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 M 1 -like polarization, secreting variable amounts of pro-inflammatory cytokines and causing insulin resistance. Piperine has been proven to have excellent anti-inflammatory activity and has therapeutic effects on a variety of inflammatory diseases. Therefore, we investigated the effect of piperine on adipose tissue inflammation and insulin resistance in obese mice.

Methods: In this study, the monosodium glutamate (MSG) obese mice model was used. 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. Meanwhile, the 6-month-old normal mice without MSG treatment were selected as normal controls.

Results: When the obesity model was successfully established, obesity degree, insulin resistance, fasting blood glucose(FBG) and serum lipid profiles were significantly increased. Our results showed that the 10-week administration of piperine (40 mg/kg/d) not only significantly decreased the elevated FBG, 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-1β (IL-1β). Furthermore, piperine clearly down-regulated the mRNA levels of pro-inflammatory cytokines and the protein levels of M 1 -like polarization marker 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 M 1 phenotype.

Conclusions: In summary, these findings demonstrated that piperine could significantly inhibit body weight gain, reduce fat accumulation, 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.

1. Background

Obesity and type 2 diabetes mellitus (T2DM) have been a great threat worldwide in recent years. According to the Global Diabetes Atlas (9th edition, International Diabetes Federation), the global incidence of diabetes was 9.3%, and the total number of patients was 463 million in 2019. By 2045, these two figures will increase to 10.9% and 700 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 M_1-polarized macrophages, and the ratio of M_1 to M_2 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 M_1 to M_2-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 M_1-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 visceral 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 that 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. On the one hand, the obese mice show hypothalamic lesions, and neuro-endocrine changes have been observed in the insulin and leptin signaling [15]. On the other hand, MSG mice is characterized by sloth and more closely resembles human central obesity and type 2 diabetes [16, 17]. The mice gradually develop obvious centripetal obesity, oral glucose intolerance, metabolic inflammation and insulin resistance from 2 months, and high fasting blood glucose 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 [18, 19].

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 [20-22]. 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 [23-25].

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.

Therefore, the aim of this study was to explore the changes of (i) body weight, fat accumulation, daily food intake and visceral index; (ii) plasma glycolipid levels; (iii) glucose tolerance and insulin sensitivity; (iv) histology of liver and abdominal adipose; (v) inflammatory cytokines in serum and abdominal adipose tissue. (vi) M_1 macrophage polarization in LPS-stimulated RAW 264.7 cells. Based on this, the investigation will help us understand the role of piperine and find implications in the treatment of obesity-induced metabolic dysfunction and prevention of related chronic low-grade inflammation.

2. Methods

2.1. Animal model establishment and experimental design

Pregnant ICR mice were purchased from Vital River Laboratory Animal Technology (Beijing, China). On the day of birth, the newborn mice were randomly divided into two groups. On postnatal Day 2, MSG-treated pups were injected subcutaneously (SC) with MSG (4g/kg/d, Sigma-Aldrich, USA) for 7 consecutive day. Normal pups were SC injected with equivalent volumes of 0.9% physiological saline solution [17, 26]. Weaning after 21 days, MSG and normal mice were separated by gender. Biochemical and physiological determinations were performed in the two groups at 1, 2, 4 and 6 months of age. The mice were allowed free access to water and a chow diet. All mice were kept in the Laboratory Animal Center of Qingdao University at an ambient temperature of 25 ± 2℃, 12 h light /dark cycles and humidity of 40–60%. After 24 weeks, 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: (i) the Model group which was administered an equal volume of normal saline. (ii) the Piperine group and Metformin group were treated with 40 mg piperine/kg/day (Sigma-Aldrich, USA) or 150 mg metformin/kg/day(Sigma-Aldrich, USA) by gavage for 10 weeks, respectively. There were 8 mice per group. The food intake and body weights of the mice were recorded weekly during the 10-week period of the treatment. 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, Sigma-Aldrich, USA). 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 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 with a one Touch Ultra glucose meter(ACCU-CHEK Performa Nano, Roche Diabetes Care GmbH). The area under the curve (AUC) was calculated according to the equation:
AUC=1/4(PG0min+PG30min)+1/4(PG30min+PG60min)+1/2(PG60min+PG120min). Where, PG0min, PG30min, PG60min, and PG120min 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 4h 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(250mg/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, novolin, Novo Nordisk, Danmark) 4h 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 one Touch Ultra Meter.

2.7 Enzyme-linked immunosorbent assays

Serum insulin was determined by using a mouse ultrasensitive insulin ELISA kit (American Laboratry Products Company, USA). LPS produced by the Gram-negative bacteria in the intestine, M_1-like pro-inflammatory cytokines (IL-1β and Gal-3) and M_2-like anti-inflammatory cytokine (IL-10) in the serum samples were measured by ELISA kits (Mlbio, Shanghai, China).

2.8 Semiquantitative reverse transcriptase polymerasechain reaction

Total RNA was extracted from the adipose tissues or cultured cells homogenized in Trizol (CWBIO, 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-ΔΔCT method. The primer sequences are presented in Table 1.

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% CO_2 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 (1µg/mL, Sigma-Aldrich, USA) was added afterward for 24 h to induce M_1 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

The software GraphPad Prism 7.00 was used for data analysis. 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 Effects of piperine on body weight, mesenteric fat accumulation, dietary intake and Lee's index

To explore the effect of piperine on the established obesity, the body weight, mesenteric fat accumulation, 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 (Model: 70.2 ± 2.54) than normal mice (Normal 48.09 ±1.43, $P<0.0001$). 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 ± 2.88g, significantly different from that of the MSG-obese mice (70.2 ± 2.54g, $P<0.001$). Additionally, the body weight of metformin-treated

mice declined to 58.6 ± 4.18 g. The effect of a 40 mg/kg B.W dose of piperine was better than metformin. No significant differences of daily food intake and Lee's index were observed between the model and piperine-treated groups, metformin also has no significant effect on these two indicators (Fig. 1C-D).

3.2 Effects of piperine on visceral organ indexes

The relative weights of the abdominal fat, pancreas, liver, and kidney were calculated after the mice were sacrificed. Compared to the normal group (visceral index of abdominal adipose: 2.58 ± 0.24%; Pancreas index: 0.566 ± 0.016%; Kidney index:1.242 ± 0.041%; Liver index: 4.198 ± 0.095%), the results show that the MSG-obese mice had a higher visceral index of abdominal adipose(8.11 ± 0.56%) and lower pancreas(0.282 ± 0.0126%), kidney (0.724 ± 0.028%), liver index (2.709 ± 0.130%), the differences between groups were significant ($P < 0.001$). As expected, the piperine treatment completely reduced the visceral index of the abdominal adipose(6.273 ± 0.606%, $P<0.05$) (Fig. 2A) and increased the visceral index of the pancreas(0.3393 ± 0.014%, $P<0.05$) 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 kidney (Fig. 2C) and liver (Fig. 2D) ($P>0.05$). We also found that metformin reduced index of the abdominal adipose (6.149 ± 0.358%, $P<0.05$) in MSG mice, the effect was quantitatively similar to piperine. Furthermore, metformin significantly increased liver index(3.13 ± 0.12%, $P<0.05$), but there was no changes on pancreas and kidney indices ($P>0.05$).

