

Anti-inflammatory effect of piperine ameliorates insulin resistance in monosodium glutamate–treated obese mice

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Abstract

Background: Metabolic inflammation has been considered as an essential event in obesity-induced diabetes and insulin resistance. In obesity, an increasing number of macrophages recruited into visceral adipose tissues undergo significant M₁-like polarization, secreting variable amounts of pro-inflammatory cytokines and causing insulin resistance. **Methods:** In this study, we investigated the effect of piperine on adipose tissue inflammation and insulin resistance in monosodium glutamate (MSG)-induced obese mice. The 6-month-old MSG mice were divided into three groups, which were treated with piperine (40 mg/kg/day), metformin (150 mg/kg/day) and vehicle for successive 10 weeks, respectively. Normal mice at the same age as the normal control. **Results:** Our results showed that the 10-week administration of piperine (40 mg/kg/d) not only significantly decreased the elevated fasting blood glucose, serum TC and TG levels, but also enhanced infusion rate in hyperglycemic clamp experiment and improved the oral glucose intolerance as well as abnormal insulin tolerance in adult MSG obese mice. Additionally, piperine significantly decreased the total and differential white blood cell (WBC) count and the serum level of lipopolysaccharide (LPS), pro-inflammatory cytokines such as galectin-3 (Gal-3), interleukin-1 β (IL-1 β). Furthermore, piperine clearly down-regulated the mRNA levels of pro-inflammatory cytokines and the protein levels of CD11c and Gal-3 in adipose tissues. In addition, the in vitro study showed that piperine inhibited LPS-stimulated polarization of RAW 264.7 cells toward the M₁ phenotype. **Conclusions:** In summary, these findings demonstrated that piperine could significantly rectify glycolipid metabolism disorders, improve severe insulin resistance and ameliorates systemic metabolic inflammation in MSG obesity mice. Our study indicates that piperine, as a potential natural alkaloid, can be used in the treatment of obesity-associated diabetes by delaying the progression of obesity-induced insulin resistance. **Keywords :** Insulin resistance; Piperine; Chronic low-grade inflammation.

1. Background

Obesity and type 2 diabetes mellitus (T2DM) have been a great threat worldwide in recent years. According to the Global Diabetes Atlas (8th edition, International Diabetes Federation), the global incidence of diabetes was 8.8%, and the total number of patients was 425 million in 2017. By 2045, these two figures will increase to 9.9% and 629 million, respectively [1]. Obesity is one of the main causes of metabolic diseases including T2DM, steatohepatitis, fatty liver diseases, and many cardiovascular diseases [2–5]. Studies have revealed that both obesity and T2DM are chronic low-grade inflammatory diseases [6, 7]. It is worth noting that macrophages play an essential role in obesity and T2DM. Firstly, studies have shown that the number of macrophages is significantly increased in the adipose tissue of both rodents and humans [8]. Further studies show that the increase of the macrophages is mainly because of the increase of pro-inflammatory macrophages, i.e., the M₁polarized macrophages, and the ratio of M₁ to M₂ macrophages is also increased, which causes inflammations in adipose tissues and insulin resistance [9, 10]. These pieces of evidence indicate that modulation of the conversion of M₁ to M₂-like polarized state of macrophages, either by genetic or pharmacological methods, is a promising approach for the treatment of obesity-induced insulin resistance and diabetes.

Pro-inflammatory cytokine galectin-3 (Gal-3) plays a vital disease-exacerbating role in autoimmune/inflammatory diseases including obesity-associated diabetes [11]. Gal-3, an approximate 31-kDa protein with a specific carbohydrate recognition domain and a conserved N-terminal domain, functions as an inflammatory mediator [12], which is mainly secreted by M₁-like macrophages in visceral adipose tissues and can directly enhance macrophage chemotaxis. A previous study has shown that the expression of Gal-3 is significantly decreased in the CD11c⁺ macrophages of the adipose tissues when the obese mice are fed a normal chow diet, and the inflammatory reaction and insulin resistance are both ameliorated [13]. Gal-3 inhibits the downstream signaling of the insulin receptor (IR) by directly binding with IR, leading to systemic insulin resistance [14]. These studies indicate Gal-3 may be a potential target for diabetes. Insulin sensitivity may be improved, and glucose tolerance may be achieved by the inhibition of Gal-3.

In this study, the obese mice were obtained by monosodium glutamate (MSG) neonatal intoxication. The obese mice show hypothalamic lesions, and neuro-endocrine changes have been observed in the insulin and leptin signaling [15]. The mice gradually develop obvious centripetal obesity, severe metabolic inflammation, disorders of glycolipid metabolism, and T2DM after 4 months. Adult MSG obese mice have typical properties of obesity associated with insulin resistance and T2DM. Hence, this model is suitable for the investigation of obesity-related metabolic dysfunctions [16, 17].

Piperine is the major alkaloid presented in black pepper (*Piper nigrum*), long pepper (*Piper longum*), and many other piper species. Piperine exhibits a wide range of biological properties, such as immunomodulatory, anti-oxidant, anti-lipid metabolism disorder, and anti-inflammatory [18–20]. Among these pharmacological activities, what attracts us most is its excellent modulatory effect on immune-inflammation in disease models such as clone diseases, arthritis, as well as ulcerative colitis [21–23]. However, the function of piperine in metabolisms is yet to be understood. In addition, it is unclear whether piperine can improve insulin resistance by inhibiting the inflammatory reaction in obesity. The investigation in this study will help us understand the role of piperine and find implications in the treatment of T2DM.

2. Methods

2.1. Chemicals and Animal model

Monosodium glutamate (MSG), glucose, metformin, piperine, and LPS were purchased from Sigma Chemical Co. (St Louis, MO, USA). Male ICR mice (newborn) were purchased from Vital River Laboratory Animal Technology (Beijing, China). MSG was administered via the subcutaneous injection route at 4 g/kg/day for 7d [24]. Normal mice in the control group were injected with 0.9% physiological saline solution. The mice were weaned and kept in the Laboratory Animal Center of Qingdao University at an ambient temperature of 25 ± 2°C, 12 h light /dark cycles and humidity of 40–60%. The mice were allowed free access to water and a chow diet. The food intake and body weights of the mice were recorded weekly during the 10-week period of the treatment. Compared with the normal mice, the MSG mice showed

significant centripetal obesity after adulthood, accompanied by elevated serum TC, TG, and insulin levels. Insulin resistance was increased as well. Based on the body weight, Lee's index, serum TC, TG and fasting blood glucose, the 6-month-old MSG mice were divided into three groups, which were treated with piperine (40 mg/kg/day), metformin (150 mg/kg/day) and vehicle for successive 10 weeks, respectively. All animal protocols conformed to the Guidelines for the Care and Use of Laboratory Animals prepared and approved by the Animal Care and Use Committee of the Affiliated Hospital, Qingdao University and approved by the Animal Experimental Ethical Committee of Affiliated Hospital, Qingdao University, Shandong Province, China.

2.2 Measurement of visceral organ indexes and the collection of serum

The mice were euthanized by opening the heart under anesthesia with pentobarbital (50 mg/kg). Then, a laparotomy was performed to collect and weigh the weight of abdominal adipose, pancreas, liver, and kidney and then frozen above organs in liquid nitrogen before further analysis. RNA extraction and real-time PCR analysis were performed using these tissue samples. Blood samples were prepared by centrifuge at 4000 rpm for 10 min at 4 °C. The samples were stored at -80 °C for later analyses. Throughout the experiments, all mice were provided with housing that allows the expression of species-specific behaviours, using appropriate anaesthesia and analgesia to minimise pain, and training mice to cooperate with procedures to minimise any distress. Thus, the experimentally induced stress was minimized. All experimental procedures conformed to the European Guidelines for the care and use of Laboratory Animals (directive 2010/63/EU).

2.3 Oral glucose tolerance test (OGTT)

Before the last week of the experiment, 4-h-fasted mice received glucose (2 g/kg body weight) by gavage. A total of 3.5 µL blood was collected from the tip of the tail vein at different time points (0, 30, 60, and 120 min) after glucose load, which was used for blood glucose determination on an one Touch Ultra glucose meter. The area under the curve (AUC) was calculated according to the equation: $AUC = \frac{1}{4}(PG_{0min} + PG_{30min}) + \frac{1}{4}(PG_{30min} + PG_{60min}) + \frac{1}{2}(PG_{60min} + PG_{120min})$. Where, PG_{0min}, PG_{30min}, PG_{60min}, and PG_{120min} is the blood glucose level at 0, 30, 60, and 120 min after glucose load, respectively.

2.4 Hyperglycemic clamp experiment

At the last week of the experiment, mice fasted for 4 h were anesthetized with (50 mg/kg i.p.) of pentobarbital and perform tracheotomy. 30 minutes after the operation when the animals were stable, the hyperglycemic clamp experiment was performed. First, injected an initial dose of glucose(250 mg/kg B.W.) through a jugular vein tube to quickly increase blood glucose to a higher level within 5 minutes, and then a variable rate of glucose (20%, w/v) was infused and blood glucose levels were measured at 5-min intervals until the blood glucose level reached to steady state (blood glucose levels at 13.5–14.5 mmol/L). After steady state, the average glucose infusion rate at 5 time points was taken as the glucose infusion rate (GIR) at steady state.

