Osteocalcin prevents insulin resistance, hepatic inflammation and autophagy associated with high-fat diet induced fatty liver hemorrhagic syndrome in aged laying hens

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Keywords: high-fat diet, fatty liver hemorrhagic syndrome, osteocalcin, metabolic disorder

Posted Date: March 23rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-18321/v1

Abstract

Background: Osteocalcin (OCN), as an energy-regulating hormone, involves in preventing nonalcoholic steatohepatitis. Laying hens have been used as an animal model for investigating liver function and related metabolic disordersas that the synthesis of fat in laying hens is much faster than in mammals with limited adipose tissue. The aim of this study was to investigate the effects of OCN on fatty liver hemorrhagic syndrome (FLHS) in aged laying hens.

Methods: Thirty 68-week-old White Plymouth laying hens were randomly assigned into conventional single-bird cages, and the cages were randomly allocated into one of three treatments: normal diet (ND + vehicle , ND+V), high-fat diet (HFD + vehicle, HFD+V), and HFD + OCN (3 μg/bird, 1 time/2 days, i.m.) for 40 days. At experimental day 30, oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) were performed. At the end of experiment, the hens were euthanized followed blood collection. The plasma aspartate transaminase (AST), alkaline phosphatase (ALP), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using an automatic biochemistry analyzer. Pathological changes in the liver were examined under both light and transmission electron microscopes. The plasma inflammatory factors including interleukin-1 (IL-1), IL-6, and tumor Necrosis Factor-alpha (TNF-α) were analyzed by ELISA, and the gene expressions of these inflammatory factors in the liver were analyzed by Real-time PCR. And oxidative stress was evaluated using Malondialdehyde (MDA) and Glutathione peroxidase (GSH-Px) assay kits.

Results: The results showed HFD hens had more severe liver haemorrhage and fibrosis than ND hens. The ultra-microstructural examination showed that hepatocytes of HFD hens appeared necrotic pyknosis associated with great intracellular electron, mitochondrial swelling, shrunk nucleus and absence of autolysosomes. OCN mitigated these pathological changes by improved HFD hens' insulin resistance via alleviating the glucose intolerence and improving insulin sensitivity; inhibited HFD-induced oxidative stress as evidenced by decreased liver concentrations of MDA but increased GSH-Px; and reduced the inflammatory reaction with reducing blood IL-6 and TNF-α concentrations and mRNA expressions.

Conclusion: These results suggest a high-fat diet promotes the FLHS development in aged hens, while OCN prevents the FLHS process through inhibiting insulin resistance, inflammatory reaction, oxidative stress and fibrosis, and acting autophagy.

Introduction

Fatty liver hemorrhagic syndrome (FLHS) is a critical noninfectious metabolic disease, causing sudden death of birds resulted from liver rupture and hemorrhage [1, 2]. The syndrome is often observed in female hens as one of the main reasons of caged hen death [3]. It is associated with approximately 40% of the necropsied hens, and can be up to 74% in the caged laying hens [2]. Several factors have been relate to FLSH, such as nutritional, metabolic, hormonal, environmental, and genetic factors [1, 2], but

excess energy intake is the fundamental player as FLHS can be induced by a high-fat diet (HFD)[4–8] or a high fat and low protein (HFLP) diet [1, 9–12].

Hen liver plays a major role in the synthesis and metabolism of fat. In contrast to mammals, the synthesis of fat in birds is greater in the hepatic tissue and very limited in the adipose tissue [13]. In addition, commercial hens have been selected for high production and feed efficency, by which hen liver is more susceptible to metabolic disorder-induced injury compared with mammals. Hen FLHS has been used as a model of mammal nonalcoholic fatty liver disease (NAFLD) [8, 14–17]. Although the pathophysiological mechanism of hen FLSH remains unclear, but it has some similar with NAFLD, such as insulin resistence (IR), hepatic oxidation stress, inflammatory reaction and autophagy [15–18].

Osteocalcin (OCN), a major non-collagenous protein in bone matrix, is mainly synthesized by osteoblasts [19, 20]. A fraction of OCN is released in blood, presented as uncarboxylated OCN (ucOCN) and carboxylated OCN (cOCN) [21, 22]. The total circulation OCN is approximately 300 ng/mL in adult wild-type mice, while the ucOCN is only 5 ~ 10 ng/mL[23, 24]. However, ucOCN has been known as the “active form” with an energy-regulating function in rodent [23, 25, 26] and clinical research [27–29]. In humans, it has been used for preventing the metabolic diseases including type 2 diabetes [23, 30] and nonalcoholic fatty liver disease (NAFLD) [26, 31, 32]. In mice, ucOCN (3 or 30 ng/g/day) significantly increases glucose tolerance and insulin sensitivity in mice fed a regular die. In addition, injection of 30 ng/g/day OCN can be partially restored insulin sensitivity and glucose tolerance in HFD fed mice, and prevents the development of type 2 diabetes [30]. OCN also prevents NAFLD in mice fed a western-style high-fat high-cholesterol diet, resulting from protecting against IR and reducing hepatic damage including steatosis, ballooning degeneration, and fibrosis as well as synthesizing proinflammatory and profibrotic genes through the regulation of the nuclear factor-like 2 (Nrf2) and or c-Jun N-terminal kinase (JNK) pathways [31, 32].

In hens, the OCN concentration is affected by the sexual maturation process and egg production. It is 200 ~ 300 ng/mL at 6-wk-old, decreases to about 150 ng/mL at 16-wk, and then sharply declines to below 50 ng/mL after hens starting to lay eggs, even cannot detect in some individual birds [33].The average of total OCN concentration is only 10 ng/mL ~ 30 ng/mL at 70-wk or older laying hens [20, 33]. The circulating total OCN concentration of adult hens is far lower than adult mice (about 300 ng/mL) [24].Therefore, it is possible that hens OCN have some specific function which is not similar with mice. Previously, we have shown that the alteration of serum OCN concentration is correlated with the fatty liver disorder in high-fat and low-protein diet fed hens [12]. In the current study, the mechanism of OCN in promoting liver health of high-fat and low-protein diet fed laying hens was further investigated.