3.3 Effects of piperine on the changes of glycolipid metabolism parameters

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 FBG (Model: 6.45 ± 0.409; Normal: 4.32 ± 0.276, $P<0.01$), serum TC (Model: 5.663 ± 0.657; Normal:3.668 ± 0.197, $P<0.01$) and TG (Model: 1.407 ± 0.082; Normal: 1.117 ± 0.047, $P<0.05$). In contrast, the piperine treatment dramatically reduced the FBG (4.717 ± 0.441, 27.0% reduction compared with the model mice, $P<0.01$), serum TC (3.554 ± 0.299, 37.3% reduction, $P<0.01$) and TG levels (0.935 ± 0.0534, 33.6% reduction, $P<0.001$) (Fig. 3A-C). Additionally, the MSG mice showed obvious hyperinsulinemia (Model:4.498 ± 0.865; Normal: 1.276 ± 0.172, $P<0.01$). Piperine administration also had a certain relieving effect on the serum insulin level (3.373 ± 0.934). However, it is not statistically different (Fig. 3D) ($P>0.05$). Metformin also significantly lowered FBG (4.488 ± 0.292, 30.5% reduction, $P<0.01$), TC (3.747 ± 0.280, 33.9% reduction, $P<0.01$), and TG (1.091 ± 0.063, 22.1% reduction, $P<0.01$). 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 Effects of piperine on oral glucose tolerance test hyperglycemic clamp experiment and insulin tolerance test

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 and metformin 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 B.W compared with metformin, the effect of piperine was similar.

The result of hyperglycemic clamp experiment showed that the GIR of MSG mice was significantly lower than that of the normal group (Model: 6.564 ± 0.391; Normal: 17.49 ± 0.505, $P<0.0001$), 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% (11.95 ± 0.486, $P<0.0001$) and 57.6% (15.49 ± 0.408, $P<0.0001$), 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 Effects 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. These effects of 40 mg/kg B.W of piperine were better than those observed with the metformin group (Fig. 5B-C). These data indicate that piperine plays a vital role on regulating lipid metabolism in the abdominal adipose and liver, both are the main targets of insulin.

3.6 Effect of piperine on improving 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 (Model: 413.2 ± 19.59; Normal: 250.2 ± 12.16, P<0.001) , IL-1β (Model: 28.78 ± 0.495; Normal: 15.24 ± 1.259, P<0.01) and Gal-3 (Model: 2.527 ± 0.070; Normal: 1.115 ± 0.058, P<0.0001) were elevated in the MSG mice compared with the normal mice. At the end of the 10-week period, the serum level of LPS (311.2 ± 11.01, P<0.001), IL-1β (22.62 ± 0.877, P<0.01) and Gal-3 (1.479 ± 0.068, P<0.0001) 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 (20.6 ± 0.782) was lower than that in the normal mice (29.61 ± 1.345, P<0.01) , the administration of piperine did not restore this indicator (19.6 ± 1.286, P>0.05) (Fig. 6D). These effects of piperine were similar to those observed with the metformin group, so above results demonstrated that the piperine treatment suppressed the obesity-enhanced inflammatory responses in obese mice.

3.7 Effects 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 M_1-like macrophage marker CD11c and related inflammatory cytokines at the mRNA level. qRT-PCR showed that the mRNA level of CD11c, IL-1β, Gal-3 and TNF-a were significantly increased in the adipose tissue in MSG obese mice. In contrast, these genes were markedly decreased in the piperine-treated group (Fig. 7A-D). The results also demonstrated that significant reductions in IL-1β and Gal-3 were observed in the metformin-treated group compared to the model group.

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 M_1-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, M_1-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 M_1 macrophage polarization was evaluated using an inflammatory cell culture model. As expected, LPS treatment increased mRNA expressions of TNF-α, IL-1β and M_1

marker CD11c, while piperine inhibits LPS-induced TNF-α, IL-1β and CD11c expression in a concentration-dependent manner in RAW 264.7 cells (Fig. 9A-C). We also found the LPS-stimulated IL-1β production was inhibited by piperine and also their combination (Fig. 9D). Furthermore, we examined the TLR-4, CD11c and IL-1β with a Western blot analysis. The results showed that piperine (20, 40, and 80μM) inhibited the expression of TLR-4, IL-1β, 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 metabolic inflammation [2, 3, 27]. 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 degree of the MSG mice was greatly reduced by 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, fat accumulation, adipocyte sizes and index of abdominal adipose compared with the MSG mice. Secondly, piperine down-regulated the mRNA expression of CD11c, Gal-3, IL-1β 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 macrophages infiltration and polarization in the adipose tissue.

Previous studies have shown that insulin resistance and type 2 diabetes (T2D) can be induced by abnormal immune cell activation [28]. In present study, obese mice develop systematic insulin resistance, which may be associated with the systematic insulin resistance in the MSG obese mice [29]. 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 [30]. ATMs not only undergo quantitative increases in adipose tissue, but also undergo qualitative changes in their activated state to promote metabolic inflammation during obesity [31]. $CD11c^+$ ('M_1') 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 (M_2-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 [32]. On the contrary, obesity triggers the accumulation of classically activated macrophages (M_1-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-1β, TNF-α and Gal-3 are up-regulated, which impair the insulin signaling pathways [9, 14, 28, 33-35]. 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-L_1 adipocytes reduced the expression of insulin receptor substrate-1 and GLUT4 [36]. 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 M_1 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 produced by the inflamed immune cells in visceral adipose tissue. LPS is a strong stimulation to trigger the several cytokines associated with systemic insulin resistance [37]. 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 [38, 39]. 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 [40]. LPS-treated mice developed inflammation, as the expression of pro-inflammatory cytokines genes, IL-1β, 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 [41]. Conversely, TLR-4 overexpression led to some extent of adipose insulin resistance [42-44]. 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 [41]. 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 [45]. 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-1β (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-1β, 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 [46]. 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 [20]. 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. For example, single dose of piperine was used; therefore, we could not explore the effects of different doses of piperine on T2DM. Furthermore, the study firstly focuses on studying the therapeutic effect of piperine on obese-related T2DM. As to further research, we are investigating the underlying mechanism of piperine-induced alleviation on glycolipid disorder, metabolic inflammation, M_1-like polarity of macrophages and the malignant induction between adipocytes and macrophages on obese and T2DM mice models. In addition, in vitro experiments, we only performed the inhibitory effect of piperine on LPS induced macrophage polarization, but did not explore the related signaling pathway of this inhibition. We will study the effects of piperine on the direct or indirect induction between macrophage and adipose cells. In the near future, we will make up for these limitations and explore the molecular biological mechanism by which piperine regulates glycolipid disorder and metabolic inflammation.