2.5 Insulin tolerance test (ITT)

The mice were administered with insulin (0.4 unit/kg) 4 h after fasting via intraperitoneal injection. The blood glucose levels were calculated using blood samples collected at 0, 40 and 90 min after insulin injection. The percentage of blood glucose reduction at 40 min was calculated accordingly.

2.6 Routine blood test and biochemical analysis

Detection of routine blood of mice by blood cell analyzer. Serum total cholesterol (TC) and triglyceride (TG) were assayed by using the enzymatic colorimetric methods provided by the commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). Fasting blood glucose was detected by an one Touch Ultra Meter (Roche Acompany, Nano, Switzerland).

2.7 Enzyme-linked immunosorbent assays

Serum insulin was determined by using a mouse ultrasensitive insulin ELISA kit (American Laboratory Products Company, USA). LPS produced by the Gram-negative bacteria in the intestine, M₁-like pro-inflammatory cytokines (IL-1 β and Gal-3) and M₂-like anti-inflammatory cytokine (IL-10) in the serum samples were measured by enzyme-linked immunosorbent assay kits (Mibio, Shanghai, China).

2.8 Semiquantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from the adipose tissues or cultured cells homogenized in Trizol (CW BIO, Beijing, China), which was used for the synthesis of cDNA. qRT-PCR amplification (TaKaRa Bio, Shiga, Japan) was performed on a BioRad CFX96 detection system (BioRad, Hercules, CA, USA) using the primers obtained from the GeneBank. The expression levels of the genes were measured relative to the β -actin level and evaluated using the 2- $\Delta\Delta CT$ method. The primer sequences are presented in Table 1.

Table 1
Primer sequences for qRT-PCR.

Gene	Primer sequences
CD11c	Forward:5'-ACACAGTGTGCTCCAGTATGA-3' Reverse:5'-GCCAGGGATATGTTCACAGC-3'
IL-1 β	Forward:5'-AAATACCTGTGGGCCTGGGC-3' Reverse:5'-CTGGGATCCACACTCTCCAG-3'
TNF- α	Forward:5'-CCAGACCCTCACACTCAGATC-3' Reverse:5'-CACTTGGTGGTTGCTACGAC-3'
Galectin-3	Forward:5'-TCCTGGAGGCTATCCTGCTG-3' Reverse:5'-TGTTTGCCTGGGTTCACTG-3'
β -actin	Forward:5'-AAGAGAGGGCATCCTGACCC-3' Reverse:5'-TACATGGCTGGGTGTTGAA-3'

2.9 Morphologic and immunohistochemical analysis

The abdominal adipose and liver were collected at the end of the experiment, fixed with 4% polyformalin in PBS solution for over 24 h and embedded in paraffin. Hepatic fat lesions and adipocyte diameters in abdominal adipose were observed by HE staining for morphologic analysis. Paraplast-embedded sections (5 µm) were subject to immunoperoxidase staining. The sections were incubated with anti-mouse CD11c(1:250, Affinity, USA) and Galectin-3 (1:250; Abcam, Cambridge, MA, USA) antibody. Amplification and staining were performed using an avidin-biotin-complex and 3,3-diaminobenzidine method, respectively.

2.10 Cell culture and treatment

RAW 264.7 macrophages were obtained from ATCC, USA and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) in a 5% CO₂ humidified cell incubator at 37 °C. Piperine was added to the cell culture to a final concentration of 0, 20, 40, and 80 µM for 12 h, respectively. LPS (1 µg/mL; Sigma) was added afterward for 24 h to induce M₁ macrophage polarization. After treatment, cell-free supernatants were collected to determine the IL-1β using ELISA kit assay, and cells were extracted for RNA as described above. The piperine solution was prepared by dissolving piperine in DMSO. The final concentration of DMSO in the cell culture should not exceed 0.05%.

2.11. Western blot analysis

The stimulated cells were homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors (Slarbio, Beijing, China) after being washed with phosphate buffered saline (PBS) buffer twice. The homogenate was subject to 10–12% SDS-PAGE electrophoresis before transferred to a PVDF membrane, which was then incubated with the primary antibody CD11c, Toll like receptor-4 (TLR-4) (1:1000;Affinity, USA) and IL-1β (1:2000; Abcam Cambridge, MA, USA) overnight at 4 °C, followed by incubation with the HRP-linked secondary antibody at 25 °C for 2 h. An eECL western blot kit (CWBIO, Beijing, China) was used to detect the proteins.

2.12 Statistical analyses

All values are reported as means ± SD. An unpaired t-test was used for comparisons between two groups. One-way ANOVA was used for comparisons among three or more groups. p < 0.05, p < 0.01, p < 0.001 or p < 0.0001 indicates significant difference.

3. Results

3.1 Effect of piperine on body weight, dietary intake and Lee's index

To explore the effect of piperine on the established obesity, the body weight, Lee's index, glycolipid metabolism, and insulin sensitivity were assessed in MSG-obese insulin resistant mice upon piperine treatments. The results show that MSG caused more mesenteric fat accumulation and body weight gain in MSG-obese insulin resistant mice than normal mice. In contrast, the piperine treatment relieved mesenteric fat accumulation and body weight gain (Fig. 1A-B). It was found that from the 4th week onwards, the body weight of the piperine-treated mice began to decrease and eventually reached 53.0 ± 2.88 g, significantly different from that of the MSG-obese mice (70.2 ± 2.54 g). Additionally, the body weight of metformin-treated mice declined to 58.6 ± 4.18 g. No significant differences of daily food intake and Lee's index were observed between the model and piperine-treated groups (Fig. 1C-D).

3.2 Effect of piperine on visceral organ indexes

The relative weights of the abdominal fat, pancreas, liver, and kidney were calculated after the mice were sacrificed. The results show that the MSG-obese mice had a higher visceral index of abdominal adipose and lower pancreas, liver, and kidney index. As expected, the piperine treatment completely reduced the visceral index of the abdominal adipose (Fig. 2A) and increased the visceral index of the pancreas in the MSG obese mice (Fig. 2B). The data suggest that piperine may protect the pancreas to a certain extent and restore the relative weight of the pancreas. Besides, we found that the piperine treatment did not change the relative weight of the kidney (Fig. 2C) and liver (Fig. 2D) ($P > 0.05$).

3.3 Effect of piperine on the changes of glycolipid metabolism parameters in MSG mice

Obesity is a main factor causing glycolipid metabolism disorders. The regulatory effect of piperine on glycolipid metabolism was examined in this section. As assumed, the obese mice had higher levels of serum TC, TG, insulin and FBG. In contrast, the piperine treatment dramatically reduced the FBG (27.0% reduction compared with the model mice), serum TC (37.3% reduction), and TG levels (33.6% reduction) (Fig. 3A-C). Additionally, the MSG mice showed obvious hyperinsulinemia. Piperine administration also had a certain relieving effect on the serum insulin level. However, it is not statistically different (Fig. 3D) ($P > 0.05$). Metformin also significantly lowered FBG (30.5% reduction), TC (33.9% reduction), and TG (22.1% reduction). The results suggest that the effect of piperine on glucose metabolism is similar to metformin, but piperine may have a better effect on lipid metabolism compared with metformin.

3.4 Effect of piperine on oral glucose tolerance test □ hyperglycemic clamp experiment and insulin tolerance test in MSG mice

The effects of piperine on insulin sensitivity was evaluated using the insulin tolerance test (ITT). Oral glucose tolerance test (OGTT) and hyperglycemic clamp experiment were used to assess the effects of

piperine on glucose tolerance. Administration of MSG led to significant insulin resistance and glucose intolerance in the ICR mice, which were markedly attenuated in the piperine-treated MSG mice.

Glucose tolerance results are summarized in Fig. 4A. The results show that the glucose level in the MSG mice significantly increased in the first 30 min after glucose load (9.91 ± 0.63 mM vs. 12.4 ± 1.46 mM). However, the glucose levels were 25% lower in the piperine treated mice compared with that of the control mice (9.28 ± 0.65 mM vs. 12.4 ± 1.46 mM). The results also showed that the integrated glucose level was greatly lowered in the piperine-treated mice compared with that of the control mice (Fig. 4B). The glucose level in the MSG mice could be completely recovered by piperine at 40 mg/kg body weight. Compared with metformin, the effect of piperine was similar.

The result of hyperglycemic clamp experiment showed that the GIR of MSG mice(Model group) was significantly lower than that of the normal group, indicating that there was significant glucose intolerance in MSG-obese mice. After 10 weeks of administration, compared with the model group, both piperine and metformin treatment increased the GIR of MSG mice by 45.1% and 57.6%, respectively, suggesting that piperine is beneficial to improve the sensitivity of islet β cells to glucose stimulation in obese mice, that is, it can improve the function of islet β cells(Fig. 4C).

The ITT results show that after 40 min of insulin injection, blood glucose in the piperine group decreased by 33.02%, which is significantly higher than that in the MSG group (15.26%), indicating that the piperine treatment improved systemic insulin sensitivity in the MSG-obese mice (Fig. 4D-E).

