

Flavonoid compound from Agrimonia Pilosa Ledeb improves adipose insulin resistance by alleviating oxidative stress and inflammation

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Abstract

Background

Researches and practice of Chinese medicine indicated that *Agrimonia Pilosa Ledeb* could improve insulin resistance (IR) and treat type 2 diabetes (T2DM). Flavonoid component (FC) was collected from *Agrimonia Pilosa Ledeb*. The aim of this study was to elucidate the effects of FC on glucose metabolism to improve IR by suppressing oxidative stress and inflammation.

Methods

By overdosed glucose and insulin or high-fat diet to establish adipocytes or mice IR model. The uptake of 2-NBDG and glucose consumption were measured to verify insulin sensitivity in vitro and vivo. Reactive oxidative species (ROS) were detected by flow cytometry, activity of superoxide dismutase (SOD) and content of malondialdehyde (MDA) were also measured. Meanwhile, factors related to insulin signal pathway such as PPAR γ , insulin receptor substrate-1 (IRS-1) and GLUT4, factors related to oxidative stress such as NF-E2-related factor 2 (Nrf2), and expression and secretion of inflammatory cytokines such as NF- κ B, IL-1 β , IL-6 and TNF- α were tested. Furthermore, the JNK/PI3K/Akt signal pathway were also explored.

Results

FC ameliorated the impaired glucose metabolism significantly. Further study indicated that FC could promote the expression related to insulin signal pathway to improve insulin resistance. What's more, it could not only upregulated PPAR γ with the similar efficacy as pioglitazone (Piog) straightway. But FC also decreased the endogenous ROS, lowered the content of MDA, increased the activity of SOD and expression of Nrf2 to facilitate oxidative homeostasis. It also attenuated expression and secretion of inflammatory cytokines obviously. At last, the underling mechanism JNK/PI3K/Akt pathway was improved in adipocytes and adipose tissue.

Conclusion

FC could ameliorate glucose metabolism and improve IR. It exerted these effects by suppressing oxidative stress and inflammation. FC from *Agrimonia Pilosa Ledeb* has a good prospect to be drugs or functional foods for IR and T2DM.

Bcakground

Type 2 diabetes mellitus (T2DM) affected 536.6 million 20-79-year-olds in 2021 which would reach to 738.2 million in 2045, especially for middle-income countries. China will have the most people with

diabetes over 174 million by 2045 [1]. It is gaining considerable attention, so the pre-treatment and prevention of this disease is urgently required. As a chronic metabolic disorder, it involves several pathogenic processes, mainly refers to insulin resistance (IR) and usually relative (rather than absolute) insulin deficiency. The impaired secretion and deficient action of insulin often coexist.

Oxidative stress has been thought to be the main factor in the onset and progression of IR. Common Soil Hypothesis declares oxidative stress is the pathogenic mechanism underlying IR and T2DM [2, 3]. Reactive oxidative species (ROS) derived from oxidative stress can decrease glucose consumption and disorder insulin signaling pathway by disturbing GLUT to act on glucose transporting and accelerating serine phosphorylation of insulin receptor substrate-1 (IRS-1). ROS also triggers inflammatory cytokines to worsen IR [4]. Consequently, anti-oxidative therapy can be an approach in preventing T2DM [4]. Furthermore, hyperglycemia and hyperinsulinemia are accompanied with systemic and tissue specific inflammation [5, 6]. For instance, elevated TNF- α and IL-6 have been reported in various IR states. Following c-Jun N-terminal kinase (JNK) and NF- κ B mainly [5], inflammatory cytokines directly affect the insulin signal pathway such as disturbing IRS-1 and GLUT. By the way, they can also activate accumulation of free fatty acid reciprocally to worsen IR. In addition, JNK, as the common point of oxidative stress and inflammation, its phosphorylation can trigger the serine phosphorylation of IRS-1 and lead to IR. These series of reports clearly provide evidence that ROS and inflammatory factors can phosphorylate JNK [7], and controlling JNK may play an indispensable role in preventing IR [8]. Taken all of these together, alleviating oxidative stress and inflammation may be efficient and reliable in preventing IR and T2DM.