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. Further clinical trials need to be conducted to confirm these effects.

Abbreviations

MSG: Monosodium glutamate; B.W: body weight; TC: Total cholesterol; TG: Total triglycerides; WBC : White blood cell; LPS : Lipopolysaccharide; Gal-3: Galectin-3; IL-1β: Interleukin-1β; 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; WAT: White adipose tissue; TNF-αTumor necrosis factor-α; FFA: Free fatty acidGLUT-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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Tables

Table 1: Primer sequences for qRT-PCR.

Gene	Primer sequences
CD11c	Forward:5′-ACACAGTGTGCTCCAGTATGA-3′
	Reverse:5′-GCCCAGGGATATGTTCACAGC-3′
IL-1β	Forward:5′-AAATACCTGTGGGCCTTGGGC-3′
	Reverse:5′-CTGGGATCCACACTCTCCAG-3′
TNF-α	Forward:5′-CCAGACCCTCACACTCAGATC-3′
	Reverse:5′-CACTTGGTGGTTTTGCTACGAC-3′
Galectin-3	Forward:5′-TCCTGGAGGCTATCCTGCTG-3′
	Reverse:5′-TGTTTGCGTTGGGTTTCACTG-3′
β-actin	Forward:5′-AAGAGAGGCATCCTGACCCT-3′
	Reverse:5′-TACATGGCTGGGGTGTTGAA-3′

Table 2 Routine blood test in animals

	Normal	Model	Metformin	Piperine
WBC (9×10^9 /L)	5.23±1.02	8.2±1.09$^{###}$	5.73±1.76**	4.78±0.98***
LYMPH(9×10^9 /L)	3.79±1.26	6.02±1.13$^{##}$	3.87±0.83**	3.77±0.53***
NEUT(9×10^9 /L)	1.32±0.25	1.26±0.055	1.23±0.13	1.18±0.11
MONO(9×10^9 /L)	0.259±0.101	0.625±0.151$^{####}$	0.232±0.157****	0.145±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±2.58$^{##}$	23.67±5.45*	25.17±2.27**
MONO%	4.73±1.81	7.98±1.93$^{#}$	4.36±2.94*	3.14±1.12**
HGB□g/L)	124.1±16.35	112.8±17.13	118.7±12.8	111.7±8.47
RBC(9×10^{12} /L)	8.89±1.2	7.58±1.43	8.14±1.13	7.24±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±4.31$^{#}$
MCH(pg)	13.8±0.5	14.42±0.79	14.63±0.91	15.68±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 ± 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.

Figures

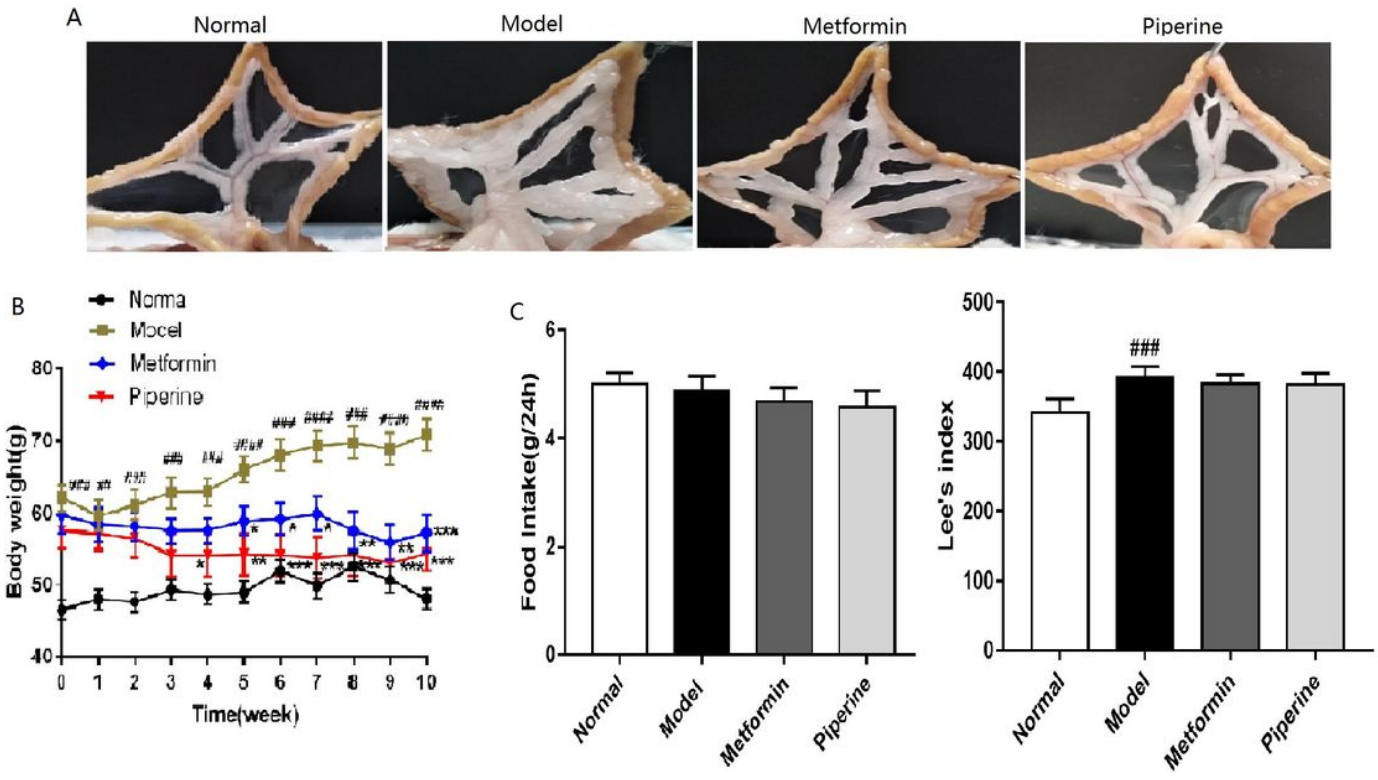

Figure 1

Piperine reduced body weight but not food intake and lee's index. (A) Piperine reduced mesenteric fat accumulation. (B) Piperine inhibited the MSG-induced body weight gain. (C-D) Piperine did not change the daily food intake and Lee's index. Data are expressed as mean ± 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.