Materials And Methods

Experimental design

The study was conducted under the guidelines approved by the Animal Ethics Committee of the Southwest University.

Thirty 68-week-old White Plymouth laying hens were randomly assigned into conventional single-bird cages (40 cm × 35 cm × 35 cm each). After 2 weeks for hens to adapt their rearing environment, the cages were randomly assigned into one of three treatments (n = 10): normal diet + vehicle (ND + V), high-fat diet (HFD) + vehicle (HFD + V) (Table 1), and HFD + OCN treatment (3 μg/bird, 1 time/2 days, i.m.) (HFD + OCN) for 40 days. The selected dose was based on the studies conducted in mice [23, 31, 32]. The recombinant chicken ucOCN solution (Mybiosource, San Diego, USA) was freshly diluted in saline solution at a concentration of 6 μg/mL. During the experimental period, hens received light for 16 h/day. Feed and water were provided ad libitum.

Table 1
Laying hen feeding recipes

Ingredient	Normal Diet	High-fat diet
Corn (%)	62.6	49
Soybean (%)	25.7	28.5
Shell powder (%)	7.4	7.4
Soybean oil (%)	1.3	9.8
Zeolite powder	0	2.3
3% premix[1] (%)	3	3
Nutrition composition		
Energy (MJ/kg)	11.2	13.0
Crude protein (%)	16.5	16.5
Calcium (%)	3.5	3.5
Premix supplied the following per kilogram of feed: Vitamin A: 220 000-330 000 IU, Vitamin D_3: 55 000–85 000 IU, Vitamin E: ≥320 mg, Vitamin K_3: 40–140 mg, Vitamin B_1: ≥75 mg, Vitamin B_2: ≥155 mg, Vitamin B_6: ≥75 mg, Thiamine nitrate: ≥ 80 mg, Calcium pantothenate: ≥ 155 mg, Nicotinamide: ≥ 850 mg, Iodine: 5–15, Iron 2000–6000 mg, Zinc: 2400–4830 mg, Manganese: 2930–4820 mg, Copper: 267–667 mg, Selenium: 5–15 mg, Calcium: ≥ 8%, Total phosphorus: ≥3.3%, Sodium chloride: 7–14%, Methionine: ≥2.3%.		

Sample collection

Body weight was measured individually at day 0, 20, and 40. Feed intake was recorded and calculated on days 19–20 and 39–40. At the end of the experiment, each hen was anesthetized with pentobarbital sodium (30 mg/mL) within 2 minutes after removed from its cage. A 10 mL of blood sample from each

hen (n = 10) was collected via cardiac puncture into a plasma separator tube with EDTA and then centrifuged at 3 000 × g for 15 min at 4 ℃ for collecting plasma. After blood collection, the chickens were euthanized immediately.

Followed gross liver pathological examination (please see below), the whole abdominal fat pad, liver and pancreas weight of each hen were collected, and relative weight of the abdominal fat pad, liver and pancreas mass was calculated using the following formula: relative weight = tissue (organ) weight (g)/body weight (kg). A piece of the left lob liver (1 mm × 1 mm × 1 mm) sample was collected from each hen and fixed with 2.5% glutaraldehyde for transmission electron microscope analysis. The rest of left lob liver was fixed in 10% formalin until analysis. And, a piece of right lob liver sample was stored at -80 °C for real-time PCR and western-blotting analyses. The rest of right lob live was stored at -20 °C for liver fat content analysis by the Soxhlet method (Jiang et al., 2013).

Liver hemorrhage score

The liver haemorrhage was scored from 0 to 5: 0, indicating normal liver; 1, less than 10 subcapsular petechial or ecchymotic haemorrhages; 2, more than 10 subcapsular petechial or ecchymotic haemorrhages; 3, more than 1/3 but less than 1/2 of liver has subcapsular ecchymosis or ecchymosis;4, more than 1/2 liver but less than 3/4 of liver has subcapsular ecchymosis or ecchymosis; 5, more than 3/4 of liver has subcapsular ecchymosis or ecchymosis [1, 34].

Metabolic analysis

At day 30, Oral glucose tolerance tests (OGTT) were performed following 16 h overnight fasting. Five birds per group take orally 2 g/kg dose of glucose, and glucose of comb blood was measured at 0, 15, 30, 60, 120 and 240 min by blood glucose meter. Insulin tolerance tests (ITT) were performed on the rest five birds per group after 16 h of fasting. 100 μg/kg insulin was administered by abdominal subcutaneous injection (s.c.), and glucose of comb blood was measured at 0, 15, 30, 60, 120 and 240 min. The concentration of plasma insulin was measured on day 40 in 80-wk laying hens by ELISA kit (Xiamen Huijia Biotechnology Co., Ltd, Fujian, China).

Blood Parameters analysis

The concentrations of plasma aspartate transaminase (AST), alkaline phosphatase (ALP), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using the relative commercial kits via an automatic biochemistry analyzer (Olympus AU400, Japan). And the concentrations of interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor Necrosis Factor- alpha (TNF-α) were analyzed using relative ELISA kits (Xiamen Huijia Biotechnology Co., Ltd, Fujian, China).

Liver microstructural and ultra-microstructure analyses

The light microscopic analysis. The liver samples were processed by followed a routine tissue preparation procedure. Briefly, the samples were dehydrated with serially diluted ethanol, transparentized

with benzene, embedded in paraffin, and then sectioned at 3 μm using a sliding microtome (Leica RM2235, Leica microsystems, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (HE) or Masson's trichromatic staining for light microscopic examinations (Leica DM500, Leica microsystems, Wetzlar, Germany). The Masson's trichromatic stained fibrosis areas were analyzed by Image J software (National Institutes of Health, USA).