Agrimonia Pilosa Ledeb is a kind of herb for tonic function and used to treat T2DM in traditional Chinese medicine. It includes flavonoids and flavone glycosides [9, 10], triterpenoids [11, 12] and agrimonolides. We found flavonoid component (FC) was one of the most abundant components. After tremendous endeavors, studies showed flavonoids had anti-oxidative activity [13, 14], acted on immune system to exert anti-inflammatory effect [15], and also had a positive effect on insulin sensitivity [16]. What's more, it has been widely documented that *Agrimonia Pilosa Ledeb* provided a considerable resistance to hyperglycemia and insulin resistance in insulin incubated-HepG2 cells [17] or high-fat diet-fed rats [18, 19]. Our previous study also indicated that extracts from *Agrimonia Pilosa Ledeb* could reinforce insulin sensitization through PPAR γ [12]. Therefore, in order to clarify whether FC from *Agrimonia Pilosa Ledeb* has the therapeutic effect on IR, we collected FC and investigated its effects on 3T3-L1 adipocytes and C57BL/6 mice with IR which was induced by overdosed glucose and insulin. We detected the glucose consumption and uptake, determined the disturbed factors related to insulin signal, oxidative stress and inflammation, explored JNK/PI3K/Akt signal pathway. This study will illuminate the mechanism of FC on improving IR and FC's edible values or medicinal benefits.

Methods

Materials

Agrimonia Pilosa Ledeb obtained from Western Medicine City (Chongqing, China). The extraction process represented in reference [20]. FC had a level of total flavonoids with a value of 316.53 ± 6.37 mg/g and was composed of quercetin, quercitrin, vitexin, isovitexin, hyperoside, rutin, kaempferol, tiliroside, apigenin, catechinic acid, taxifolin and digitoflavone-7-O-glucoside mainly. The powder was stored at 4°C.

3T3-L1 pre-adipocytes were purchased from ATCC. C57BL/6 mice were purchased from Children's Hospital of Chongqing Medical University (China). The low-fat diet (LFD, 19% protein, 4% fat, 67% carbohydrate, and 10% of calories from fat) and high-fat diet (HFD, 24% protein, 24% fat, 41% carbohydrate, and 45% calories from fat) were purchased from Shanghai SLAC Laboratory Animal CO. LTD. (Shanghai, China). DMEM, penicillin-streptomycin and FBS were purchased from HyClone. Bovine calf serum (BCS) was purchased from Gibco and BSA from Equitech-Bio. Paraformaldehyde, Oil Red O, 3-isobutylmethylxanthine (IBMX), dexamethasone (DEX) and insulin were purchased from Sigma-Aldrich (St Louis, MO). 2-(N-[7-Nitrobenz-2-oxa-1, 3-diazol-4-yl)Amino]-2-Deoxyglucose (2-NBDG) was purchased from Invitrogen. Glucose oxidase assay, ROS assay kit, total superoxide dismutase (SOD) assay kit with WST-1 and lipid peroxidation malondialdehyde (MDA) assay kit were purchased from Beyotime (Jiangsu, China). Trizol reagent was purchased from Invitrogen and reverse transcription system from Takara. SYBR Green was obtained from BIO-RAD. ELISA kits for insulin, IL-1 β , IL-6 and TNF- α were purchased from 4A Biotech (China). The antibodies of β -actin, p-IRS-1, GLUT-4, PPAR γ , Nrf2, p-IkB α , NF- κ B p65, t-/p-JNK 1/2, PI3K and p-Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and adipocyte differentiation

3T3-L1 pre-adipocytes were induced to adipocytes based on the protocol [21]. Cells maintained in DMEM (25.0 mmol/L glucose) supplemented with 10% BCS and 1% penicillin-streptomycin. At day 0, after reaching confluence, treating with differentiation medium: DMEM (25.0 mmol/L glucose) supplemented with 0.5 mM IBMX, 1 μ g/ml insulin, 0.25 μ M DEX and 10% FBS for 48 h. For the next 48 h, treating with DMEM (25.0 mmol/L glucose) supplemented with 1 μ g/ml insulin and 10% FBS. Then treating with DMEM (25.0 mmol/L glucose and 10% FBS) for 6 days until more than 90% of cells were differentiation. After differentiation at 11th day, 3T3-L1 adipocytes could be used for Oil Red O staining or induced to establish IR model subsequently.