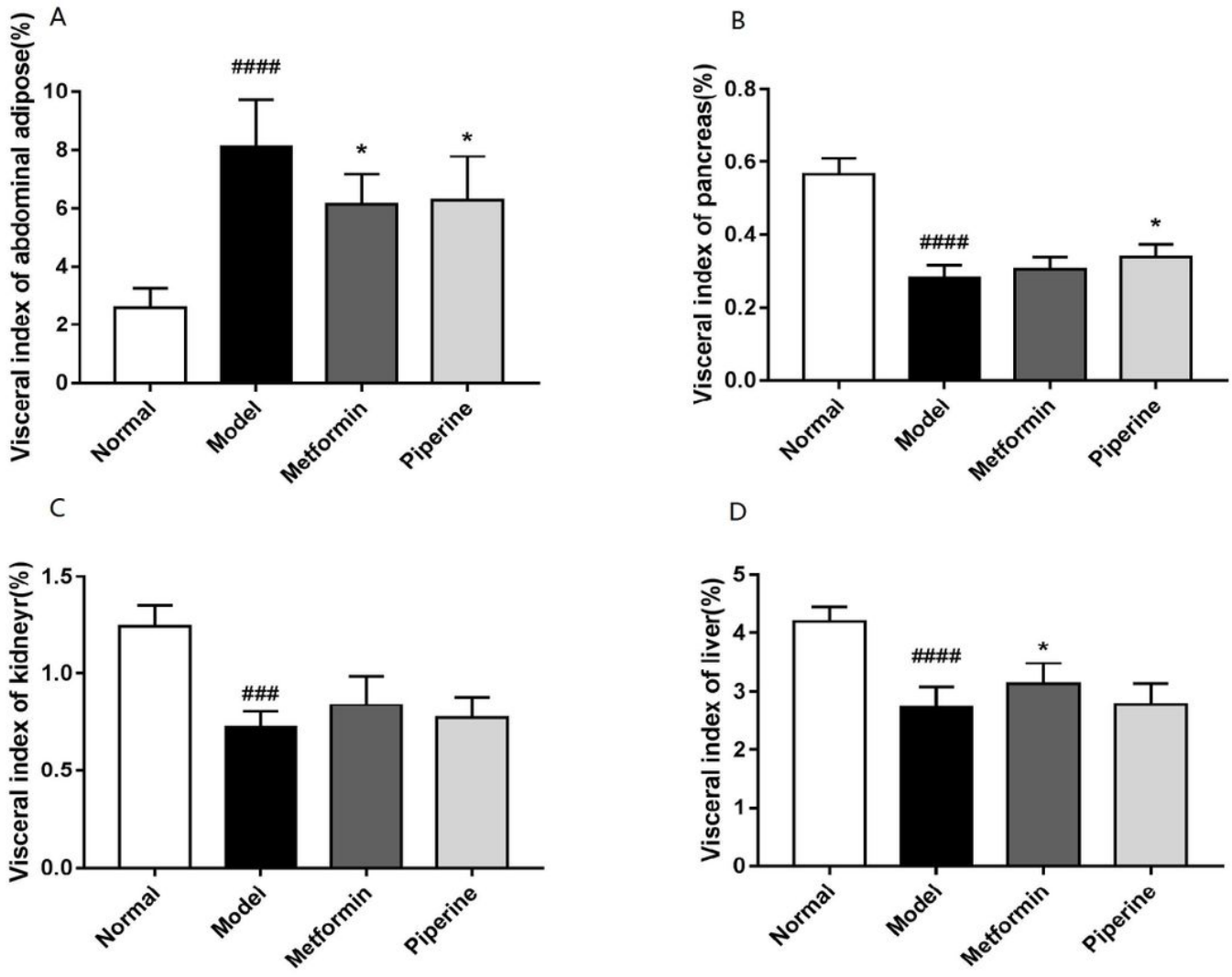

Figure 2

Effect of piperine on visceral organ indexes. (A) Piperine decreased the visceral index of abdominal adipose. (B) Piperine increased the visceral index of pancreas. (C-D) Piperine did not change visceral index of kidney and liver. Data are expressed as mean ± SD, n=6-8. ###P<0.001, ####P<0.0001 vs. Normal group; *P<0.05 vs. Model group.

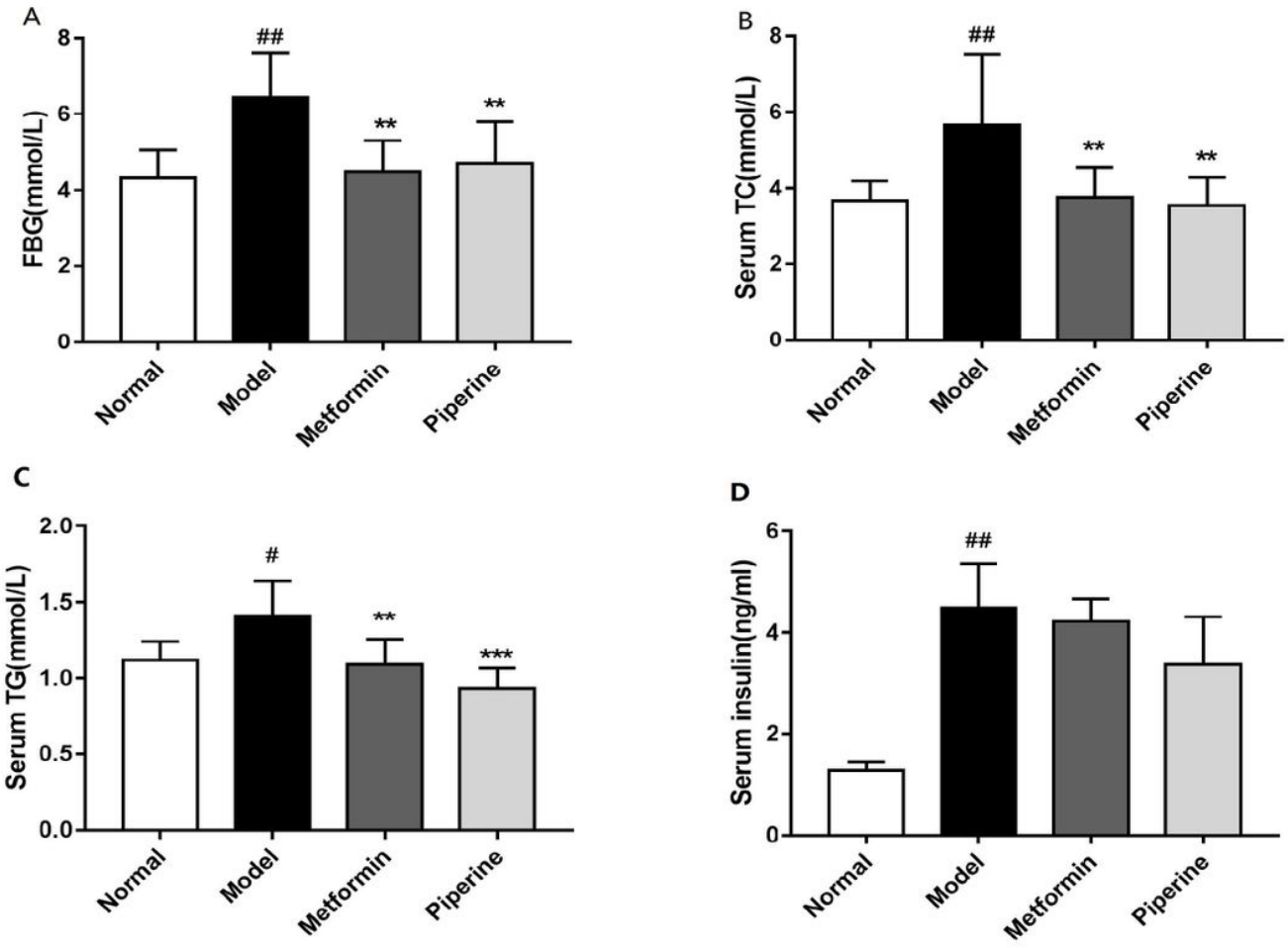

Figure 3

Piperine regulated glycolipid metabolism in MSG mice. (A) Piperine decreased the level of fasting blood glucose(FBG). (B-C) Piperine decreased the level of serum TC and TG. (D) Piperine did not reduced the concentration of serum insulin. Data are expressed as mean ± SD, n=6-8. #P<0.05, ##P<0.01 vs. Normal group; **P<0.01,***P<0.001 vs. Model group.