The electronic microscopic analysis. The ultrathin sections of liver samples were prepared and analyzed at the Wuhan Servicebio technology CO., LTD (Wuhan, China). Briefly, Liver samples were fixed with 2.5% glutaraldehyde, and rinsed by 0.1M phosphoric acid buffer (PB, pH7.4) for 15 min, and repeated 3 times, then fixed again with 1% osmic acid·0.1M PB. The fixed sections were dehydrated with serially diluted ethanol, permeated with acetone and SPI-pon812 embedding agent (1:1, SPI Supplies, West Chester, PA, U.S.A), and embedded with SPI-Pon812 embedding agent, then sectioned at 60–80 nm using ultra microtome (Leica UC7, Leica microsystems, Wetzlar, Germany). The slices were stained using double staining method: 2% saturated alcohol solution of uranium acetate and lead citrate, 15 min for each. The specimens were analyzed under the transmission electron microscope (HT7700, Hitachi, Japan).

Oxidative damage factors concentration of liver

The concentrations of malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) in the liver were measured by using MDA and GSH-Px assay kit, respectively, (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Real-time PCR

Total RNA of each liver samples was extracted using Trizol reagent (Invitrogen); and its quality was examined using NanoPhotometer (P330, Implen, Munich, Germany). The cDNA of IL-1, IL-6, and TNF-α and GAPDH [35] was synthesized from 1 μg of total RNA using NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge) (Novoprotein Scientific Inc., Jiangsu, China). The GAPDH was used as a housekeeping gene. The quantities of mRNA expression of IL-1, IL-6, and TNF-α relative to GAPDH mRNA expression were determined using the $2^{-\Delta Ct}$ method by fluorescent quantitative real-time PCR, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$. The sequences of primers were designed using the Primer 5.0, synthesized by the Invitrogen Biotechnology (Shanghai, China), and presented in Table 2.

Table 2

Real-time PCR primers and amplified PCR product size

Gene	GenBank ID	PCR Primers sequence (5′ to 3′)	PCR Products (bp)
IL-1	NM_204524.1	F: GGTCAACATCGCCACCTACA R: CATACGAGATGGAAACCAGCAA	86
IL-6	NM_204628.1	F: AAATCCCTCCTCGCCAATCT R: CCCTCACGCTCTTCTCCATAAA	106
TNF-α	NM_204267.1	F: GGACAGCCTATGCCAACAAG R: ACACGACAGCCAAGTCAACG	168
GAPDH	NM_204305.1	F: TTGACGTGCAGCAGGAACAC R: ATGGCCACCACTTGGACTTT	124

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor- alpha.

Statistical analyses

The data were analyzed by using SPSS 20.0 (IBM Corp., U.S.A). A One-way ANOVA was used to analyze the differences between treatments. Post hoc multiple comparisons were performed using LSD's test. Data normality was checked. Values were expressed as mean ± SEM, and P-value < 0.05 was considered statistically significant.

Results

OCN effect on HFD-induced gross organ changes and liver damage

Compared with ND + V birds, both HFD + V and HFD + OCN birds had higher absolute and relative abdominal fat pad weights ($P < 0.01$; Table 3), while HFD birds but not HFD-OCN birds also had a lower relative liver weight ($P < 0.05$). There were no treatment effects on body weight, feed intake, pancreas weight, and liver fat content ($P > 0.05$; Table 3). However, compared to controls, OCN administration alleviated HFD induced hen liver hemorrhage damage ($P = 0.05$).

Table 3

The effects of osteocalcin on body weight, feed intake, abdominal fat pad, pancreas and liver weights in high-fat diet fed 80-wk-old laying hens

Item	ND + V	HFD + V	HFD + OCN	SEM	P
0d BW (kg)	1569.8	1602.9	1607.7	0.06	0.81
20d BW (kg)	1603.0	1665.1	1673.6	0.07	0.59
40d BW (kg)	1557.8	1687.7	1655.9	0.08	0.23
20d feed intake (g)	95.66	87.50	88.18	8.11	0.56
40d feed intake (g)	97.51	89.43	91.05	8.26	0.91
Fat pad weight (g)	37.37^{B}	67.32^{A}	62.97^{A}	8.48	0.003
Relative fat pad mass (g/kg)	23.61^{B}	39.19^{A}	37.66^{A}	4.11	0.001
Liver weight (g)	40.90	37.85	40.72	3.03	0.54
Relative liver mass (g/kg)	26.19^{a}	22.31^{b}	24.71^{ab}	1.44	0.04
Pancreas weight (g)	3.46	3.28	3.46	0.27	0.76
Relative pancreas mass (g/kg)	2.22	1.94	2.09	0.14	0.17
Liver hemorrhage	0.8^{a}	1.67^{b}	1.0^{ab}	0.92	0.05
Fat content of liver (%)	21.11	17.06	18.20	0.8	0.15

$^{a, b}$ Mean ± SEM with different small letter in the same row differ significantly (n = 10, $P < 0.05$); $^{A, B}$ Mean ± SEM with different capital letter in the same row differ significantly (n = 10, $P < 0.01$).

HFD + V: high-fat diet + vehicle; HFD + OCN: high-fat diet + osteocalcin; ND + V: normal diet + vehicle.

OCN effect on biochemical parameters of liver function and blood lipids

Both HFD + V and HFD + OCN hens had higher plasma ALP activity than control hens ($P < 0.01$; Table 4); however, there were no treatment effects on plasma AST activity and TC, TG, LDL-C and HDL-C concentrations ($P > 0.05$).

Table 4
The effects of osteocalcin on liver function and lipid biochemical indexes in high-fat diet fed 80-wk-old laying hens

Parameters	ND + V	HFD + V	HFD + OCN	SEM	P
AST (U/L)	157.40	147.06	142.41	9.82	0.32
ALP (U/L)	285.1^{B}	1139.7^{A}	978.3^{A}	42.79	0.008
TC (mmol/L)	2.75	2.07	2.97	0.64	0.35
TG (mmol/L)	12.06	11.98	12.59	2.28	0.96
LDL-C (μmol/L)	312.08	325.45	321.27	9.77	0.40
HDL-C (μmol/L)	198.15	202.86	200.73	5.80	0.73
$^{A, B}$ Mean ± SEM with different capital letter in the same row differ significantly (n = 10, P < 0.01).					
AST: aspartate transaminase; ALP: alkaline phosphatase; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; TC: total cholesterol; TG: Triglyceride; HFD + V: high-fat diet + vehicle; HFD + OCN: high-fat diet + osteocalcin; ND + V: normal diet + vehicle.					