3.5 Effect of piperine on pathological changes in the abdominal adipose and liver

Heavy accumulation of fat in the liver was observed by histomorphological analysis, indicating there were severe pathological changes of nonalcoholic fatty live disease (NAFLD) in the MSG mice. The livers of the mice in the model group showed heavy hepatic steatosis, whereas, in the MSG mice, the steatosis was partially relieved by the piperine treatment (Fig. 5A). In some obese patients, insulin resistance occurs due to the accumulation of "dysfunctional" adipose tissues, which are characterized by "large" lipid-laden adipocytes. Our results show that the adipocyte size was greatly increased by MSG, while the hypertrophic adipocyte was ameliorated by piperine treatment (Fig. 5B-C). These data indicate that piperine plays a vital role in regulating lipid metabolism in the abdominal adipose and liver, both of which are the main target of insulin.

3.6 Effect of piperine on improves systemic inflammation

The routine blood test results showed that WBC, Lymphocyte, and Monocyte in the piperine-treated group were significantly lower than the mice in the model group (Table 2). Besides, we found that serum pro-inflammatory cytokines such as LPS, IL-1 β and Gal-3 were elevated in the MSG mice compared with the normal mice. At the end of the 10-week period, the serum level of LPS, IL-1 β and Gal-3 were significantly reduced in the piperine-treated mice compared with that in the control mice (Fig. 6A-C). Additionally,

although the serum anti-inflammatory cytokine IL-10 in the model mice was lower than that in the normal mice, the administration of piperine did not restore this indicator (Fig. 6D) ($P > 0.05$). These results demonstrated that the piperine treatment suppressed the obesity-enhanced inflammatory responses in obese mice.

Table 2
Routine blood test in animals

	Normal	Model	Metformin	Piperine
WBC ($9 \times 10^9 /L$)	5.23 ± 1.02	$8.2 \pm 1.09^{###}$	$5.73 \pm 1.76^{**}$	$4.78 \pm 0.98^{***}$
LYMPH($9 \times 10^9 /L$)	3.79 ± 1.26	$6.02 \pm 1.13^{##}$	$3.87 \pm 0.83^{**}$	$3.77 \pm 0.53^{***}$
NEUT($9 \times 10^9 /L$)	1.32 ± 0.25	1.26 ± 0.055	1.23 ± 0.13	1.18 ± 0.11
MONO($9 \times 10^9 /L$)	0.259 ± 0.101	$0.625 \pm 0.151^{####}$	$0.232 \pm 0.157^{****}$	$0.145 \pm 0.04^{****}$
LYMPH%	68.76 ± 6.75	75.84 ± 3.74	71.97 ± 6.45	71.58 ± 3.35
NEUT%	26.5 ± 6.56	$16.18 \pm 2.58^{##}$	$23.67 \pm 5.45^{*}$	$25.17 \pm 2.27^{**}$
MONO%	4.73 ± 1.81	$7.98 \pm 1.93^{#}$	$4.36 \pm 2.94^{*}$	$3.14 \pm 1.12^{**}$
HGB(g/L)	124.1 ± 16.35	112.8 ± 17.13	118.7 ± 12.8	111.7 ± 8.47
RBC($9 \times 10^{12} /L$)	8.89 ± 1.2	7.58 ± 1.43	8.14 ± 1.13	$7.24 \pm 0.44^{#}$
HCT%	47.46 ± 5.23	42.72 ± 7.04	46.3 ± 3.94	45.02 ± 3.35
MCV(fL)	52.91 ± 2.88	56.83 ± 5.68	57.47 ± 6.64	$60.1 \pm 4.31^{#}$
MCH(pg)	13.8 ± 0.5	14.42 ± 0.79	14.63 ± 0.91	$15.68 \pm 0.6^{###}$
MCHC(g/L)	261.1 ± 8	255.2 ± 21.84	256.2 ± 15.37	245.3 ± 9.16
PLT(fL)	946.1 ± 88.14	889.2 ± 159.5	793.3 ± 131.5	734.2 ± 150.4
MPV(fL)	7.67 ± 0.377	7.88 ± 0.511	7.78 ± 0.794	8.2 ± 1.14

Routine blood test in animals. Data are expressed as mean \pm SD, n = 6–8 per group, $^{*}p < 0.05$; $^{##}p < 0.01$; $^{###}p < 0.001$; $^{####}p < 0.0001$ vs. Normal group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ vs. Model group.

3.7 Effect of piperine on inflammatory mediator gene and protein expressions in the adipose tissue

In order to detect the inflammatory status of adipose tissue in each group of mice, we examined the expression of M₁-like macrophage marker CD11c and related inflammatory cytokines at the mRNA level.

qRT-PCR showed that the mRNA level of CD11c, IL-1 β , Gal-3 and TNF- α were significantly increased in the adipose tissue in the model mice. In contrast, these genes were markedly decreased in the piperine-treated group (Fig. 7A-D).

We simultaneously measured the protein expression of CD11c and Gal-3. Immunohistochemistry results showed that both CD11c and Gal-3 were over-expressed in the adipose tissue of the MSG group. Piperine treatment reduced the level of both key proteins, CD11c and Gal-3, in the adipose tissue (Fig. 8A-D). Together, these results indicated that piperine alleviated obesity enhanced M₁-like macrophage polarization and the secretion of pro-inflammatory cytokines in the abdominal adipose tissue, which is consistent with serum pro-inflammatory cytokine levels. In fact, M₁-like macrophage polarization in visceral adipose tissue is the source of systemic inflammation.

3.8 Effect of piperine on in vitro macrophage polarization

The inhibitory effect of piperine on M₁ macrophage polarization was evaluated using an inflammatory cell culture model. As expected, LPS treatment increased mRNA expressions of TNF- α , IL-1 β and M₁ marker CD11c, while piperine inhibits LPS-induced TNF- α , IL-1 β and CD11c expression in a concentration-dependent manner in RAW 264.7 cells (Fig. 9A-C). We also found the LPS-stimulated IL-1 β production was inhibited by piperine and also their combination (Fig. 9D). Furthermore, we examined the TLR-4, CD11c and IL-1 β with a Western blot analysis. The results showed that piperine (20, 40, and 80 μ M) inhibited the expression of TLR-4, IL-1 β , and CD11c in the RAW264.7 cells after LPS treatment (Fig. 10A-B).

4. Discussion

In diabetes patients, the severity of insulin resistance is closely associated with the degree of inflammation [2, 3, 25]. Insulin resistance and T2DM may be cured or alleviated by direct inhibition of inflammatory responses. In this study, we investigated the effect of piperine on obesity-induced inflammation and insulin resistance. The results show that obesity of the MSG mice was greatly reduced by the piperine treatment, and the inflammation in adipose tissue macrophages (ATM) was altered, hence protecting MSG mice from insulin resistance. Firstly, the piperine-treated mice had lower body weight, adipocyte sizes and index of abdominal adipose compared with the MSG mice. Secondly, piperine down-regulated the mRNA expression of CD11c, Gal-3, IL-1 β and TNF- α and the protein expression of CD11c and Gal-3 in the abdominal adipose tissue of MSG mice. Thirdly, the administration of piperine alleviated glycolipid metabolism disorder and insulin resistance induced by severe obesity. In addition, the anti-diabetic effect of piperine was associated with the decrease of immune cell infiltration in the adipose tissue.

Previous studies have shown that insulin resistance and type 2 diabetes (T2D) can be induced by abnormal immune cell activation [26]. In present study, obese mice develop systematic insulin resistance, which may be associated with the systematic insulin resistance in the MSG obese mice [27]. Correlations between body mass and cell numbers of the ATMs indicate that macrophages and chronic low-grade

inflammation might play a key role in obesity-induced insulin resistance [28]. ATMs not only undergo quantitative increases in adipose tissue, but also undergo qualitative changes in their activated state to promote metabolic inflammation during obesity [29]. CD11c⁺ ('M₁') was increased in the adipose tissue, which contributes to the elevated metabolic inflammation and causes insulin resistance through paracrine mechanisms [9, 10]. In healthy/lean adipose tissues, the initially activated macrophages (M₂-like) were altered, which only express CD11b and F4/80 on their surface and secrete anti-inflammatory cytokines such as IL-4 and IL-10. These anti-inflammatory cytokines play a key role in maintaining the sensitivity of adipocytes to insulin, thereby inhibiting the lipolysis process [30]. On the contrary, obesity triggers the accumulation of classically activated macrophages (M₁-like) induced by FFA or LPS. In these macrophages, many factors, such as CD11c, CD11b and F4/80, and pro-inflammatory cytokines such as IL-6, IL-1 β , TNF- α and Gal-3 are up-regulated, which impair the insulin signaling pathways [9, 14, 26, 31–33]. It is ascribed to their architectural organization in which metabolic cells are in close proximity to immune cells. For example, co-cultured macrophages and 3T3-L₁ adipocytes reduced the expression of insulin receptor substrate-1 and GLUT4 [34]. We found that the elevated expression of CD11c and TLR-4 induced by MSG were greatly alleviated by the piperine treatment through direct suppression of the LPS-stimulated M₁ polarization in RAW264.7 cells.