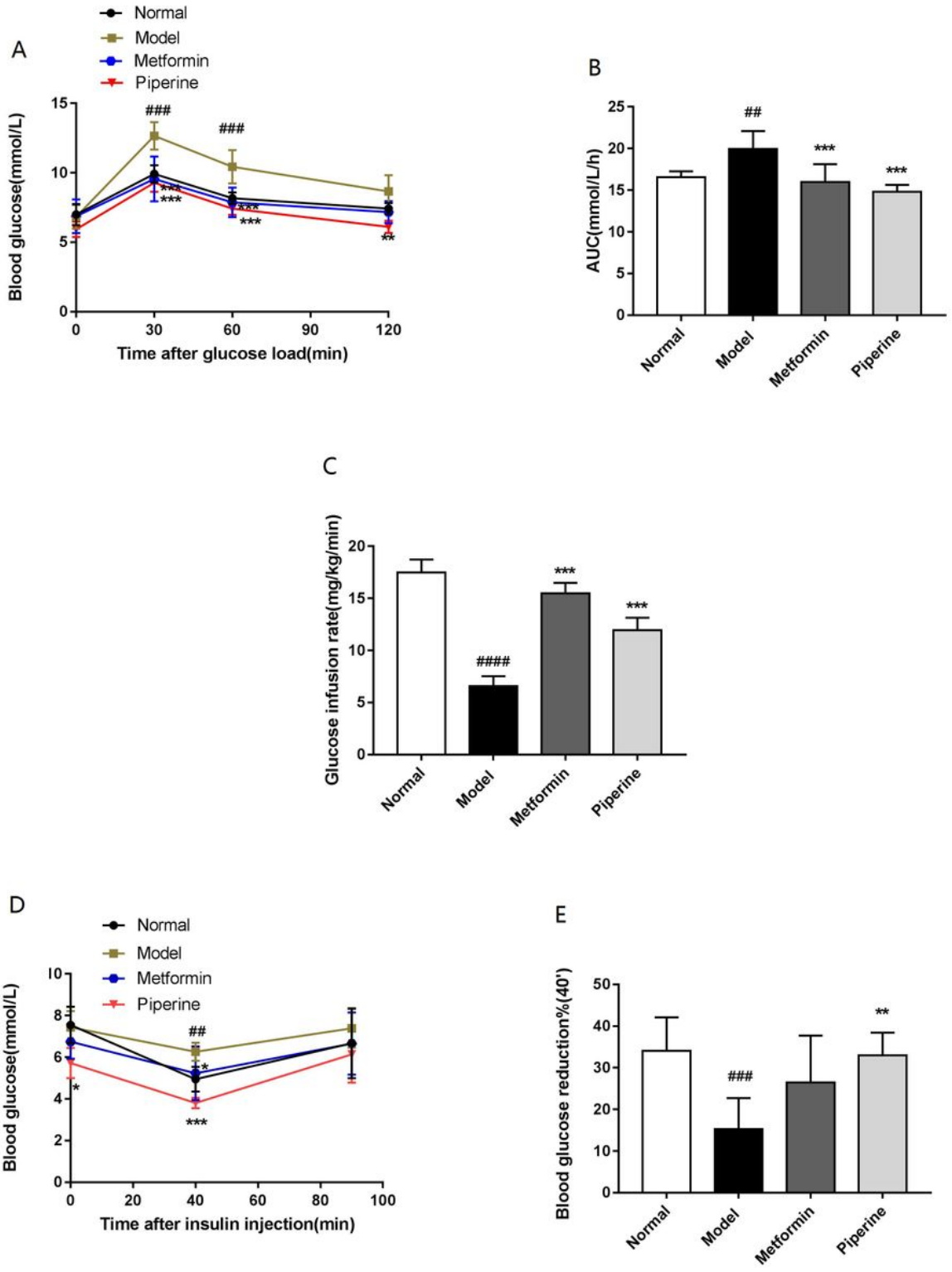

Figure 4

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 ± 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.