OCN effect on hepatic fibrosis, pyknosis, and autophagy

There was no treatment effect on the liver microstructure observed with HE staining (Fig. 1A-C) except that the fibrosis areas, excessive amount of scar tissue, were significantly increased (P < 0.01) in HFD + V group compared with ND + V group (Fig. 1D-G), which the HFD negative effect was reduced by OCN administration although without significance (P = 0.14).

The transmission electron microscopic image observation indicated compared with ND + V (Fig. 2A, a) controls, the HFD + V (Fig. 2B, b) hens' hepatocytes had more cellular damage with hepatocyte apoptosis evidenced as nuclear pyknosis, higher intracellular electron, and great mitochondrial swelling with decreased or disappeared of crista and increased dilated rough endoplasmic reticulum. OCN administration prevented the damage effects of HFD on hepatocytes autolysosomes were existed in ND + V and HFD + OCN (Fig. 2C, c) hens' hepatocytes, which was replaced by lysosome in HFD + V hens' hepatocytes.

OCN alleviates HFD-induced insulin resistance

The treatment significantly affected the OGTT results, showing as its unique time-dependent changed pattern of blood glucose concentrations. In ND + V group, the blood glucose concentration was sharply increased at 15 minutes of oral glucose challenge, a maximum (12.48 mmol/L) at 30 minutes, maintained at the similar level until 60 minutes, then sharply reduced at 120 minutes and maintained at the level up to 240 minutes (Fig. 3A). In the HFD + V group, the blood glucose concentration was continuously increased from 15 minutes and reached a peak (13.16 mmol/L) at 120 minutes, then

sharply diminished to the control level at 240 minutes. The effects of HFD on blood glucose concentration was decreased by administration of OCN. In HFD + OCN group, the concentration of blood glucose was relative stable, and the peak level was 10.82 mmol/L only, which was similar to the level of ND + V group but significantly lower than HFD + V group.

There were treatment effects on ITT outcomes. HFD + V hens had the highest blood glucose level from 15 to 240 min after administrated insulin among the treatments (Fig. 3B). Compared to ND + V hens, the blood glucose levels were significantly increased between 30 and 60 min in HFD + V hens ($P < 0.05$), while HFD + OCN group was intermediate. In addition, the plasma insulin concentration in HFD + V hens but not in HFD + OCN hens had a downward trend compared to ND + V hens ($P = 0.07$; Fig. 3C).

OCN effects on liver oxidative stress and inflammatory reaction

Compared to ND + V group, liver MDA concentration in HFD + OCN group was lower ($P < 0.05$; Fig. 4A) but higher GSH-Px concentration ($P < 0.05$; Fig. 4B). And, compared to HFD + V group, the liver GSH-Px was increased ($P < 0.01$) in HFD + OCN group.

Compared with ND + V group, the plasma IL-1 (Fig. 5A) concentration was increased in both HFD + V ($P < 0.05$) and HFD + OCN ($P < 0.01$) groups, but IL-6 ($P < 0.05$; Fig. 5B) and TNF-α ($P < 0.01$; Fig. 5C) concentrations were reduced in HFD + OCN group but not in HFD + V group. Moreover, compared with ND + V group, the gene expression of TNF-α in the liver was significantly decreased by OCN administration compared to ND + V ($P < 0.01$; Fig. 5c) and HFD + V ($P < 0.05$) groups. There was no treatment effect on the liver IL-1, and IL-6 mRNA expression. ($P > 0.05$; Fig. 5a-b, d).

Discussion

Hens fed a HF diet [4–7] or HFLP diet [1, 9–12] have been created for studying the pathogenesis of FLHS in laying hens or as a model for human NAFLD investigation. The pathological characters of FLHS include hepatic fat accumulation in the liver with hemorrhage or rupture. In the current experiment, however, the liver weight, especially relative liver weight, was reduced without change of fat content in hens fed HFD. These results may indicate that the HFD did not induce hepatic lipidosis in aged hens. However, HFD fed hens had severe liver hemorrhages and significant heavier abdominal fat pad. Chicken stores energy as neutral fats mainly in the adipocytes of the abdominal fat pad. Although it was not observed hepatic lipidosis in HFD-fed hens, the results from a previous study have shown that the abdominal fat weight is positively correlated with the liver fat percentage in selected broilers [36]. The different findings could be related to multiple factors including the genetic background of chickens and their age as well as the sample size. Similar to the current findings, Trott et al. [2] analyzed the 76 FLHS backyard chickens from January 2007 to September 2012 and demonstrated 48% of FLHS without hepatic fat accumulation. Rozenboim et al. [1] reported that both HFD and HFLP diets reduced liver fat content without effect on liver mass but in 100-wk-aged hens, while diet significantly increased liver fat content in 42-wk-old hens. Therefore, the hepatic lipidosis may not be generalizable in FLHS in chickens.

Further analyses, the lost liver mass in HFD + V hens may be because the hepatic apoptosis and nuclear pyknosis evidenced by the ultra-microstructural analyses, indicating severer liver damage. Ultra-microstructural analysis also revealed that HFD inhibits hepatic autophagy but OCN reversed the effect. Autophagy, a self-degradative process, is a critical biological pathway for the degradation of damaged intracellular components by lysosomes [37–40]. Hepatocytes autophagic function is affected by Hepatocytic lipid accumulation which reduces the infusion efficiency between autophagosomes and lysosomes, leading to suppressed autophagy [41]. The hepatocytes autophagy is also inhibited by insulin resistance, increasing hepatocyte oxidative stress, inflammatory reaction, and apoptosis [37, 39, 42]. Autophagy has been potentially linked to fibrogenesis [37]. The hypothesis is supported by the pathological changes with fibrosis in the livers of HFD-induced FLHS hens. In humans, similar to the pathological finding in HFD-induced FLHS in laying hens, NAFLD is an accumulating damage which includes fatty (buildup of fat), steatohepatitis, fibrosis, and cirrhosis of the liver [43]. Therefore, we considered that HFD further promotes the fat metabolic disorder-associated steatohepatitis and or fibrosis of FLHS seen in aged hens.