How could reduced CD11c⁺ ATMs by piperine lead to an improvement in insulin resistance? The main players in this interaction could be the cytokines that are produced by the inflamed immune cells. LPS is a strong stimulatory of the release of several cytokines that are key inducers of insulin resistance [35]. Studies indicate that long-term high-calorie diets changed the composition of the intestinal microbiota of the body and showed that the number of Gram-negative bacteria increased and the amount of LPS secreted by it also increased, so the plasma LPS levels in obese patients are significantly higher than normal people [36, 37]. LPS bind to complex of mCD14 and TLR-4 at the surface of the innate immune cells activate inflammatory pathway, and then triggers the secretion of pro-inflammatory cytokines consequently impact insulin action [38]. LPS-treated mice developed inflammation, as the expression of pro-inflammatory cytokines genes, IL-1 β , TNF- α , IL-6, and PAI-1 were increased in adipose, muscle, and liver. Importantly, these features occurred similarly in high-fat diet-fed mice [39]. Conversely, TLR-4 overexpression led to some extent of adipose insulin resistance [40–42]. LPS receptor deleted mice are hypersensitive to insulin, and the occurrence of obesity, insulin resistance, and T2DM is delayed in response to high-fat feeding [39]. In addition to classical pro-inflammatory cytokines, Gal-3 is also considered a key factor leading to insulin resistance. Gal-3 expression is up-regulated in CD11c⁺ macrophages isolated from the adipose tissue of insulin resistant obese mice, but not after these mice had been changed to a normal chow diet and become insulin sensitive [11]. The cytokines secreted by the inflamed immune cells may contribute to the improvement of glycolipid metabolism after the depletion of CD11c⁺ cells by the piperine treatment. Studies have shown that the sensitivity to insulin in mice returns to normal and the inflammatory markers decrease both in the adipose tissue and at the systemic level [43]. Hence, to understand whether the improvement of piperine on glycolipid metabolism disorder is associated with metabolic inflammation, we measured the blood total WBC and serum pro-inflammatory

cytokine levels and found that piperine significantly reduced blood total WBC, serum LPS (M_1 -like activated metabolite), Gal-3 and IL-1 β (M_1 -like pro-inflammatory cytokines). However, it had no significant effect on the serum level of IL-10 (M_2 -like anti-inflammatory cytokine). Visceral adipose tissue is the original source of metabolic inflammation in obesity. To further confirm our hypothesis, qRT-PCR and immunohistochemistry were performed to detect the expressions of M_1 -like polarizing biomarker CD11c and pro-inflammatory cytokines in visceral adipose tissue. The results show that piperine greatly decreased the mRNA levels of IL-1 β , Gal-3 and TNF- α in MSG obese mice. Moreover, the immunohistochemistry assay shows that the expressions of CD11c and Gal-3 in the adipose tissue of mice treated with piperine were lower than those of the MSG mice. These results indicate dietary piperine may be used to improve insulin sensitivity by regulating inflammatory states of macrophages in the adipose tissue.

It has been reported that piperine-supplemented diet could significantly reduce the body and visceral fat by 12% and 38%, respectively, compared with the mice fed with HFD [44]. Choi et al. showed that the supplement of piperine in a high-fat diet significantly reversed hepatic steatosis and insulin resistance in HFD obese mice [18]. In our study, we found that piperine is more effective compared with metformin on decreasing body weight and abdominal adipose index. Besides the decreased body and abdominal fat index, we observed the serum lipid level was also reduced by that piperine. In addition, our data revealed that piperine greatly improved insulin resistance in MSG mice. Glucose utilization was completely normalized by piperine during the 10-week period, indicating that piperine is beneficial to improve the oral glucose intolerance and the sensitivity of islet β cells to glucose stimulation in obese mice. Consistently, the FBG level was significantly lowered at the end of the experiment. The ITT data indicated that piperine could enhance insulin sensitivity in MSG obese mice.

Our results demonstrate piperine has a promising role in improving insulin sensitivity and alleviating adipose tissue inflammation in MSG obese mice. This study will pave the path for further studies of the immune-modulatory and anti-inflammatory functions of other alkaloids.

5. Conclusions

In summary, our study demonstrates that piperine caused moderate body weight loss, significantly reversed glycolipid metabolism disorders, and improved the established insulin resistance and glucose intolerance and in MSG-obese mice. The effect of piperine on obesity-associated diabetes is likely to stem from the strong inhibitory effect on systemic and adipose inflammation. Our observations prove that piperine has strong immune-modulatory and anti-inflammatory effects and therefore can ameliorate severe systemic insulin resistance in obesity.

Abbreviations

MSG : Monosodium glutamate; TC: Total cholesterol; TG: Total triglycerides; WBC : White blood cell; LPS : Lipopolysaccharide; Gal-3: Galectin-3; IL-1 β : Interleukin-1 β ; IL-4, IL-6, IL-10: Interleukin-4, Interleukin-6,

Interleukin-10; T2DM: Type 2 diabetes mellitus; OGTT : Oral glucose tolerance test; GIR: Glucose infusion rate; AUC: Under the curve; PG: Plasma glucose; ITT : Insulin tolerance test; Enzyme-linked immunosorbent assays: ELISA; FBS: Fetal bovine serum; PBS: Phosphate buffered saline; TLR-4: Toll like receptor-4; NAFLD –Nonalcoholic fatty live disease; ATM :Adipose tissue macrophages; TNF- α -Tumor necrosis factor- α ;FFA: Free fatty acid;GLUT-4: Glucose transporter-4; HFD: High fat diet; LYMPH: Lymphocyte; NEUT: Neutrophil; MONO: Monocyte; HGB: Hemoglobin; RBC: Red blood cell; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelets; MPV: Mean platelets volume.

Declarations

7.1 Ethical approval and consent to participate

All animal protocols conformed to the Guidelines for the Care and Use of Laboratory Animals prepared and approved by the Animal Care and Use Committee of the Affiliated Hospital, Qingdao University and approved by the Animal Experimental Ethical Committee of Affiliated Hospital, Qingdao University, Shandong Province, China. All efforts were made to minimize animal suffering.

7.2 Consent for publication

Not applicable.

7.3 Availability of data and materials

We wish not to share the raw data as the authors are aiming for future publications from the data. However, the data used to support the findings of this study are available from the corresponding author upon request.

7.4 Competing interests

The authors declare that they have no competing interests.

7.5 Funding

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7.6 Authors' contributions

CL is the first author; LJ and GJ designed this study; CL, YY and JZ performed the experiments; RH prepared the figures; LJ edited and revised the manuscript. All authors read and approved the final manuscript.

7.7 Acknowledgments

Not applicable.

7.8 Authors' Information

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Figures

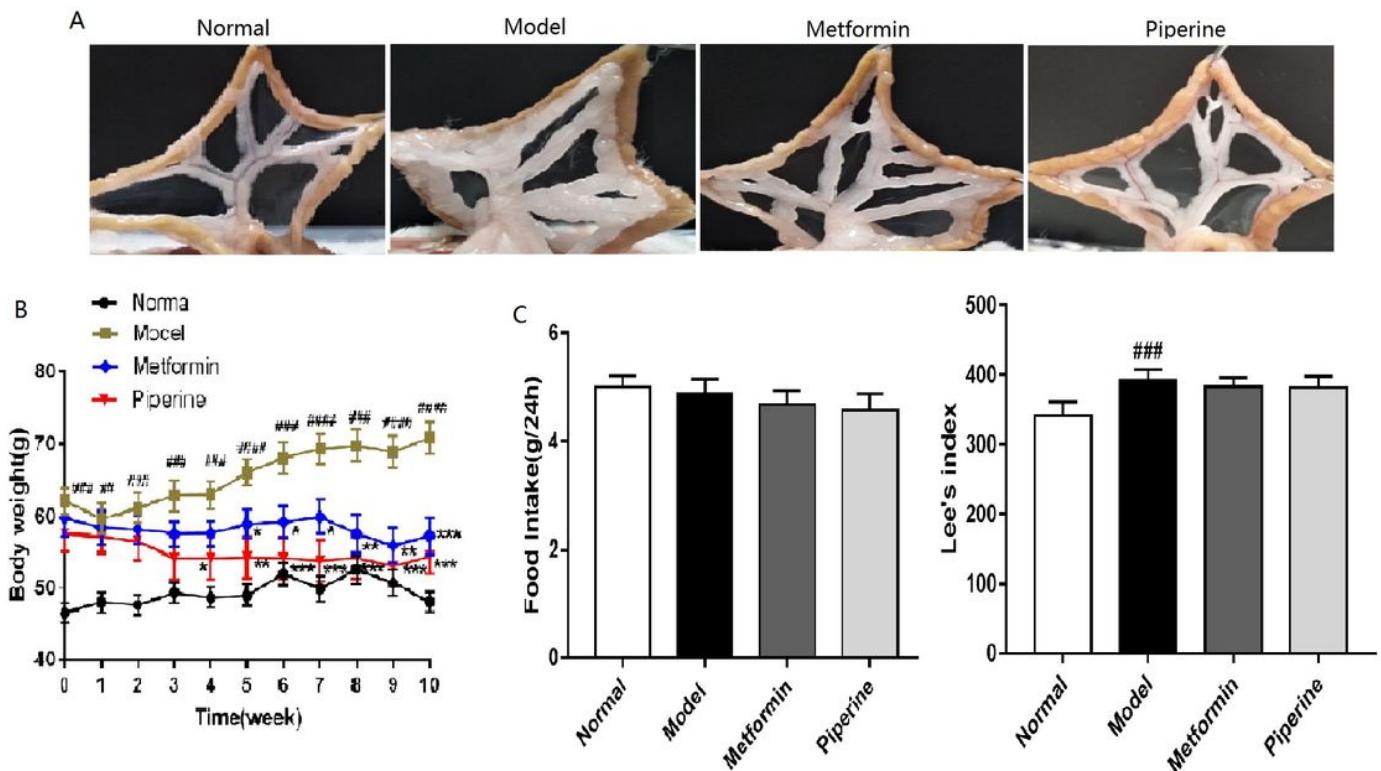


Figure 1

Piperine reduced body weight but not food intake and lee's index. (A) Picture of mesenteric fat accumulation in each group. (B) Body weight during 10 weeks treatment period. (C) Food intake. (D) Lee's index. Data are expressed as mean \pm SD, n=6-8. ##P<0.01, ###P<0.001, #####P<0.0001 vs. Normal group; *P<0.05, **P<0.01, ***P<0.001 vs. Model group.