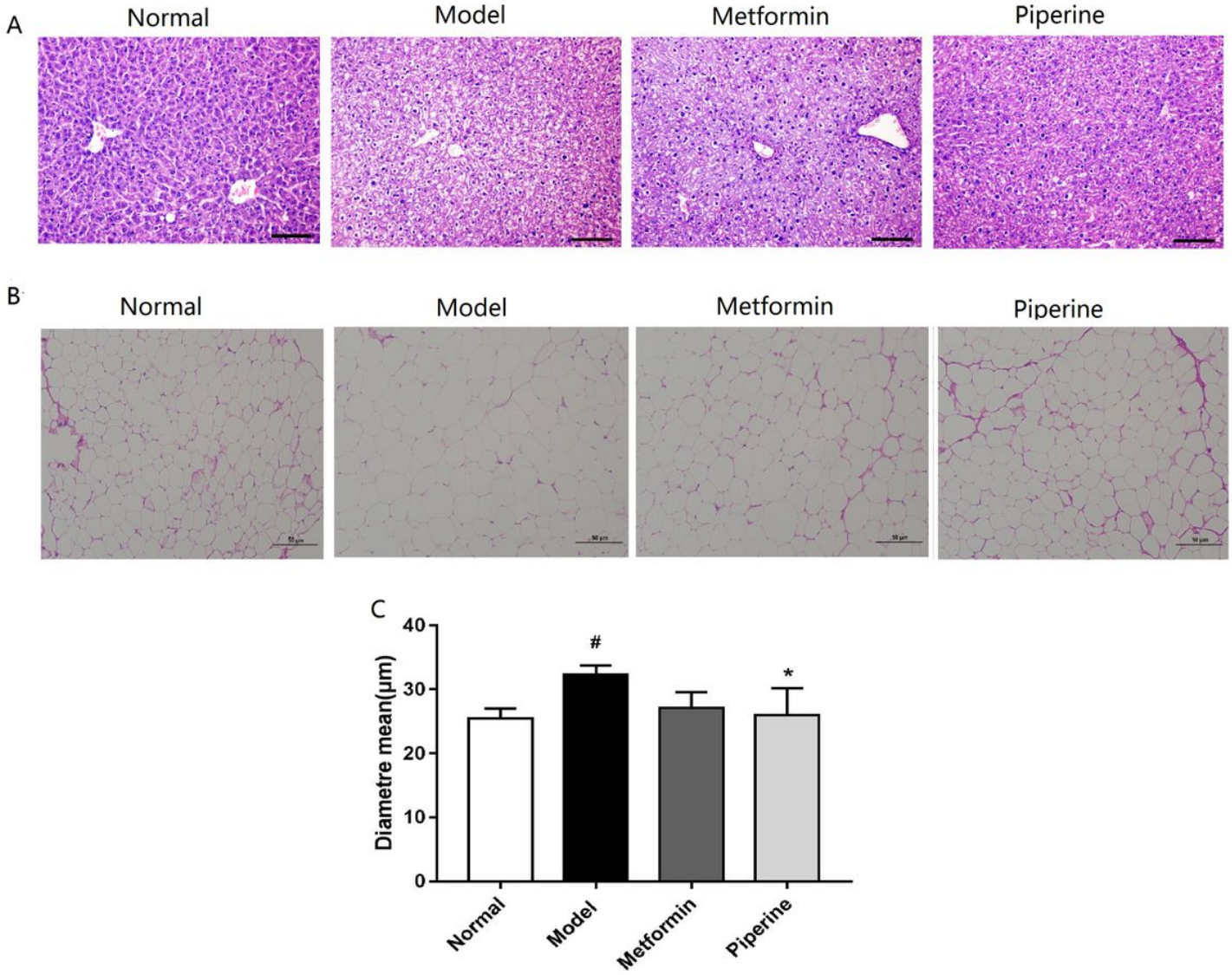

Figure 5

Protective effects of piperine on liver and adipose tissues morphology. (A) Liver sections were stained with H&E (200×), piperine treatment reduced MSG-induced fatty infiltration in liver. (B) Representative H&E staining of abdominal adipose tissue sections (100×). (C) Adipocyte diameters (mean) as measured with H&E staining, piperine treatment reduced adipocyte diameters. Data are expressed as mean ± SD, n=3. #P<0.05vs. Normal group; *P<0.05 vs. Model group.

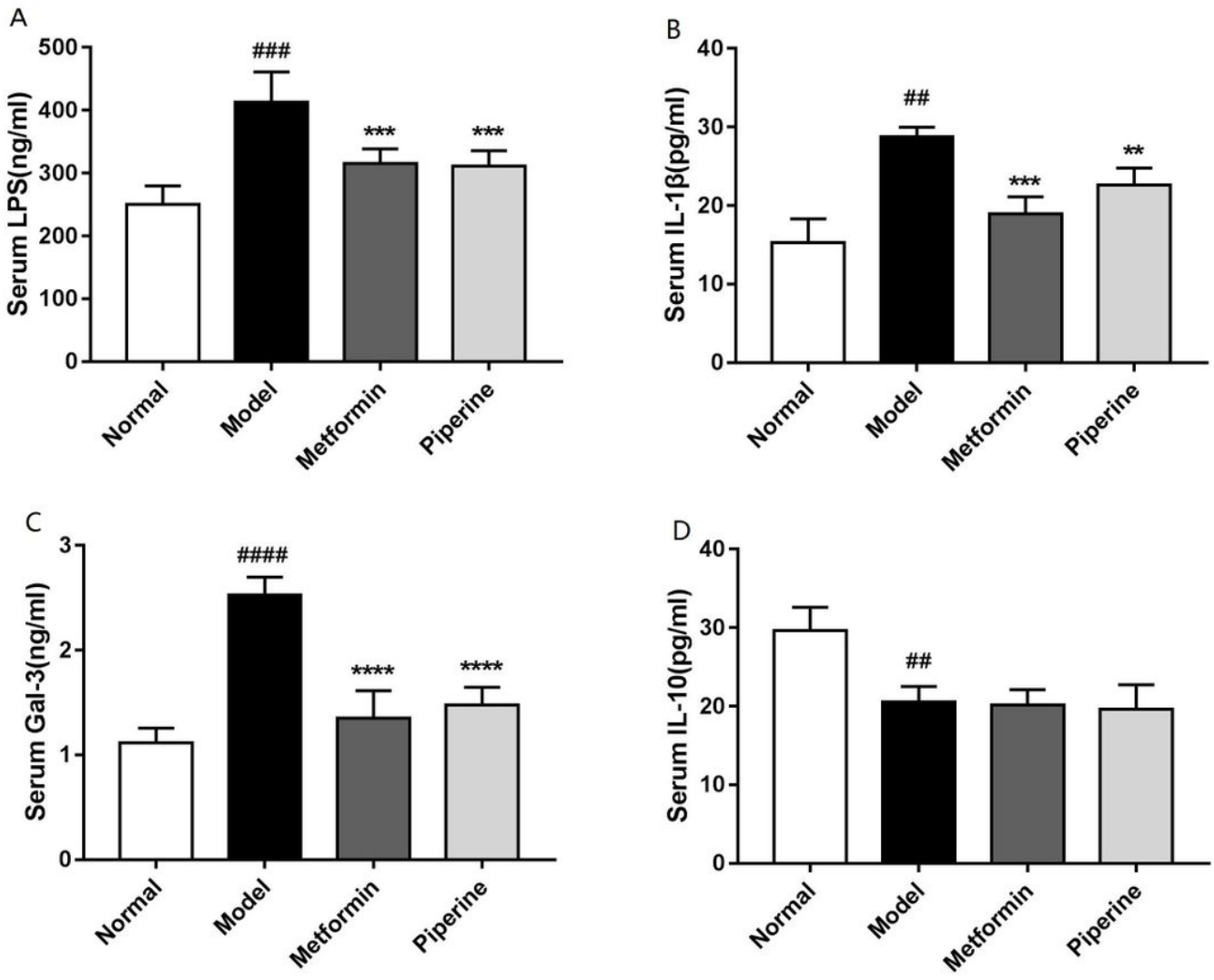

Figure 6

Piperine protected MSG obese mice against inflammatory injury in whole body. (A-C) Piperine decreased the levels of serum LPS, IL-1β and Gal-3. (D) Piperine did not change the level of serum IL-10. Data are expressed as mean ± SD, n=6-8. ##P<0.01, ###P<0.001 vs. Normal group; **P<0.01, ***P<0.001 vs. Model group.