Plasma ALP and AST activities have been used as indicators of liver damage; and plasma TC, TG, LDL-C and HDL-C are the biomarkers of blood lipid metabolism. There biological factors have been used for diagnosis of FLHS in chickens [7, 44] and NAFLD in humans [45, 46]. In the current experiment, ALP but not AST activity was increased in HFD + V hens. Similarly, Rozenboim et al. [1] analyzed the changes of ALP and AST in both 26-wk old young and 84-wk old laying hens fed HFD, LPD (Low protein diet) or HFLP at 5, 10, and 15 wks. The plasma ALP value was significantly higher in young hens at wk 5 while the value of AST became higher at wk 15 post-fed HFLP diets compared to the controls; however, the experiment diets had no difference on both ALP and AST concentrations in 84-wk old hens at any tested time points. Choi et al. [6] and Robinson et al. [9] also reported that the blood concentration of AST and ALP were not difference between control and FLSH hens. There was also no treatment effect on the concentrations of TC, TG, LDL-C, and HDL-C in our current experiment. One of the main reasons is that HFD may cause liver damage without hepatic lipidosis. These biochemistry indicators of liver function and blood lipid have been considered as limited diagnostic tools for FLHS [1].

Insulin is the only hyperglycemic hormone released by the pancreatic β cells. Insulin resistance plays a key role in both human NAFLD and hen FLHS [15, 47]. In the present study, after fed 40 days of HFD, blood glucose tolerance, insulin sensitivity, and insulin concentration were decreased in hens, but these changes were alleviated by OCN injection. Similar to our found, Zhuang et al. [15] reported that FLHS hens had a higher blood glucose than control hens detected by OGTT and ITT. In addition, HFD-induced NAFLD-like damage in mice exhibit the positive results during both OGTT and ITT, and these changes can be revised by oral administration or injection of OCN [31, 32, 48]. The changes of insulin concentrations identified in the current study may indicate that HFD causes pancreatic β cell damage, while OCN administration improves β-cell mass and insulin secretion [23, 30].

Insulin resistance will further lead to hepatic damage by triggering oxidative stress and inflammatory reaction [15, 47]. Oxidative stress is caused by reactive oxygen compounds such as MDA, which is a toxic

molecule and has been used as an index of lipid peroxidation in humans and animals [31, 49, 50]. GSH-Px is an important peroxidase, functionally as an index of anti-peroxidation ability [51]. In HFD-OCN hens, the steeply decreased MDA and increased GSH-Px evidence that OCN inhibits hepatic oxidative stress in HFD-induced FLHS hens. Interestingly, there is a similar metabolic milieu between FLHS in laying hens and NAFLD in mice and humans; and oxidative stress in NAFLD can be alleviated by OCN [31].

As pro-inflammatory factors, IL-1, IL-6, and TNF-α have been used for evaluating infectious and inflammatory reaction-associated immunity in humans and various animals including chickens [52–54]. TNF-α plays an important role in the pathogenesis of mammals' NAFLD [55] and has a close relationship with the liver steatosis, fibrosis, and apoptosis [56, 57]. In the present study, the massively reduced TNF-α concentrations in plasma and mRNA expression in the liver, along with the similar trend changes of IL-6, suggest that OCN prevents hepatic inflammatory reaction. However, OCN elevated the blood IL-1 concentration but potentially reduced liver IL-1 mRNA expression. The reasons could be that OCN does not only regulate liver's function in synthesis of inflammatory factors but also affects the function of other organs, such as adipocytes, the pancreas and the intestine, involving in immune reactions through regulating cell metabolism [58].

Conclusions

High-fat diet promotes FLHS development in aged hens, while osteocalcin alleviates HFD-induced liver damage, reducing hepatocytic haemorrhage and fibrosis, inhibits metabolic disorders including insulin resistance, oxidation stress and activates hepatocyte autophagy.

Abbreviations

AST: aspartate transaminase; ALP: alkaline phosphatase; FLHS: Fatty liver hemorrhagic syndrome; HFLP: high fat and low protein; HFD: high-fat diet; HDL-C: high density lipoprotein cholesterol; GSH-Px: glutathione peroxidase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-1: Interleukin-1; IL-6: Interleukin-6; IR: insulin resistance; ITT: insulin tolerance tests; JNK: c-Jun N-terminal kinase; LDL-C: low density lipoprotein cholesterol; LPD: low protein diet; MDA: malondialdehyde; NAFLD: nonalcohol fatty liver disease; ND: normal diet; OCN: osteocalcin; OGTT: oral glucose tolerance tests; PB: phosphoric acid buffer; TC: total cholesterol; TG: Triglyceride; TNF-α: Tumor Necrosis Factor-alpha

Declarations

Acknowledgments

Thanks for Yan Qiu help us to collect samples.

Authors' contributions

SJ designed this study, applied funding and wrote the manuscript. XW performed the experiments and analyzed the data. XZ took part in preparing tissue slices. MZ and HH took part in the animals feed and collecting data. XW guided XW, MJ guided XZ. WC, SJ and XW revised the manuscript. All authors approved the final version of the manuscript.

Funding

This research was funded by the National Natural Science Foundation of China (No.31702307).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Ethics approval

The present experiment complied with China law on the humane care and use of animals in research and the study was conducted under the guidelines approved by the Animal Ethics Committee of the Southwest University.

Conflict of Interests

The authors declare no conflict of interest.