Establishing 3T3-L1 adipocytes IR model

The model was established as described previously with some modification (Figure S1) [22]. After differentiation, the medium was replaced by DMEM (5.5 mmol/L glucose) with 1% BSA for 12 h. To establish IR model, the differentiated adipocytes were divided into LG (DMEM containing 5.5 mmol/L glucose, 1% BSA and 10^{-9} mol/L insulin), Piog (10 μ M pioglitazone and DMEM containing 25.0 mmol/L glucose, 1% BSA and 10^{-6} mol/L insulin), IR model groups which were divided into HG (DMEM containing 25.0 mmol/L glucose, 1% BSA and 10^{-6} mol/L insulin) and FC (25 μ g/ml or 100 μ g/ml or 400 μ g/ml FC

with DMEM containing 25.0 mmol/L glucose, 1% BSA and 10^{-6} mol/L insulin). By glucose oxidase assay, the glucose concentration was analyzed last 24 hours according to the instruction. After 24 hours, adipocytes were collected for further studies.

Animals and drug treatments

All procedures were in accordance with the institutional guidelines for animal experimentation and approved by the Institutional Animal Care and Use Committee of Children's Hospital of Chongqing Medical University. C57BL/6 mice (6 week) were housed under artificial lighting for 12 h/day and standard temperature (22°C–24°C). Mice were fed with LFD or HFD last 16 weeks. In the following 4 weeks, HFD mice were administered with Piog (5 mg/kg/day) or FC (5 mg/kg/day) or same volume of PBS by intragastric gavage each day. Body weight and food intake were recorded each day. After 4 weeks of drug treatment, the fed serum insulin was detected by ELISA kit, blood samples and white adipose tissue (WAT) were recovered and stored at -80°C.

Glucose uptake assay

2-NBDG, a fluorescent glucose analogue, was used to monitor the glucose uptake with the method described previously [23]. 3T3-L1 pre-adipocytes (8×10^3 /well) were plated in 96-well plate before differentiation and establishing the IR model. Each group was divided into 3 wells: 100 μ L KRB, 100 μ L 50 μ M 2-NBDG, 100 μ L 50 μ M 2-NBDG (Containing 100 nM insulin). These wells were incubated in 37°C for 30 min and detected the fluorescence intensity with the excitation wavelength at 488 nm and emission wavelength at 520 nm. Results of every 3 wells in one treating expressed as Fa, Fb, Fc. The calculation formula was $(F_c - F_b)/(F_b - F_a)$.

Intra-peritoneal glucose tolerance tests (iGTTs)

iGTTs of C57BL/6 mice were performed after fasted overnight at the end of drug treatment. At 2 g/kg, 20% glucose solution was injected, and then the blood glucose concentrations at 0, 15, 30, 60 and 120 min were measured using a glucometer (SureStep OneTouch, USA).

Detecting intracellular ROS, activity of SOD, content of MDA and inflammatory cytokines

The fluorescence intensity of DCF-DA in adipocytes was detected by flow cytometry. The activity of SOD was determined by WST-1 method at 450 nM. The intracellular content of MDA was determined at 532 nM. Both of them were detected by purchased kits with ELIASA.

The secretion of IL-1 β , IL-6 and TNF-a were detected by ELISA according to the manufacturer’s protocol.

RNA preparation and Real-Time PCR

Isolating total RNA by Trizol reagent and 1 μ g of each sample was reverse-transcribed to cDNA by the reverse transcription system. According to the manufacturer’s protocol, Real-Time PCR with iQTM SYBR Green supermix was performed. Each reaction was performed in triplicate and GAPDH was used as the internal control gene. The primers were shown in Table 1.

Table 1 Adipocyte specific primers for Real-Time PCR

Gene	Forward Primer	Reverse Primer
GAPDH	CAA GGT CAT CCA TGA CAA CTT TG	GGC CAT CCA CAG TCT TCT GG
IRS-1	ACC ATA ACC AGA GTG CCA AAG T	CGA GTA GGT GCT GAG AAG GTC T
GLUT4	CAT GGC TGT CGC TGG TTT C	AAA CCC ATG CCG ACA ATG A
JNK	ATG GAT TTG GAG GAA CGA ACT A	GAA GAC GAT GGA TGC TGA GAG
Nrf2	TCT TCC ATT TAC GGA GAC CCA	GAT TCA CGC ATA GGA GCA CTG
NF- κ B	ATT CCG CTA TGT GTG TGA AGG	TGT GAC CAA CTG AAC GAT AAC C
IL-6	CTG CAA GAG ACT TCC ATC CAG TT	AAG TAG GGA AGG CCG TGG TT
TNF-a	CTG AGG TCA ATC TGC CCA AGT AC	CTT CAC AGA GCA ATG ACT CCA AAG

Abbreviations: IRS-1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; Nrf2, NF-E2-related factor 2.

Western blot

Proteins were extracted from cell or tissue lysis and the concentration was detected by BCA assay kit. Standard protocols were used. The bands were visualized by ECL and intensities were quantified using image analyzing software (Quantity One, Bio-rad).