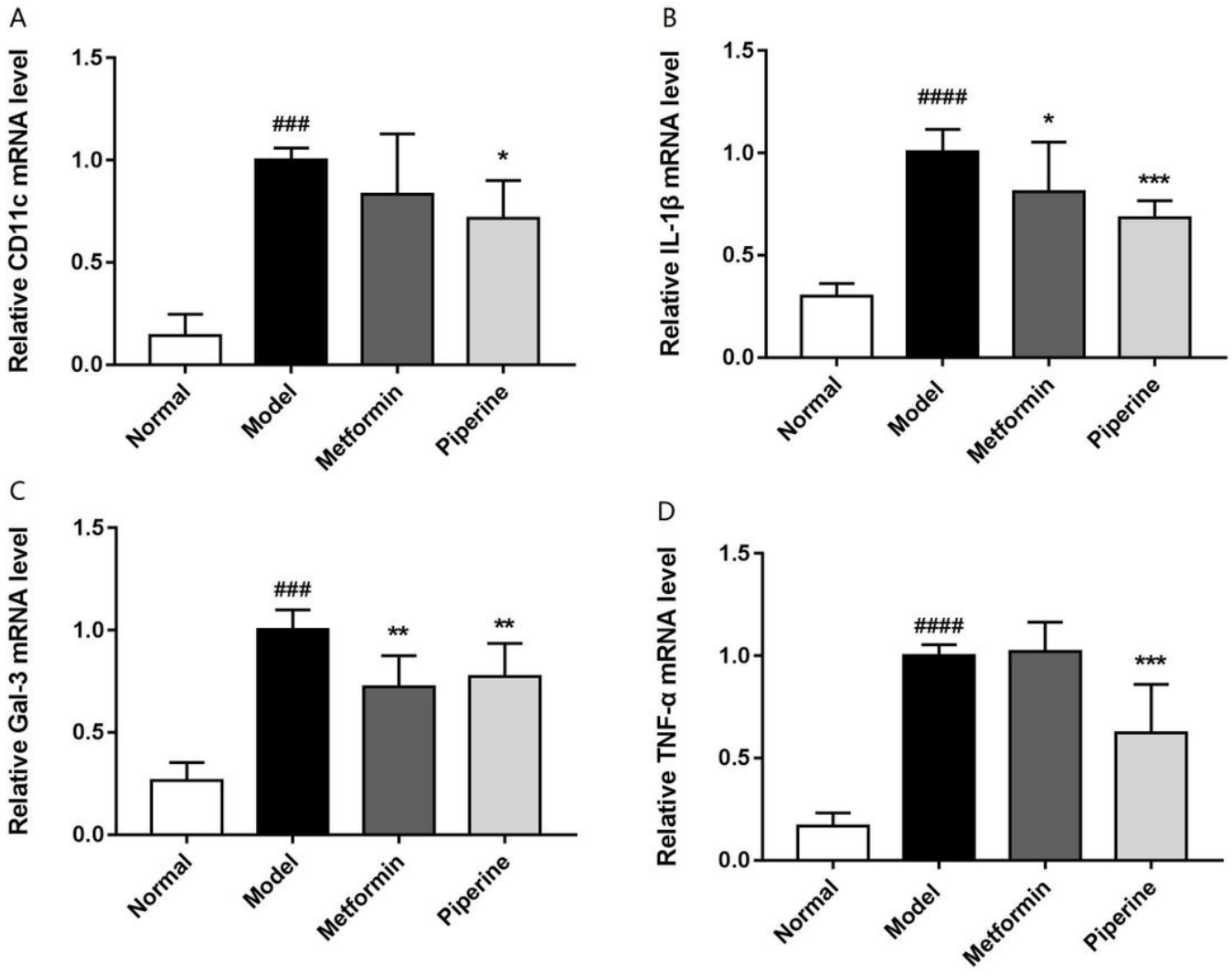

Figure 7

Piperine inhibited the inflammatory state of adipose tissue in MSG-obese mice. (A) Piperine decreased CD11c mRNA level in WAT of the MSG-induced obesity mice. (B-D) Piperine reduced IL-1β, Gal-3 and TNF-a mRNA levels in WAT of the MSG-induced obesity mice. Data are expressed as mean ± SD, n=6-8. ##P<0.01, ###P<0.001 vs. Normal group; *P<0.05, **P<0.01, ***P<0.001 vs. Model group.

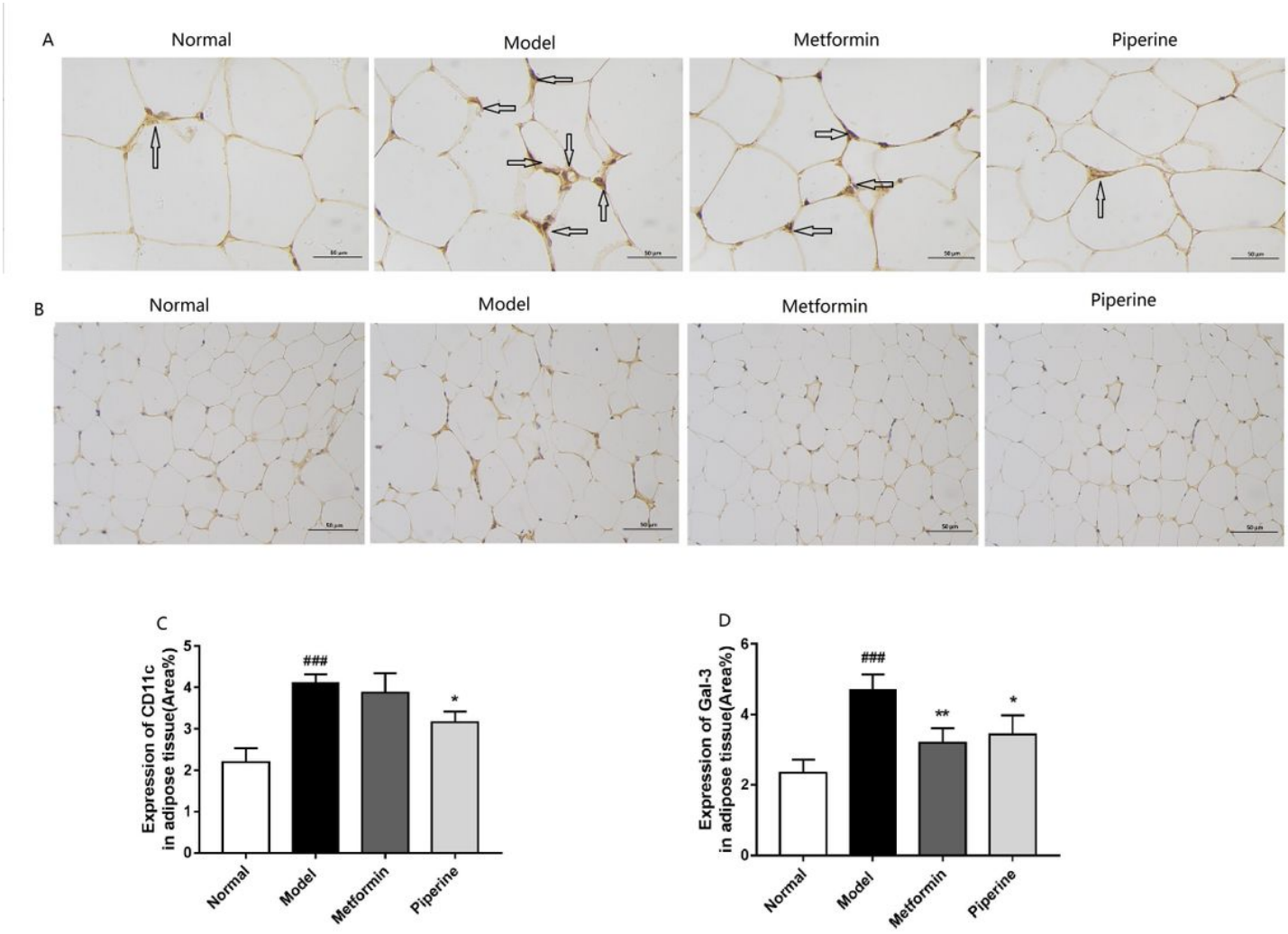

Figure 8

Immunohistochemical analyses of abdominal adipose. (A-D) The expression of CD11c(400×) and Gal-3(200×) in abdominal adipose. Data are expressed as mean ± SD, n=6-8. ###P<0.001 vs. Normal group; *P<0.05, **P<0.01 vs.Model group.