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Figures

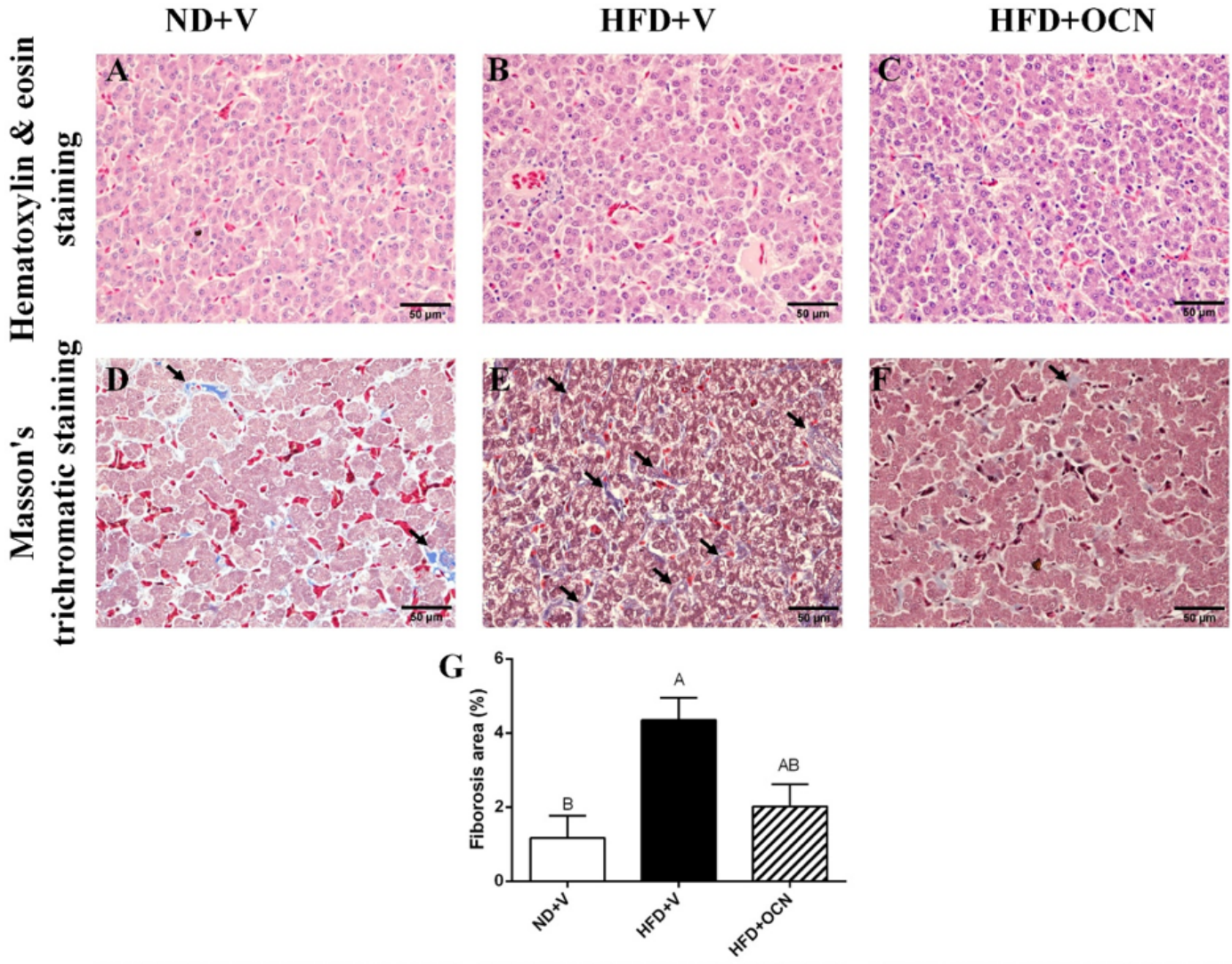

Figure 1

The effects of a high-fat diet and osteocalcin on the pathological changes of the liver in aged hens. A, B, C Examples of liver light microscopical structures were examined with HE staining; D, E, F The hepatica fibrosis change was observed by Masson's trichromatic staining; G Percentage of the fibrosis areas. Bar = 50 μm : fibrosis. HFD+V: high-fat diet + vehicle; HFD+OCN: high-fat diet + osteocalcin; and ND+V: normal diet +vehicle.