Statistical analysis

All data are presented as means $\pm$ SEM. A paired-samples T test was used for two groups and one-way ANOVA for more than two groups by SPSS 19.0 software. Difference with a value of $p < 0.05$ were considered significant statistically. All experiments were repeated at least 3 times.

Results

FC recovered the impaired insulin-stimulated glucose metabolism in 3T3-L1 adipocytes and C57BL/6 mice IR model

Pre-adipocytes were irregular form, but adipocytes turned round after differentiation (Figure S2). With the extended inducing time, cells came into round and accumulated lipid droplets gradually. We could observe more adipocytes in 10th day (Figure S2B) than those in 6th day (Figure S2A). Cells could be used to establish the IR model when the adipocytes reached to 80%-90% (Figure S2B).

As represented in Figure 1A, the overdosed glucose and insulin impaired capacity of glucose uptake and exhibited a highly significant difference compared with LG. Furthermore, to illuminate the glucose consumption in 24 h, we detected the concentration of glucose in culture media (Figure 1B). At 24 h, HG dramatically suppressed consumption in opposition to LG and Piog. But FC reversed this condition in a dose-dependent manner, especially at 100 µg/mL and 400 µg/mL, and was in accordance with the effect of Piog.

We further evaluated the effect of FC on HFD-induced insulin resistance. Compared to LG and Piog, FC was more favorable to decrease plasma glucose and area under curve (AUC) of intra-peritoneal glucose tolerance tests (iGTTs) at the dose of 5 mg/kg/day, especially at 15, 30 and 60 min after glucose loading (Figure 2A). In addition, HFD induced higher fed plasma insulin levels compared with LFD after 20 weeks. But FC and Piog decreased the induced hyperinsulinemia significantly, suggesting increased insulin sensitivity (Figure 2B). Body weight changed significantly after administration (Figure 2C), but not food intake in the last week (Figure 2D). These results clearly implied that HG and HFD had impaired glucose uptake and consumption under hyperglycemia and hyperinsulinemia, but FC could alleviate glucose metabolism and improve insulin resistance obviously.

FC improved the impaired insulin signal pathway

Insulin signal is implied in regulating glucose and insulin metabolism of adipocyte tissue, and is subjected to the tyrosine phosphorylation of IRS-1 and translocation of GLUT-4 [24]. Herein, in 3T3-L1 adipocytes IR model, the tyrosine phosphorylation (Figure 3A and 3B) and gene expression (Figure S3A) of IRS-1 were attenuated in HG group. Not surprisingly, the high level of glucose and insulin also induced the impaired protein (Figure 3A and 3C) and gene (Figure S3B) expression of GLUT-4 which suppressed glucose translocate and uptake. Consistent with the observations in vitro, the tyrosine phosphorylation of IRS-1 (Figure 3E, 3F and Figure S4A) and GLUT-4 (Figure 3E, 3G and Figure S4B) were dramatically decreased by HFD. But FC improved the disordered signal pathway markedly.

Pioglitazone, as a PPAR γ agonist, improves insulin sensitivity and metabolic profile. So Piog could not only facilitate expression of PPAR γ , but also gave rise to the elevated p-IRS-1 and GLUT-4 in IR model (Figure 3). However, FC from *Agrimonia Pilosa Ledeb* also ameliorated the suppressed expression

significantly equally with Piog (Figure 3, S3 and S4). It similarly followed with the upward glucose consumption in Figure 1 and blood glucose levels in Figure 2. Results showed that IR model had impaired expression related to insulin signal pathway, but FC had an effect on promoting their expression and enhanced insulin sensitivity to improve IR and prevent T2DM.

FC abolished the induced oxidative stress in IR model

The happening of IR promotes the augment of ROS which also aggravates IR further by disturbing insulin signal pathway. Under the excessive concentration of glucose and insulin, ROS of adipocytes in HG increased which showed a significant difference compared with LG (Figure 4A). Additionally, HG or HFD had considerable effects on the SOD activity (Figure 4B and 4G) and MDA levels (Figure 4C and 4H) in vitro and vivo. Conversely, FC abolished the induced oxidative stress compared with IR groups, although effect of Piog on these factors in glucose- and insulin-stimulated cells or mice did not reach significance.