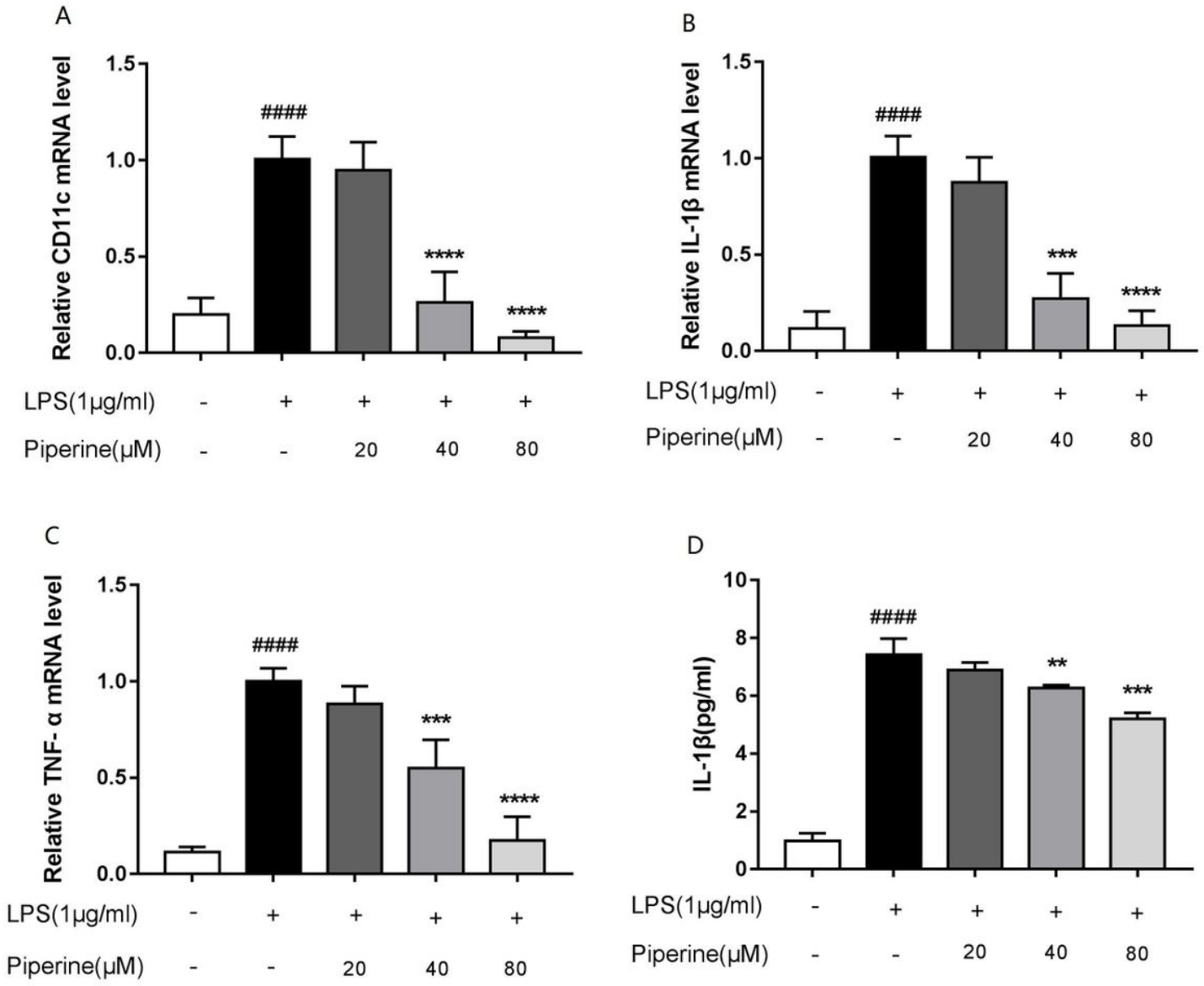

Figure 9

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

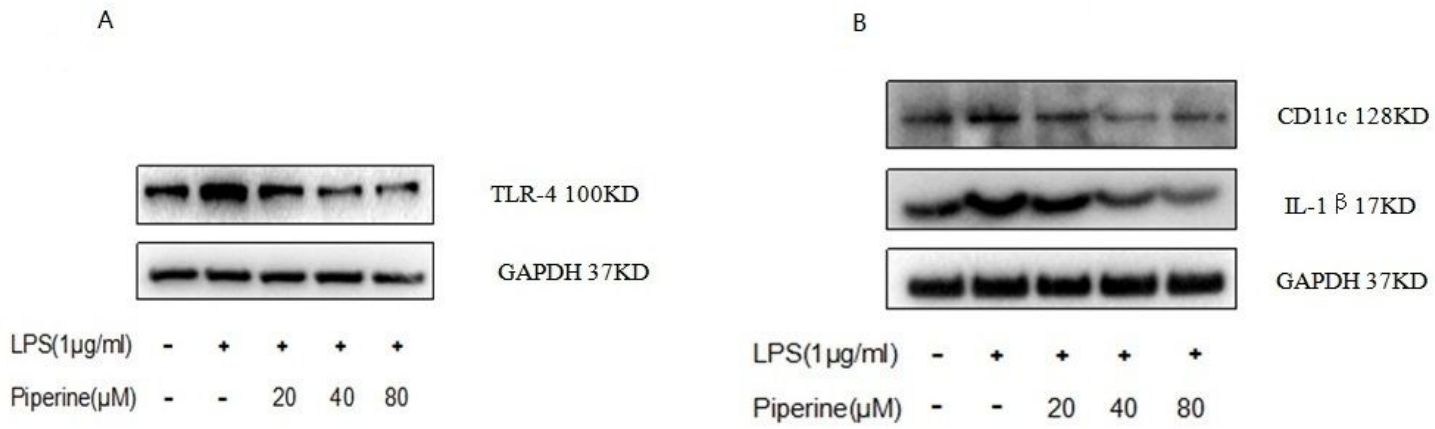

Figure 10

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.docx