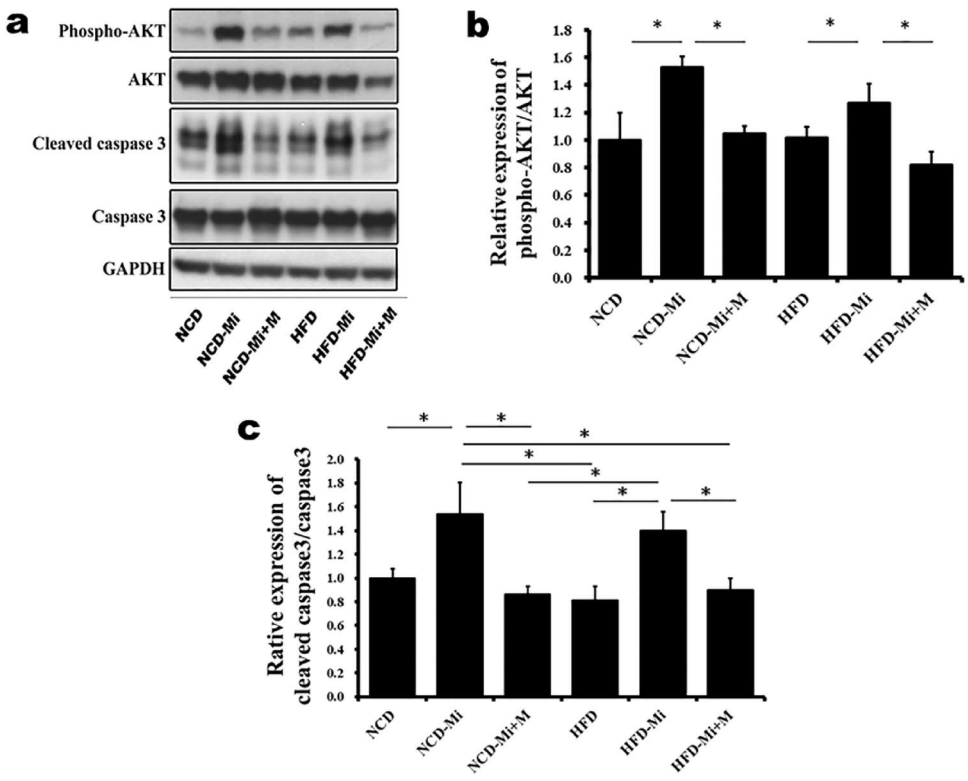

Fig. 3 Apoptosis pathway analysis. The expression of cleaved caspase 3 protein and phosphor-AKT was the highest in the high-fat diet + microplastics (HFD-Mi) group, compared with the other groups, indicating apoptosis in the HFD-Mi group. The HFD-Mi + M group exhibited lower levels of cleaved caspase 3 and phosphor-AKT than the HFD-Mi group. (a) Results of the Western blot analysis of phosphor-AKT, and cleaved caspase- 3. (b) Quantification of phospho-AKT/AKT expression. (c) Quantification of cleaved caspase-3/caspase- 3 expression. All values are expressed as mean ± standard error ($n = 36$, each group had 6 animals). *$P < 0.05$. Different letters denote the respective groups as in Fig. 2

insult from a HFD combined microplastics occurs through oxidative stress and inflammation-related mechanisms.

The use of melatonin has been suggested as a reprogramming agent [27], and its important role in pregnancy and parturition has been well established [38]. Studies have reported that melatonin can be safely administered to improve fertility rates and redox status during pregnancy [40], potentially benefiting offspring by modulating proinflammatory cytokines through redox-related mechanisms [41]. Additionally, maternal serum melatonin levels have been reported to exhibit a diurnal rhythm, serving as an important signal for the fetus to entrain the light-dark cycle of newborns after birth [42]. In our study, we also found that melatonin can reprogram the effects of a HFD combined microplastics through redox- and inflammation-related mechanisms. However, the additional increase in liver fat observed in the control group with co-exposure to melatonin in these 7-day-old pups is an interesting phenomenon that requires further investigation to be fully understood.