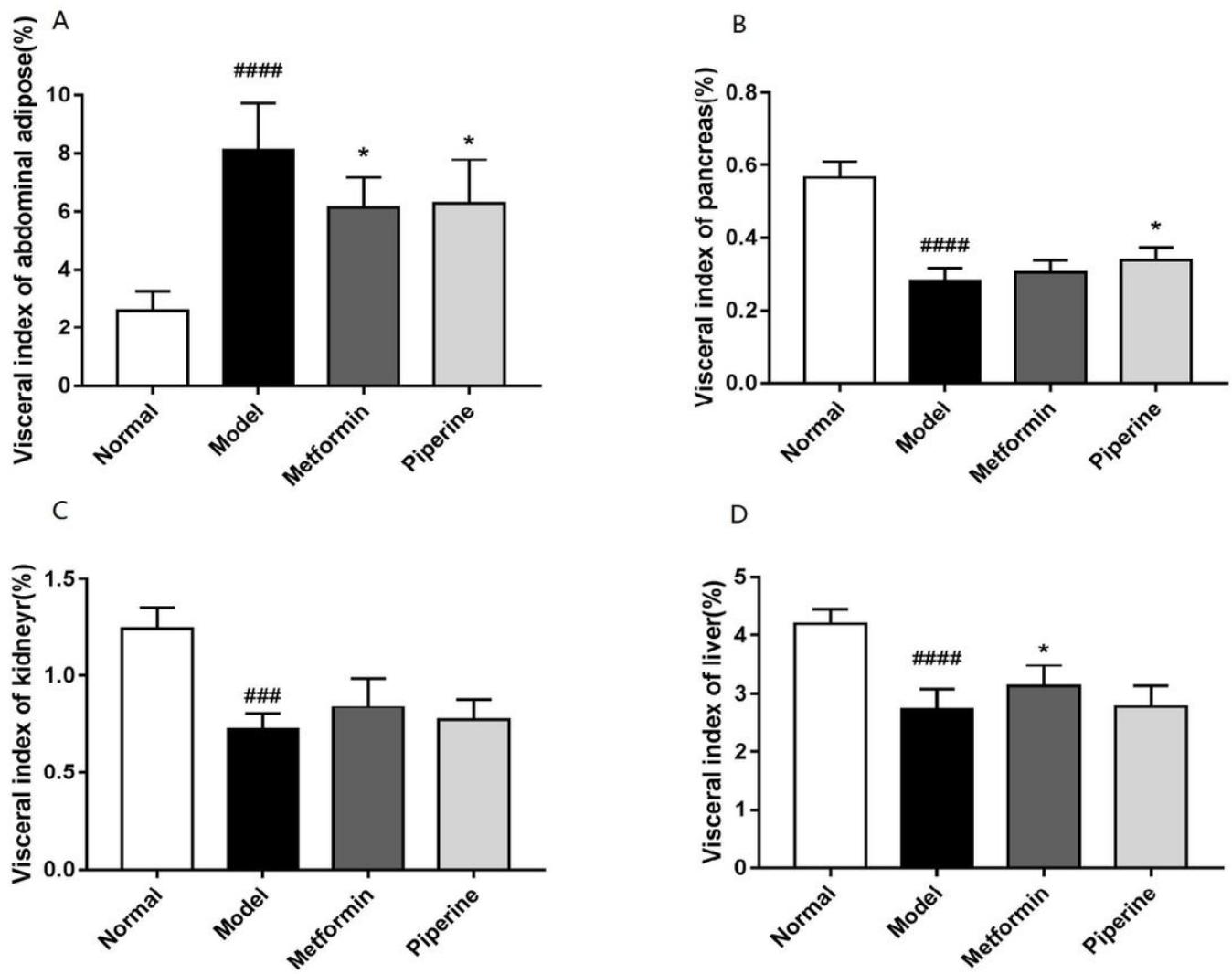


Figure 2

Effect of piperine on visceral organ indexes. (A) Index of abdominal adipose. (B) Visceral index of pancreas. (C) Visceral index of kidney. (D) Visceral index of liver. Data are expressed as mean \pm SD, n=6-8. ***P<0.001, ****P<0.0001 vs. Normal group; *P<0.05 vs. Model group.

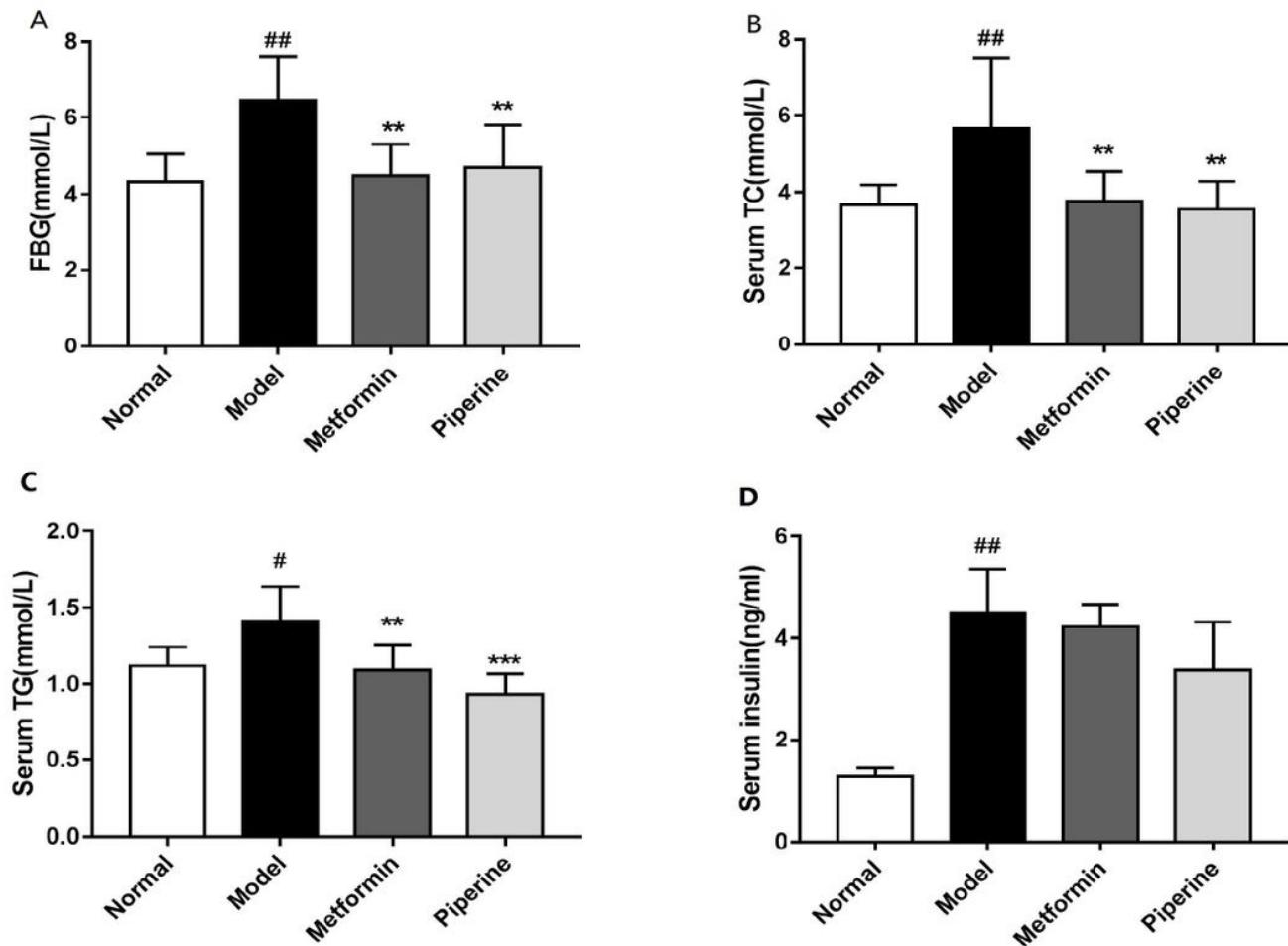


Figure 3

Piperine regulated glycolipid metabolism in MSG mice. (A) Fasting blood glucose. (B) Serum TC. (C) Serum TG. (D) serum insulin. Data are expressed as mean \pm SD, n=6-8. #P<0.05, ##P<0.01 vs. Normal group; **P<0.01, ***P<0.001 vs. Model group.