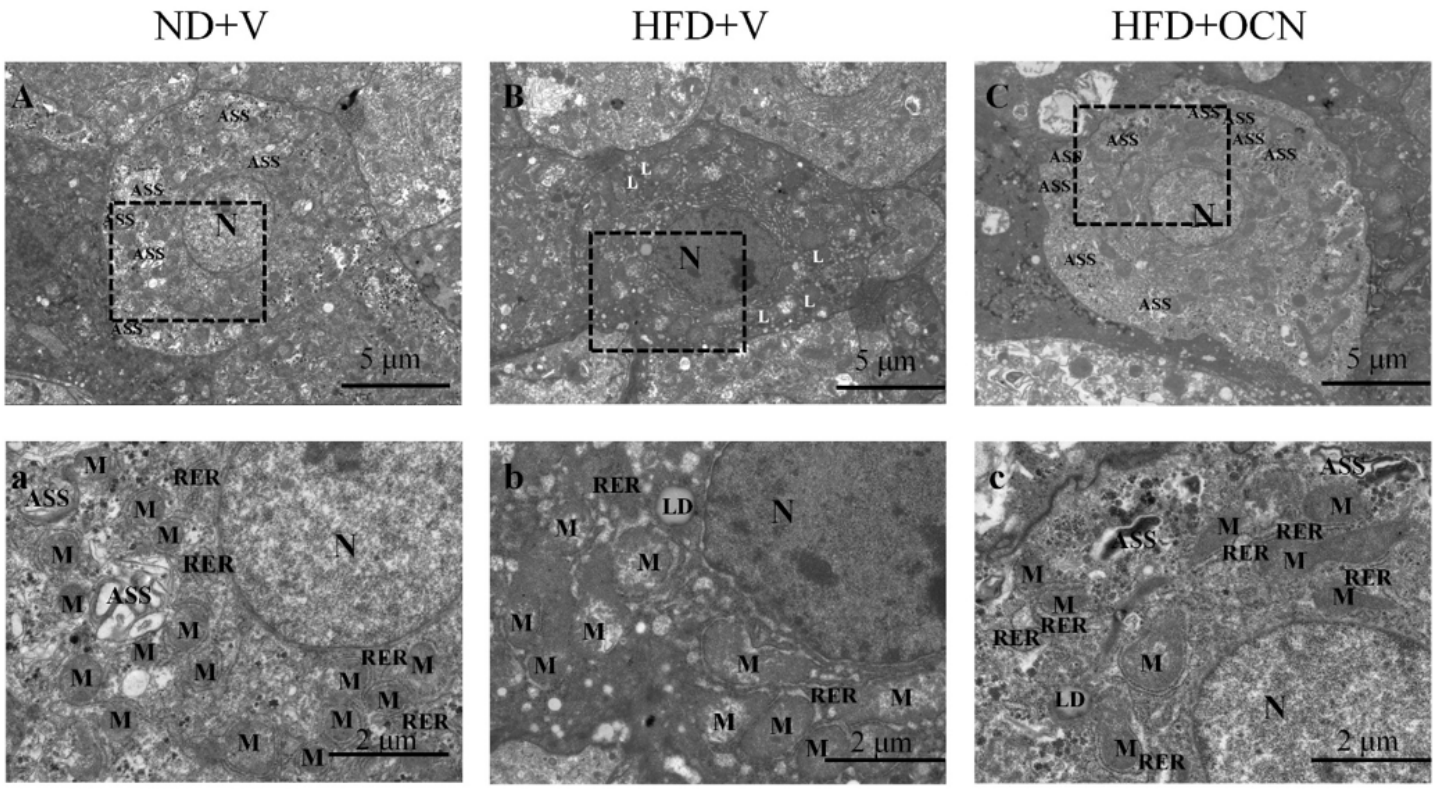

Figure 2

The effects of a high-fat diet and osteocalcin on the hepatocytic ultrastructures in aged hens. A, B, C Examples of electron micrographs showing hepatocytic ultrastructural features. a, b, c High power micrographs of the relative areas outlined by the squares. Bar = 5 or 2 μm, respectively. ASS: autolysosome; HFD+V: high-fat diet + vehicle; HFD+OCN: high-fat diet + osteocalcin; L: lysosome; LD: lipid droplet; M: mitochondria; N: nucleus; ND+V: normal diet +vehicle; RER: endoplasmic reticulum.

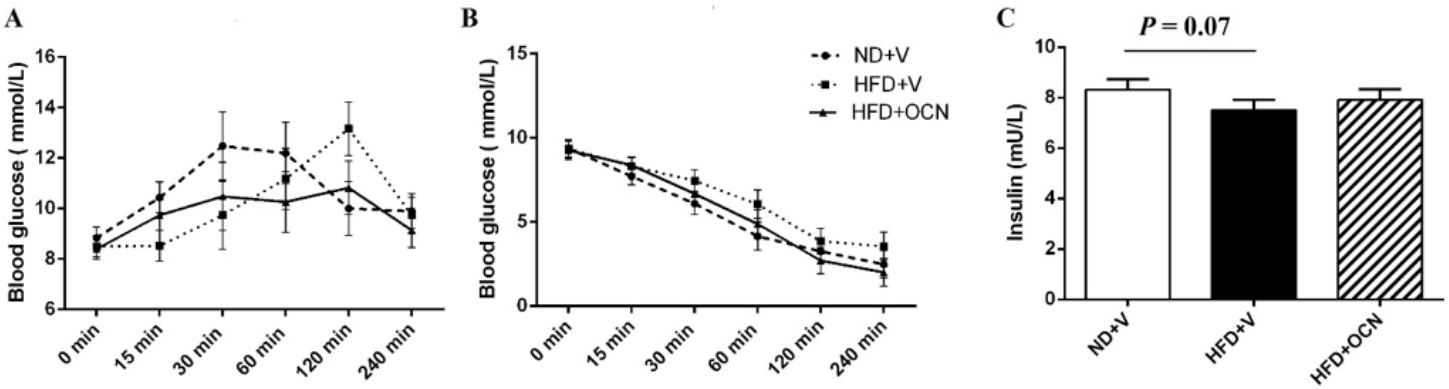

Figure 3

The effects of a high-fat diet and osteocalcin on glucose tolerance and insulin sensitivity outcomes in aged hens. A The outcomes of glucose tolerance test; B The outcomes of the Insulin tolerance test; C Plasma insulin concentrations. a, b Mean ± SEM with different small letter differ significantly (n=10, P <

0.05). HFD+V: high-fat diet + vehicle; HFD+OCN: high-fat diet + osteocalcin; and ND+V: normal diet +vehicle.