Nrf2, as a transcription factor, represents critically important cellular defense mechanisms that maintains intracellular redox homeostasis and minimizes oxidative damage. Our research exhibited that its expression was remarkably depressed in HG group (Figure 4D, 4E and 4F) and HFD group (Figure 4I, 4J and 4K), respectively. At dose of 100 and 400 µg/mL in vitro, or 5 mg/kg/day in vivo, FC could increase Nrf2 expression significantly and they were much higher than Piog. Based on these results, FC reduced intracellular level of oxidative stress and increased anti-oxidation to suppress it that accounts for the effect of improving IR and T2DM.

FC ameliorated expression and secretion of factors related to inflammation

With IR, 3T3-L1 adipocytes and C57BL/6 mice had a high level of inflammatory factors, but FC could suppress them to some extent (Figure 5, 6, S5 and S6). Particularly noteworthy is NF-κB, which plays a pivotal role in the inflammatory stress, is activated dramatically in IR models. Therefore, the threonine phosphorylation of NF-κB and IκBα were detected firstly. Hyperglycemia and hyperinsulinemia gave rise to higher elevated levels in opposition to LG and LFD group (Figure 5B, 5C, 6B and 6C). What's more, as a proinflammatory cytokine, NF-κB initiated the followed response and increased the expression and secretion of IL-1β (Figure 5D and 6D), IL-6 (Figure 5E and 6E) and TNF-α (Figure 5F and 6F) significantly. In a remarkable manner, FC decreased their expression or secretion dramatically compared with HG or HFD group, but Piog did not except for IL-6. These observations strongly supported that FC could suppress inflammation which might be the reason behind the critical role in preventing IR.

FC impeded the induced-JNK/PI3K/Akt in adipocytes and adipose tissue

The toxicity from hyperglycemia and hyperinsulinemia was inevitable responsible for the upward factors associated with inflammation and oxidative stress in the previous work and aforementioned experimental results, that strongly promoted the extension of upstream signal pathways. In this case, JNK/PI3K/Akt pathway in adipocytes and WAT was subjected to be analyzed. First of all, JNK can be induced by oxidative stress and inflammation, and is extremely extraordinary in adipocytes or adipose tissue with IR [25]. Here, the threonine phosphorylation and expression of JNK were significantly different in opposition to un-treated groups in vitro and vivo (Figure 7A, B and C) (Figure 8A, B and C). Furthermore, the disorder also has a great impact on PI3K and Akt. They were suppressed markedly compared with LG and LFD (Figure 7A, D and E) (Figure 8A, D and E). Strikingly, FC from *Agrimonia Pilosa Ledeb* prevented the over-expressed and phosphorylated JNK, and facilitated the indispensable expression of PI3K and Akt, that capacity also stronger than Piog to some extent. In conclusion, the high level of glucose and insulin contributed to the activated JNK/PI3K/Akt, but FC could alleviate this signal pathway. This might be the feasible approach to suppress oxidative stress and inflammation, even to prevent IR.

Discussion

The occurrence and development of T2DM involved many factors and signal pathways including IR, oxidative stress, inflammation and so on. Some studies had reported that oxidative stress was the causal role in IR and T2DM, and inflammation also occupied an important position in their occurrence. Therefore, the anti-oxidative and anti-inflammatory drugs may be an approach in improving IR and preventing T2DM. Looking for some alternatives, such as Chinese herbal medicine, which has the effect of anti-oxidation and anti-inflammation may reap huge fruits under hyperglycemia and hyperinsulinemia. In this study, we extracted FC from *Agrimonia Pilosa Ledeb* and researched its effects of anti-oxidative stress, anti-inflammation and improving IR in 3T3-L1 adipocytes and C57BL/6 mice with IR. Results indicated FC could ameliorate the glucose metabolism and enhance insulin sensitivity. Simultaneously, FC performed significant prevention of oxidative stress and inflammation though JNK/PI3K/Akt signal pathway in vitro and vivo.

Adipose tissue is known to confer a higher risk of IR, T2DM and cardiovascular. In the local and systemic tissue, it can secrete ROS [4, 26] and multiple inflammatory cytokines [27]. As a dominating target of IR, oxidative stress and inflammation, adipose tissue may serve as the giant battleground of prevention of IR and T2DM. Therefore, adipocytes and adipose tissue were exposed to overdosed concentration of glucose and insulin chronically to cause insult and used to establish IR model. As expected, by detecting the uptake and consumption of glucose, IR groups had significantly impaired glucose metabolism, but FC from *Agrimonia Pilosa Ledeb* could correct the disturbance. Furthermore, defects in insulin pathway, mainly PPAR γ /IRS/GLUT, which leads to disruption of insulin and glucose homeostasis have considerable effects on the development of IR. It should be noted that PPAR γ has received substantial attention owing to its effect of reducing hyperglycemia by increasing insulin sensitivity and decreasing hepatic glucose production [28]. The elevated PPAR γ significantly ameliorates insulin sensitivity in mice with high fat diets and patients with T2DM by facilitating IRS and GLUT [29]. IRS-1, a principal substrate of receptor for insulin, plays an important role in insulin signal pathway. Several of cytokines can induce