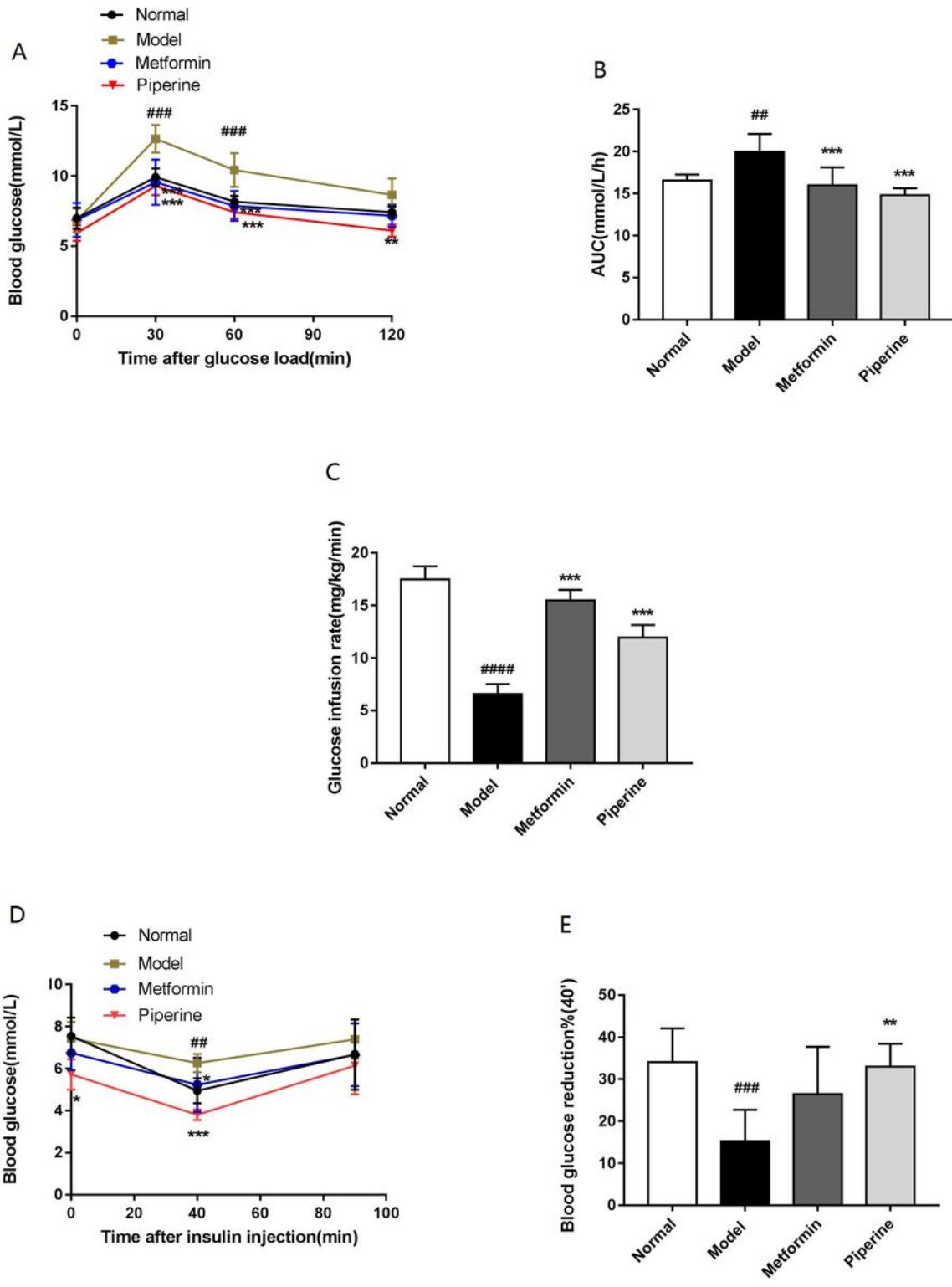


Figure 4

Piperine improved glucose tolerant and insulin sensitivity detected by OGTT and ITT respectively. (A) Plasma glucose levels were measured during a glucose tolerance test. (B) Area under the curve (AUC). (C) Glucose infusion rate in hyperglycemic clamp experiment. (D) Plasma glucose levels were measured during a insulin tolerance test. (E) Blood glucose drop percentage after 40 minutes of insulin injection.

Data are expressed as mean \pm SD, n=6-8. ##P<0.01, ###P<0.01 vs. Normal group; *P<0.05, **P<0.01, ***P<0.001 vs. Model group.

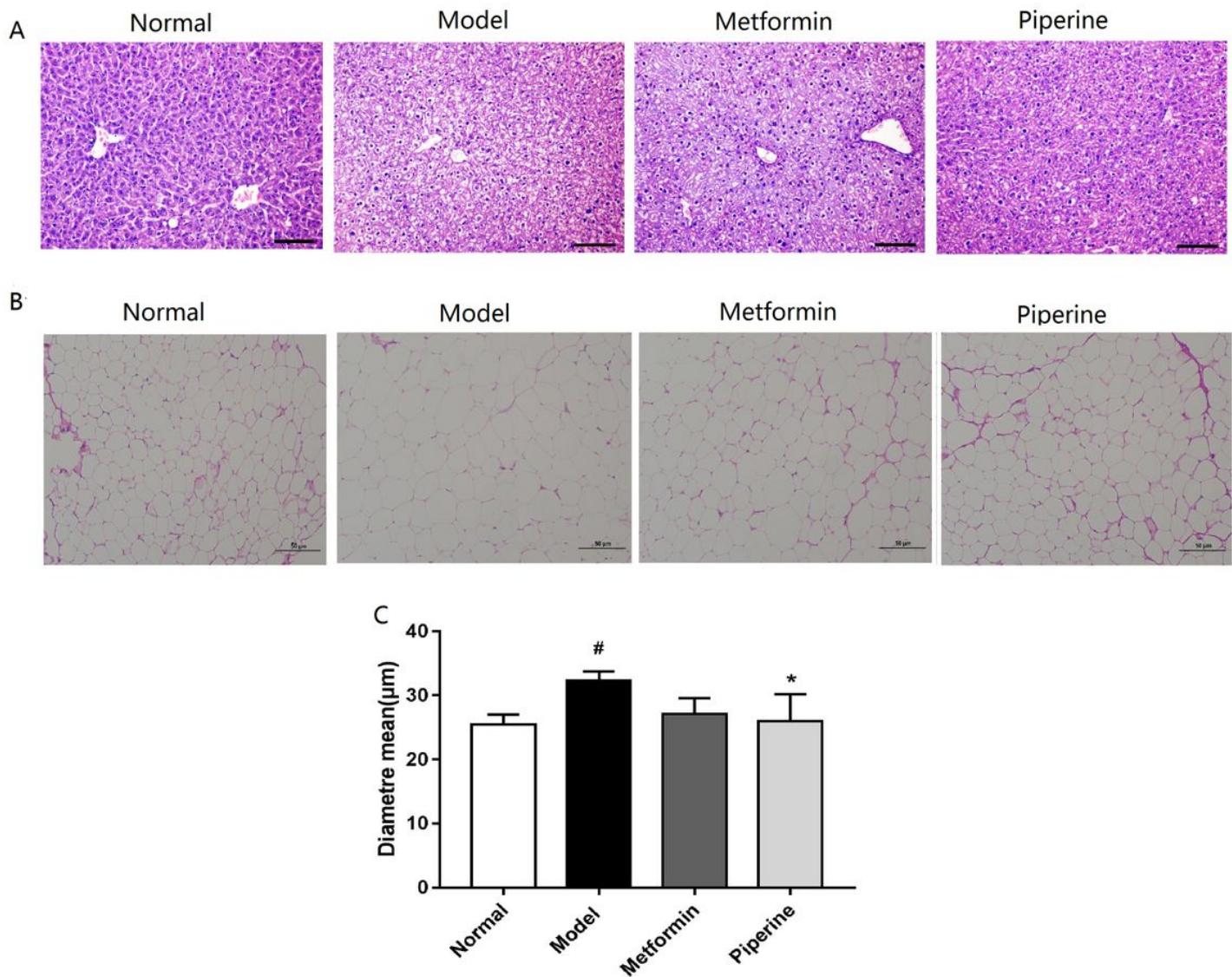


Figure 5

Protective effects of piperine on liver and adipose tissues morphology. (A) Liver sections were stained with H&E (200 \times). (B) Representative H&E staining of abdominal adipose tissue sections (100 \times). (C) Adipocyte diameters (mean) as measured with H&E staining. Data are expressed as mean \pm SD, n=3. #P<0.05vs. Normal group; *P<0.05 vs. Model group.