Furthermore, we found that microplastics induced a significant increase in phospho-AKT levels in the NCD-Mi and HFD-Mi groups, but melatonin effectively reduced phospho-AKT levels in both groups. This aligns with previous reports that phospho-AKT overexpression induces apoptosis [43, 44], it also has been shown to induce liver steatosis by suppressing the expression of downstream genes involved in fatty acid synthesis, including sterol regulatory element-binding proteins (SREBPs) [45]. Melatonin, known for alleviating apoptotic liver damage by mitigating ER stress through the modulation of unfolded protein response signaling [21, 46], demonstrated its potential to reduce liver cellular apoptosis in pups. This finding is consistent with studies on caspase 3, where microplastics increased the expression of cleaved caspase 3 associated with liver cellular apoptosis [47], and melatonin exhibited a protective effect against excessive apoptosis.

Xu et al. demonstrated that melatonin reduces weight gain, adiposity, and ectopic fat accumulation, enhances energy expenditure, and ameliorates insulin resistance and inflammation, specifically in mice fed an HFD [48]. They suggested that melatonin had no toxicity or side effects in mice fed an NCD under energy-balanced conditions [48]. These findings are consistent with the present study. Notably, in this study, melatonin was administered to pregnant rat, similar to reported in other studies [38, 49]. However, the current study involved various analyses on liver injury in the neonate offspring of rats using a combination of treatments. Despite these variations, similar positive effects were observed as those seen when melatonin was directly administered to the animals.

Microplastics have been shown to increase the expression of proinflammatory factors, including IL- 6 and TNF-α, through the NF-κB pathway [50]. Melatonin can reduce the aggregation of inflammatory cells and the release of IL- 6 and TNF-α [51]. Melatonin played key roles in reducing inflammation in both the HFD and microplastics groups in