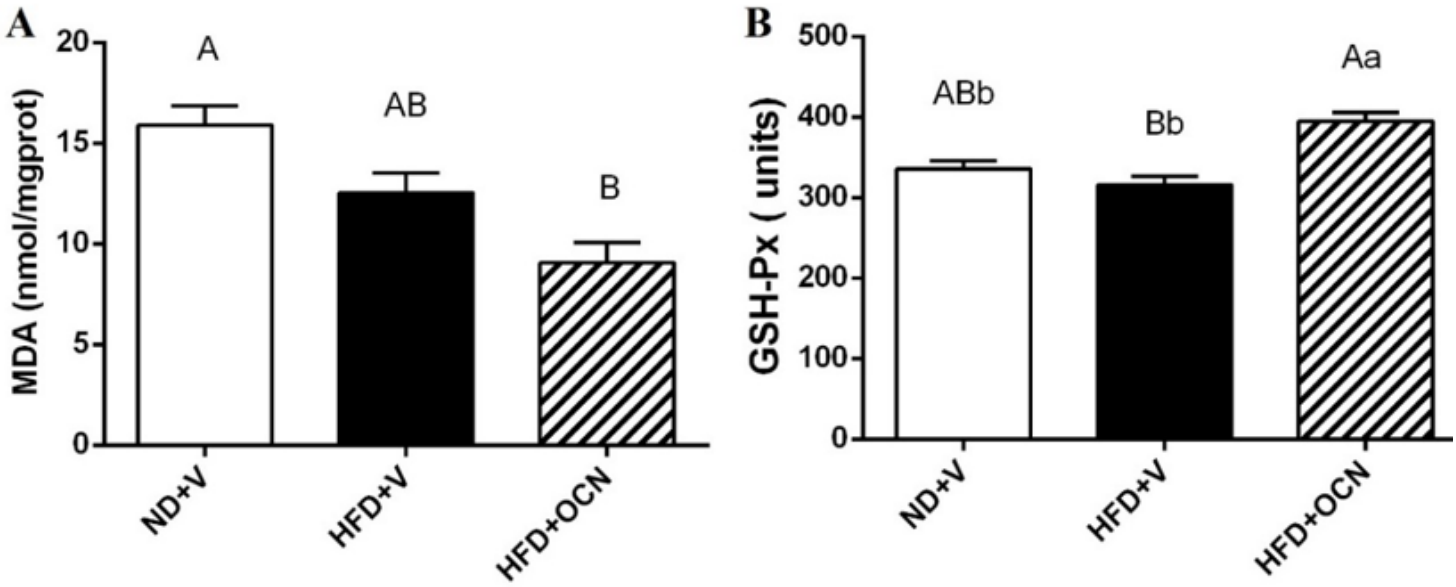

Figure 4

The effects of a high-fat diet and osteocalcin on hepatic oxidative damage in aged hens. A. The changes of malondialdehyde concentrations; B The changes of glutathione peroxidase concentrations. a, b Mean ± SEM with different small letter differ significantly (n=10, $P < 0.05$); A, B Mean ± SEM with different capital letter differ significantly (n=10, $P < 0.01$). HFD+V: high-fat diet + vehicle; HFD+OCN: high-fat diet + osteocalcin; and ND+V: normal diet +vehicle.

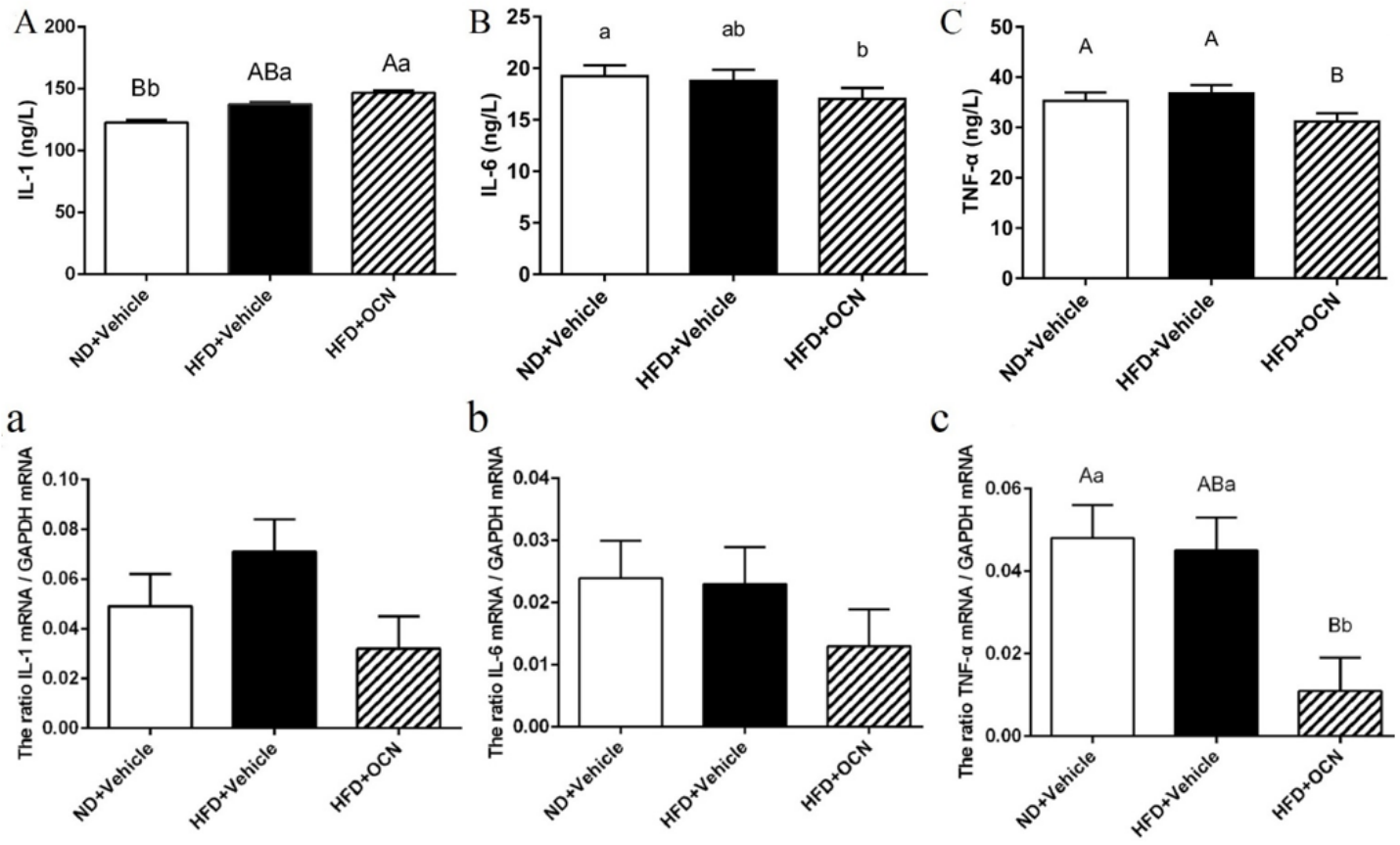

Figure 5

The effects of a high-fat diet and osteocalcin on plasma concentrations and liver mRNA expressions of inflammatory factors in aged hens. A The change of plasma IL-1; B The change of plasma IL-6; and C The change of plasma TNF-α; a The change of liver IL-1 mRNA expression; b The change of liver IL-6 mRNA expression; and c The change of liver TNF-α mRNA expression. a, b Mean ± SEM with different small letter differ significantly (n=10, $P < 0.05$); A, B Mean ± SEM with different capital letter differ significantly (n=10, $P < 0.01$). HFD+V: high-fat diet + vehicle; HFD+OCN: high-fat diet + osteocalcin; ND+V: normal diet +vehicle; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor-alpha