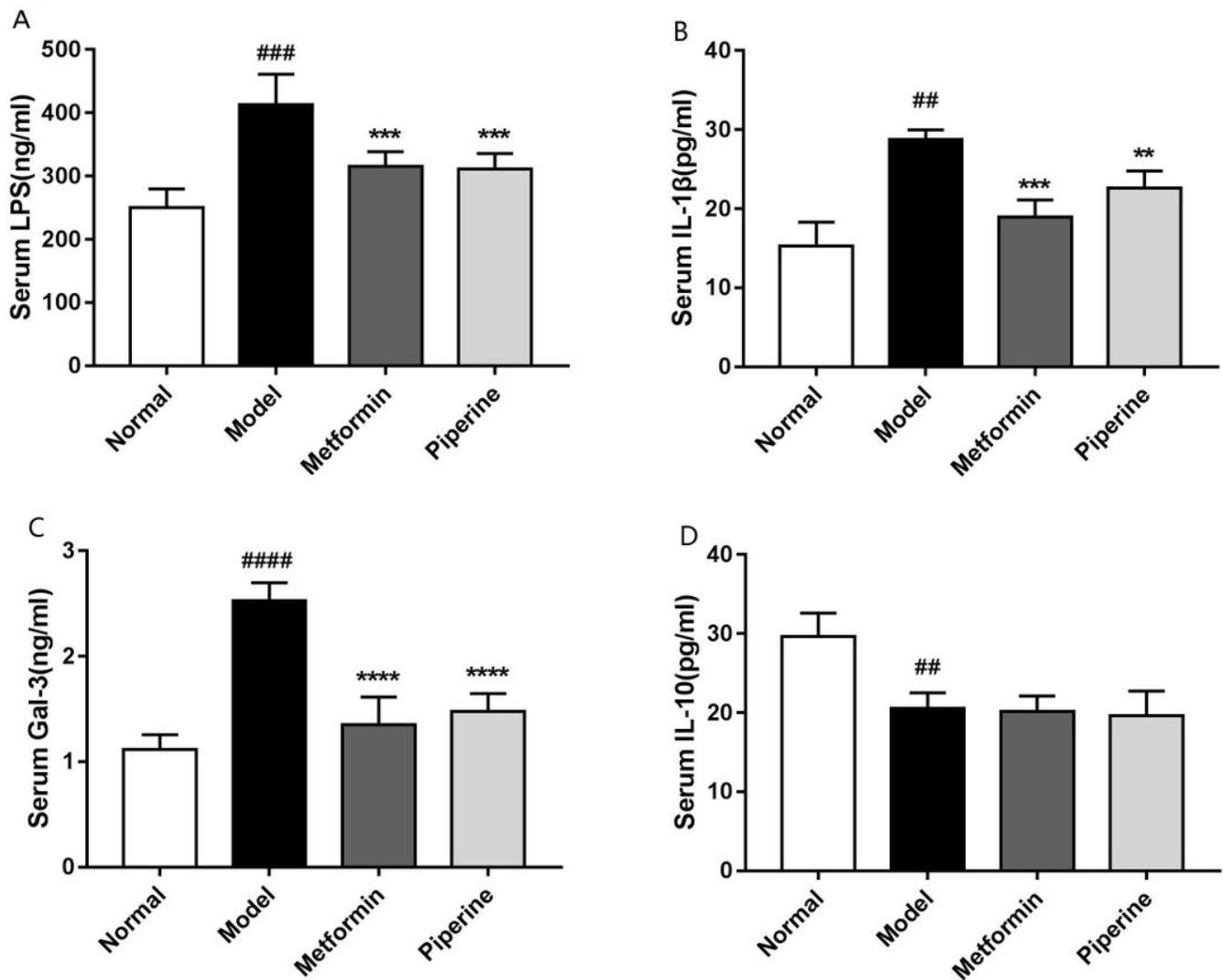


Figure 6

Piperine protected MSG obese mice against inflammatory injury in whole body. (A) Serum LPS. (B) Serum IL-1 β . (C) Serum Gal-3. (D) Serum IL-10. Data are expressed as mean \pm SD, n=6-8. ##P<0.01, ###P<0.001 vs. Normal group; **P<0.01, ***P<0.001 vs. Model group.

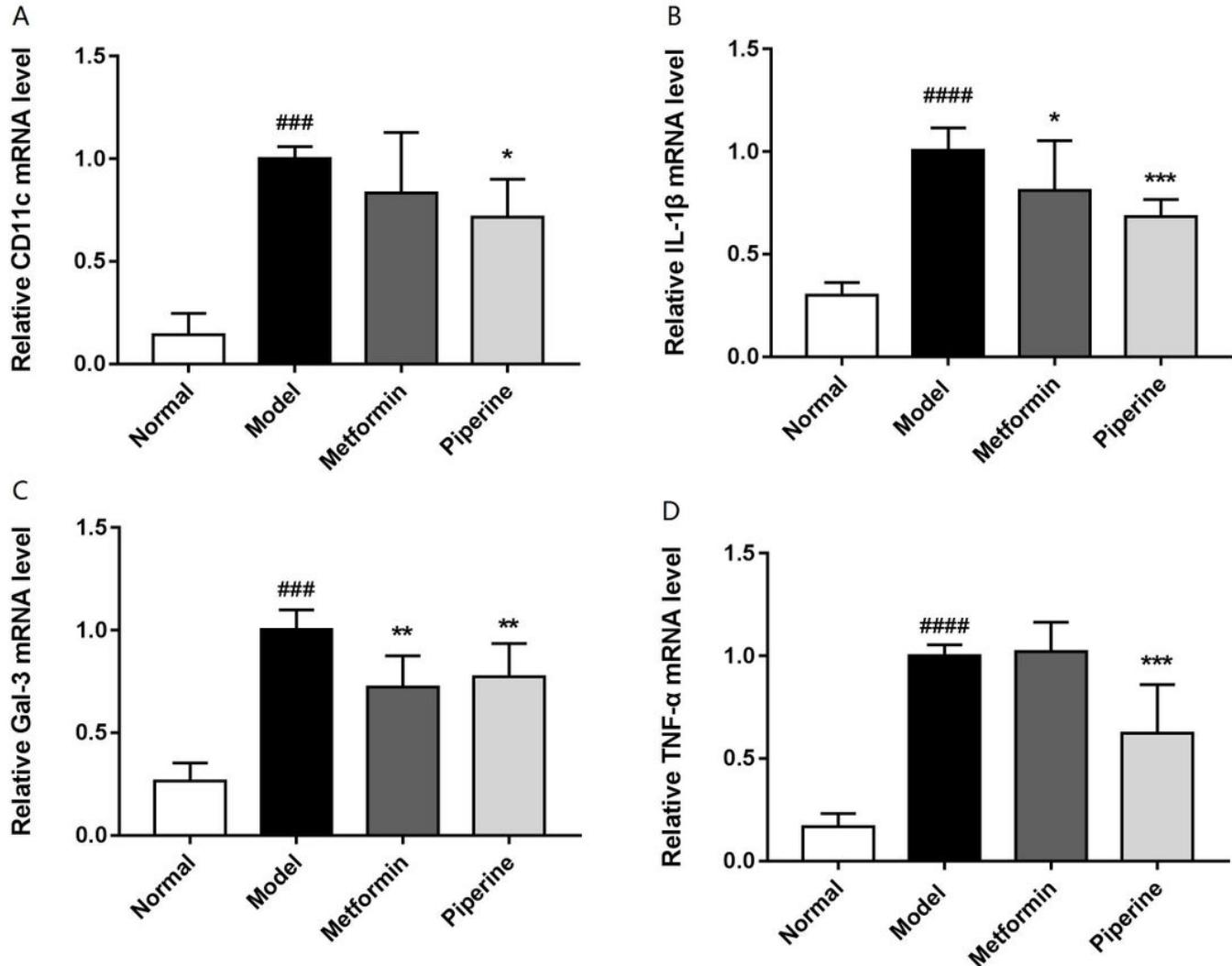


Figure 7

Piperine inhibited the inflammatory state of adipose tissue in MSG-obese mice. (A) The mRNA expression of CD11c gene. (B) The mRNA expression of IL-1 β gene. (C) The mRNA expression of Gal-3 gene. (D) The mRNA expression of TNF- α gene. Data are expressed as mean \pm SD, n=3. ##P<0.01, ###P<0.001 vs. Normal group; *P<0.05, **P<0.01, ***P<0.001 vs. Model group.