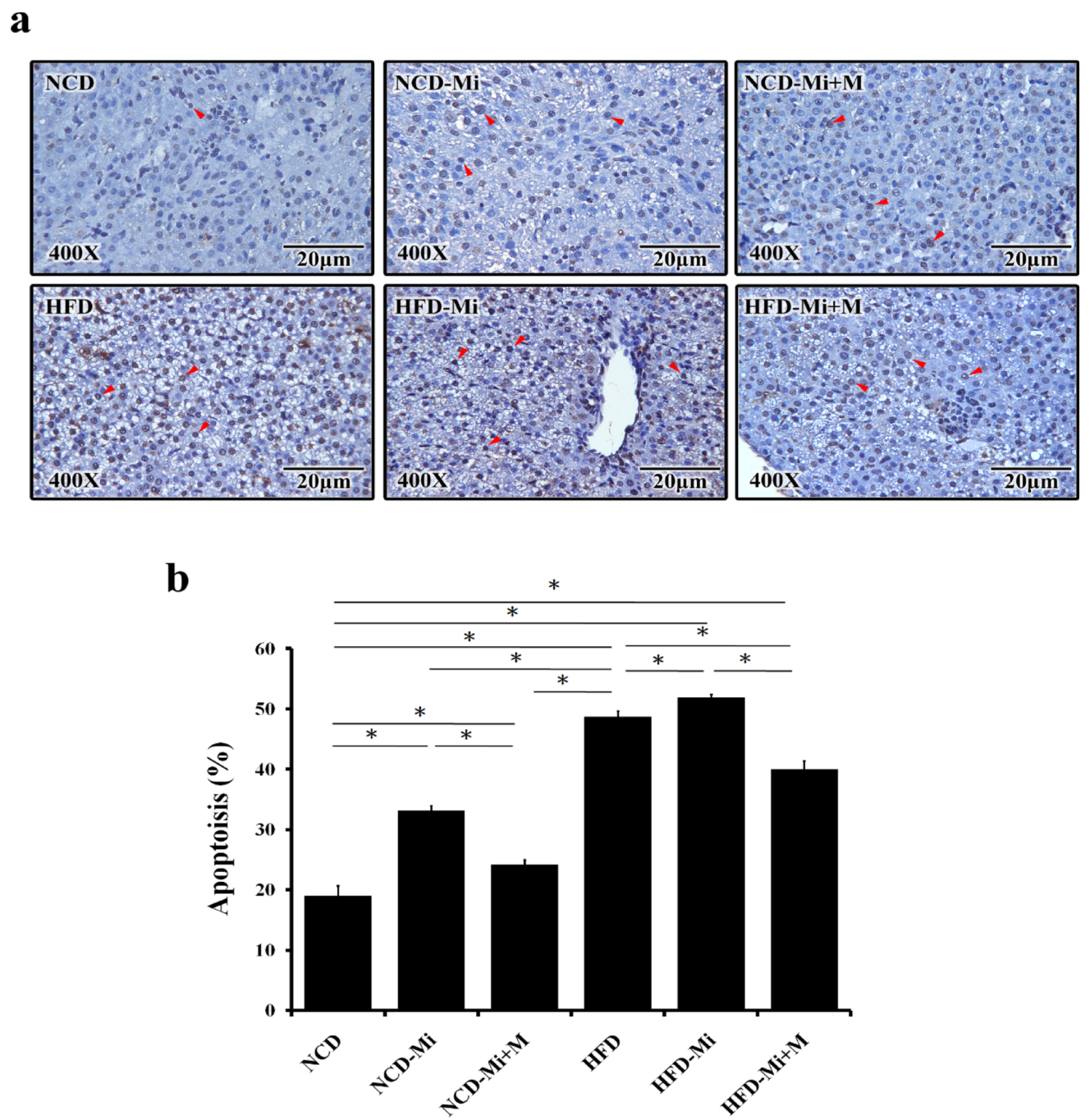

Fig. 4 Apoptosis characterized by liver cellular DNA fragmentation and late-stage apoptosis in the offspring liver. The apoptosis increased following microplastic exposure in both the NCD-Mi and HFD-Mi groups, and melatonin alleviated the apoptosis in both groups. (a) TUNEL staining in the offspring liver (b) Semi-quantitative analysis of liver TUNEL staining. *$P < 0.05$. ($n = 36$, each group had 6 animals). Different letters denote the respective groups as in Fig. 2. (original magnification 400x; bar = 20 μm)

our study. We hypothesize that melatonin can prevent liver inflammation in the pups exposed to both prenatal HFD and microplastics.

MDA is a reliable marker of oxidative stress and has been implicated in various disorders, including liver injury [52, 53]. Melatonin has been demonstrated to alleviate oxidative stress by modulating the ERK/Akt signaling pathway [54], and an HFD is known to induce oxidative stress and inflammation through the same pathway [55]. I In this study, melatonin significantly reduced MDA expression levels in the HFD-Mi + M group compared with the HFD and HFD-Mi groups. This finding may explain why melatonin effectively mitigated oxidative stress in the HFD-Mi + M group. No obvious effect of microplastics was observed in either

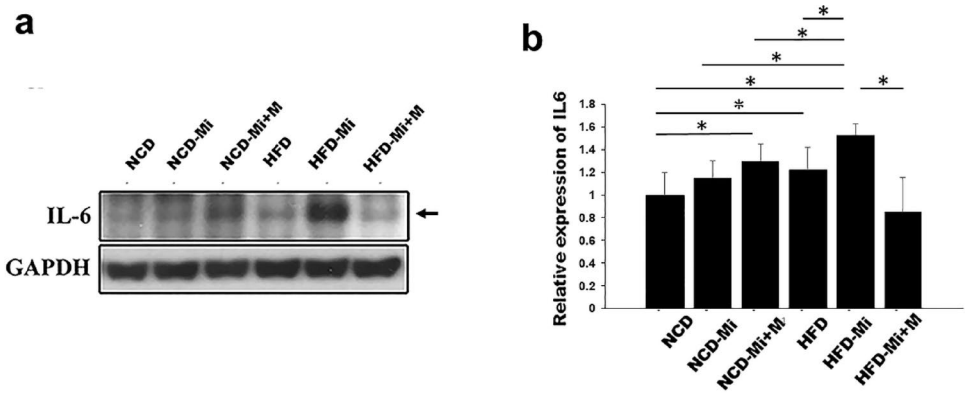

Fig. 5 Analysis of inflammatory responses. The expression of IL- 6 was the lowest in the HFD-Mi +M group. (a) Results of the Western blot analysis of IL- 6. (b) Quantification of IL- 6 expression. All values are expressed as mean ± standard error ($n = 36$, each group had 6 animals). *$P < 0.05$. Different letters denote the respective groups as in Fig. 2