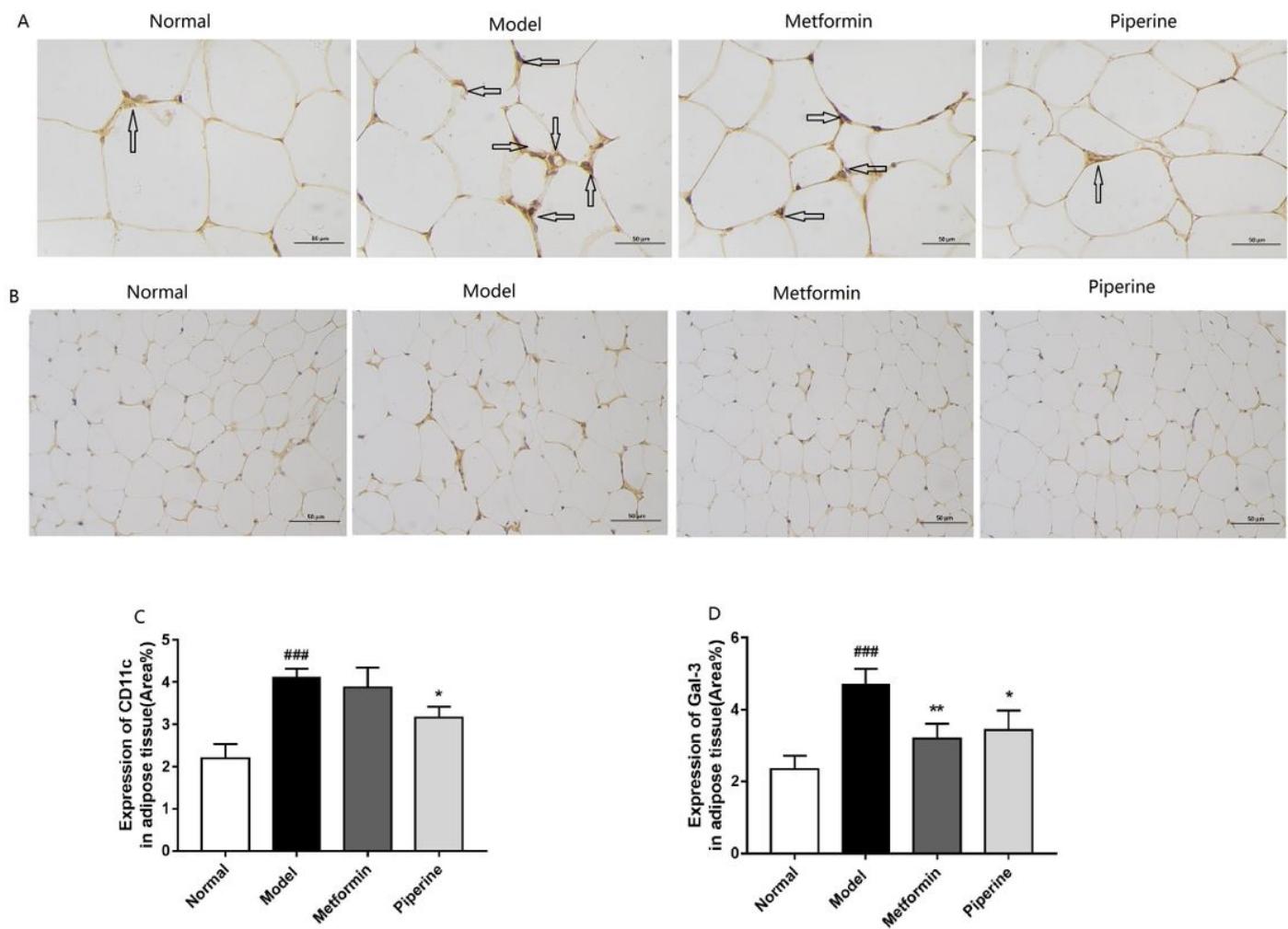


Figure 8

Immunohistochemical analyses of abdominal adipose. (A-D) The expression of CD11c(400 \times) and Gal-3(200 \times) in abdominal adipose. Data are expressed as mean \pm SD, n=3. ***P<0.001 vs. Normal group; *P<0.05, **P<0.01 vs. Model group.

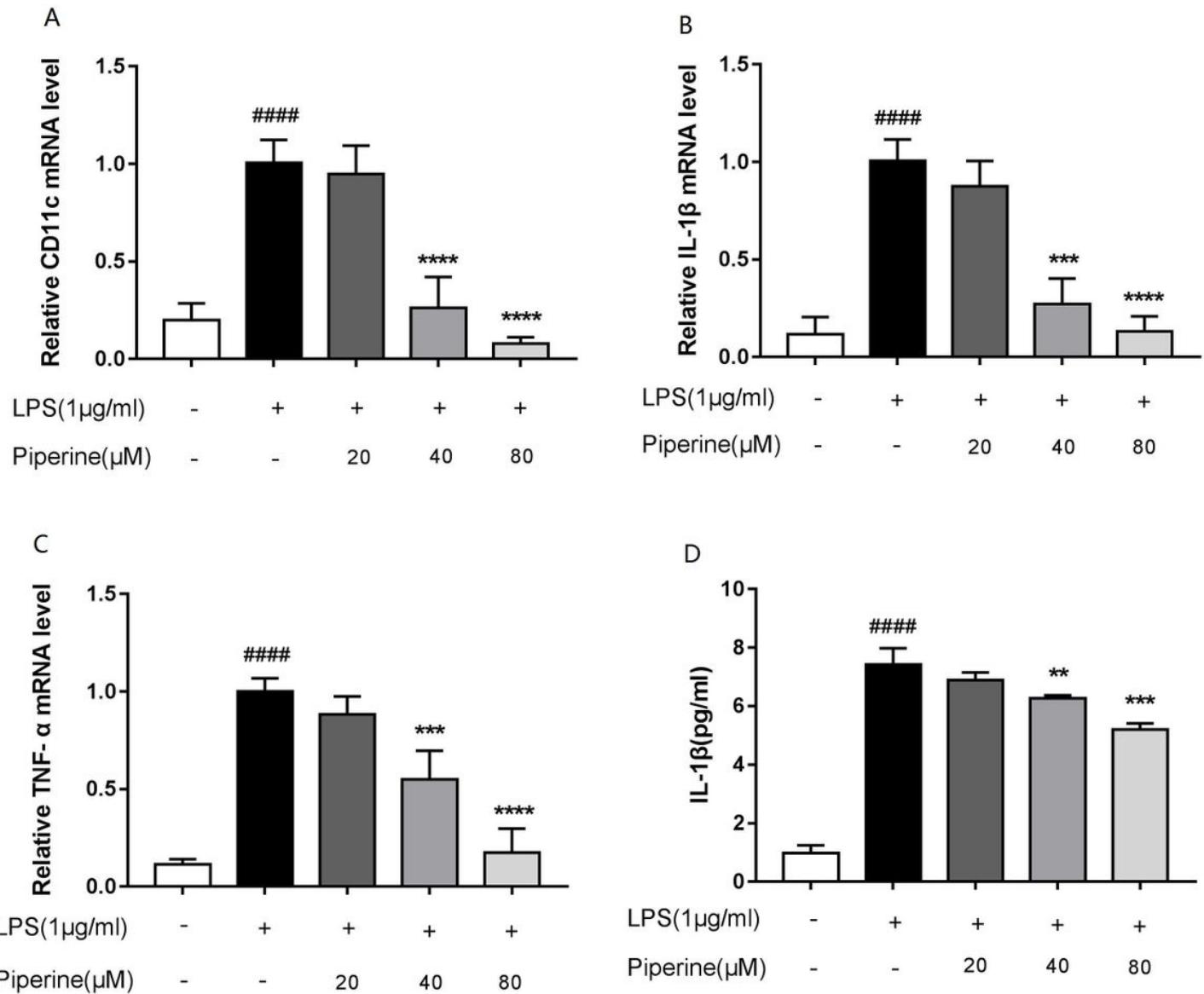


Figure 9

Effects of piperine on LPS-induced pro-inflammatory mediator gene expressions and IL-1 β production in RAW264.7 cells. RAW264.7 cells were pretreated with piperine at 20-80 μ M for 12 h and then stimulated by LPS (1 μ g/ml) for 24 h. (A-C) The mRNA expression of IL-1 β , TNF- α , CD11c were analyzed by qPCR. (D) IL-1 β level was measured by ELISA. Data are expressed as mean \pm SD, n=3. #****P<0.0001 vs. Control group; **P<0.01, ***P<0.001, ****P<0.0001 vs. LPS group.

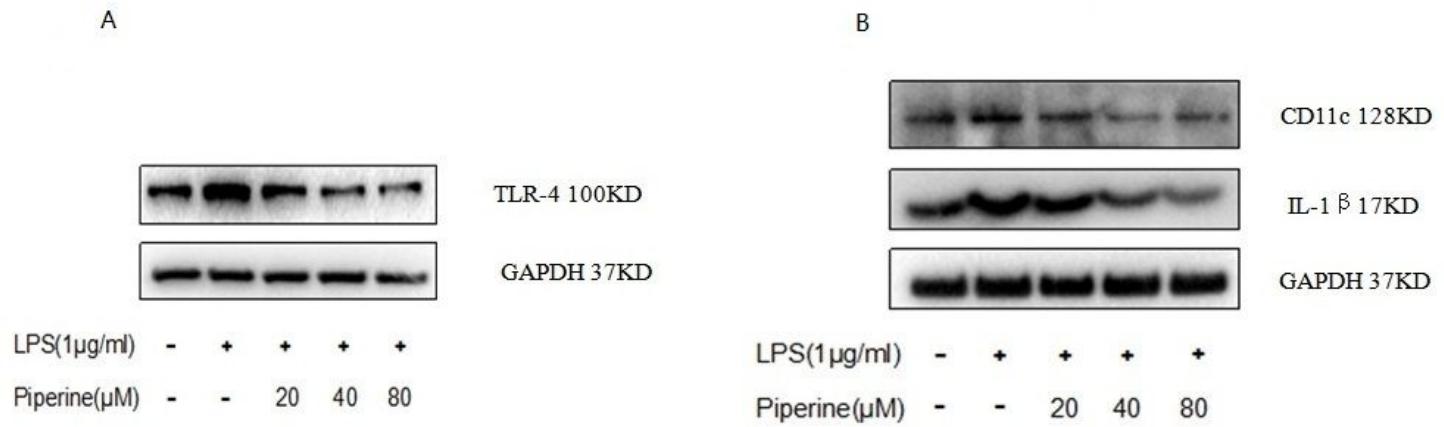


Figure 10

Piperine inhibit LPS-induced M1 polarization in RAW264.7 cells. (A-B) The protein level of TLR-4, CD11c and IL-1 β expression tested by Western Blotting.

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