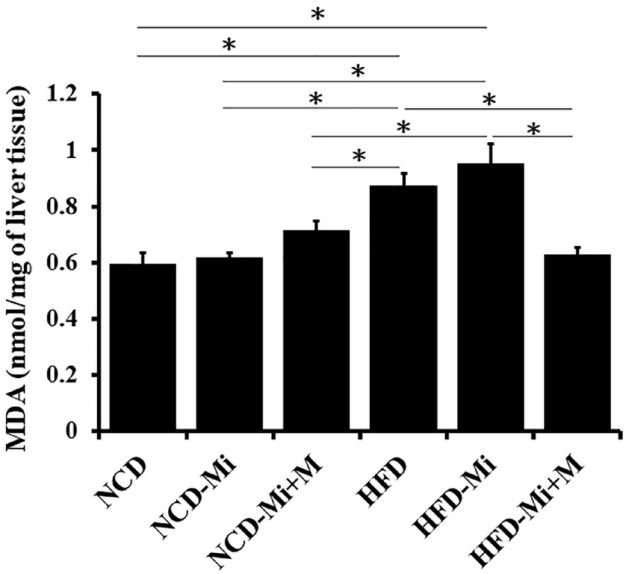

Fig. 6 Oxidative stress analysis. The expression of thiobarbituric acid reactive substances (TBARS) malondialdehyde (MDA) was lower in the HFD-Mi +M group than HFD-Mi group. All values are expressed as mean ± standard error ($n = 36$, each group had 6 animals). *$P < 0.05$. Different letters denote the respective groups as in Fig. 2

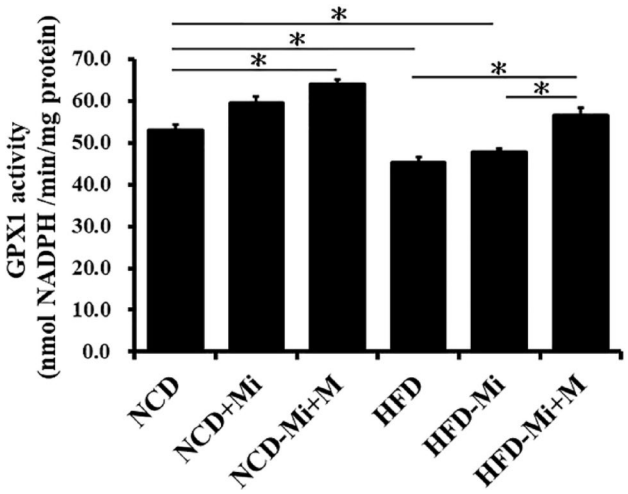

Fig. 7 Analysis of anti-free radical and antioxidant effects. The expression of GPX1 was the lowest in the HFD-Mi +M group. Results of the GPX1 assay with quantification of its expression. All values are expressed as mean ± standard error ($n = 36$, each group had 6 animals). *$P < 0.05$. Different letters denote the respective groups as in Fig. 2

the NCD-Mi or HFD-Mi groups. Since microplastics were administered during the prenatal period, maternal rat may have buffered the oxidative stress effects of microplastics on the neonate offspring.

GPX1 plays a crucial role as an antioxidant enzyme in regulating cellular redox balance by controlling ROS levels [56], and helps prevent further damage, including vascular disease [57]. Microplastics have been shown to induce oxidative stress and free radical damage [58, 59]. Melatonin directly alleviates oxidative and free radical stress in cells [60, 61]. In this study, microplastics significantly decreased antioxidant levels in both the HFD and HFD-Mi groups. Melatonin increased GPX1 levels in 7-day-old pups in the HFD-Mi +M group. This finding could be explained by the antioxidative effects of melatonin at the early developmental stage of pups.

SREBP- 1, ECSH1, and MCAD are crucial components of lipid metabolism [62, 63]. In this study, melatonin prevented the significant increase in SREBP- 1 induced by microplastics [14]. This intervention alleviated liver cell damage resulting from massive hepatic steatosis. Both melatonin and microplastics may influence SREBP- 1 through the SREBP- 1 signaling pathway, with melatonin specifically suppressing lipogenesis [59, 60]. A substantial increase in lipid accumulation was observed following microplastic exposure in both the NCD-Mi and HFD-Mi groups. However, a remarkable decrease in lipid accumulation was noted in the HFD-Mi +M group. These findings align with the changes observed in SREBP- 1 and could be explained by the combined effect of microplastics and melatonin on the SP1/SREBP- 1 signaling pathway [59, 60].

We did not study female offspring because their lipid metabolism is more sensitive to hormonal influences, leading to sex-specific differences in placental transcriptome results [64]. A HFD significantly alters the renal

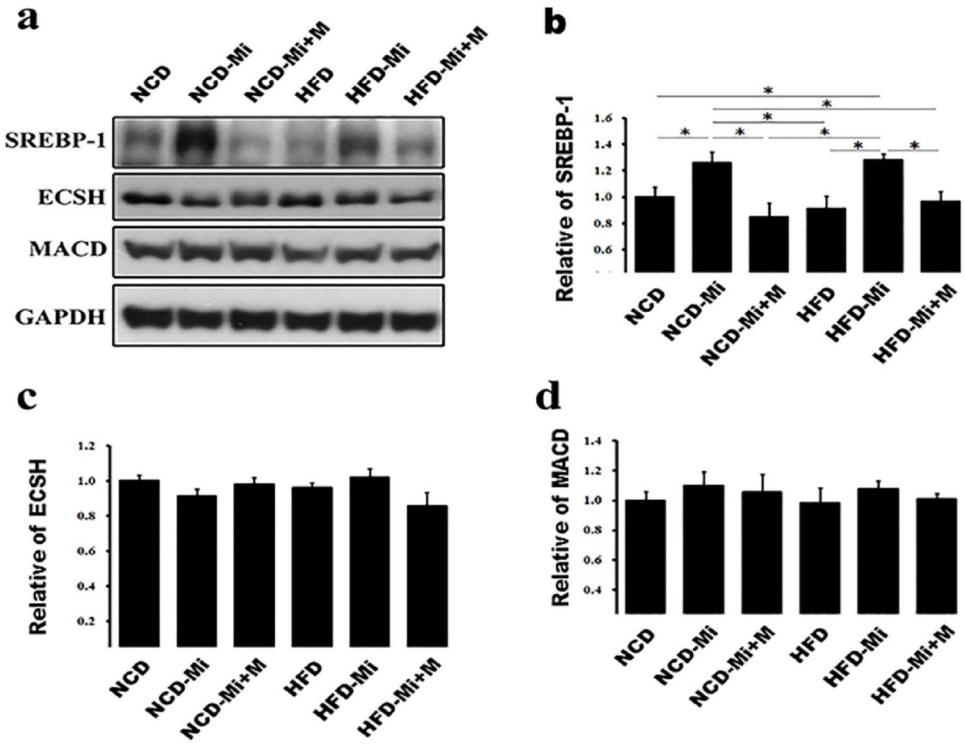

Fig. 8 Lipid metabolism study. The expression of SREBP- 1 was the lowest in the NCD-Mi + M and HFD-Mi + M groups. (a) Results of the Western blot analysis of SREBP- 1, ECSH1, and MCAD. (b) Quantification of SREBP- 1 expression. (c) Quantification of ECSH1 expression. (d) Quantification of MCAD expression. All values are expressed as mean ± standard error ($n = 36$, each group had 6 animals). *$P < 0.05$. Different letters denote the respective groups as in Fig. 2

transcriptome, with female offspring being more sensitive to HFD-related changes [65]. Maternal HFD-associated programming exhibits sexual dimorphism, with male offspring showing worse hepatic pathology, increased pro-inflammatory cytokines, and altered expression of bile acid regulators [39]. Nanoplastics exposure during pregnancy has a greater impact on female offspring than on male offspring [66]. It is possible that the impact of microplastics on offspring may vary depending on sex. Further experiments are needed to understand the differential impact of this sex difference.

Strengths

Our study elucidates the effects of prenatal exposure to an HFD, microplastics, and melatonin on offspring liver cells. The findings suggest that reducing exposure to HFD and microplastics while enhancing melatonin levels during pregnancy may benefit postnatal liver health. A key strength of this study is its focus on the combined effects of three factors: HFD, microplastics, and melatonin.

Limitations

All data were obtained from a single laboratory, ensuring consistency in experimental conditions. However, the study is limited by its associative findings and does not investigate epigenetic effects as a potential mechanism, including their impact on spermatogenesis in F1 male offspring [67–70]. Future studies should aim to clarify the cause-and-effect relationships more precisely, including potential effects on female offspring and subsequent generations.

Conclusions

Maternal HFD combined with microplastics has the potential to induce liver cellular apoptosis, inflammation, lipid accumulation, and oxidative stress in male pups. Prenatal melatonin administration may prevent liver steatosis damage in male pups.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

Institutional review board statement The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the Institutional Animal Care and Use Committee of the Kaohsiung Chang Gung Memorial Hospital (Approval No. 2021083001).

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