its serine and inhibit tyrosine phosphorylation to reduce biological effect of insulin and deteriorate IR, so the lower expression of IRS-1 predicts IR [30]. Another major factor contributing to the impaired glucose transport is the down-regulation of GLUT4 [6]. It is the most abundant facilitative glucose transporter in adipose tissue, so impaired expression also triggers IR [31]. These series of experiments clearly revealed that PPAR γ , IRS-1 and GLUT4 were significantly inhibited, but FC improved this condition in a dose-dependent manner. Taken these together, the disordered glucose and insulin contributed to the IR of adipocytes and adipose tissue. However, FC facilitated glucose intolerance and reinforced insulin sensitivity.

In the initiation and progression of IR, oxidative stress and inflammation play indispensable roles. ROS, which is secreted through oxidative stress, can conduct intervention into the insulin signal pathway which causes resistance to insulin action by accelerating serine and inhibiting tyrosine phosphorylation of IRS-1. By the way, NF- κ B also can be activated by ROS and then aggravates IR. Whereafter, the expression and secretion of inflammatory cytokines are accelerated. With the emergence of oxidative stress, antioxidant enzymes and oxidized products are altering. The content of MDA, the lipid oxidative end product, represents the level of oxidative insult. With respect to the endogenous anti-oxidative defense, SOD can decrease the oxidation stress and inflammation. Particularly, Nrf2 can achieve self-protection through nuclear accumulating and up-regulating of ARE-linked genes expression, such as SOD and CAT. So the activation of Nrf2 is indispensable to keep cellular homeostasis and prevent the initiation of IR and T2DM [32]. The obtained results clearly indicated that FC significantly depressed ROS and MDA in vitro and vivo. The activity of SOD was increased and the expression of Nrf2 was also promoted to achieve capacity of anti-oxidative stress. Increasing level of anti-oxidative stress and decreasing endogenous ROS might be essential to the effect of FC on preventing IR.

Another initiation factor, inflammation [5], can induce IR by suppressing insulin signal pathways or various of targets. NF- κ B, as a nuclear transcription factor, regulates inflammatory cytokines, adhesion molecules and chemotactic cytokines to influence inflammatory reaction and immunological processes. Activation of NF- κ B is initiated by stimuli, such as FFAs and cytokine receptors, and also depends on the threonine phosphorylation of I κ B [33]. As a consequence, the synthesis of IL-1 β , IL-6 and TNF- α is mediated by NF- κ B, but they also play vital roles in the activation of NF- κ B in turn. Meanwhile, they also can be activated by ROS and aggravate IR. As proinflammatory factors, IL-1 β and TNF- α aggravates IR further [34, 35], especially in adipose tissue, though activating various signal transduction cascades which are critical inhibitors of insulin action. For instance, they phosphorylate serine of IRS-1 and reduce insulin-induced tyrosine phosphorylation. In other respects, down-regulating protein related to glucose metabolism, such as above-mentioned GLUT4 [36] and PPAR γ [37], also occupies large proportion of induced IR. IL-6 also takes the pivotal position in IR and T2DM. It increases serine phosphorylation and inhibits the expression of GLUT4 to impair glucose consumption [38]. In this research, FC suppressed the inflammatory cytokines, these might account for the reason of ameliorating glucose homeostasis and improving IR.

Is there a cross talk between reducing oxidative stress and suppressing inflammation by FC to improve IR? We attempt to answer this by analyzing mitogen-activated protein (MAP) kinases. JNK is activated in response to various extracellular stimuli and cellular stresses, such as ROS, TNF- α and FFAs, which are known to be activated and accelerated under IR conditions. What's more, mounting evidence [25] indicates that activation of JNK is the central of mediating IR. It can bind to the serine of IRS-1 to phosphorylate IRS-1 and suppress the tyrosine phosphorylation which can induce impaired insulin signal pathway. It has been clearly demonstrated that oxidative stress can induce the activation of JNK and inhibiting JNK can prevent IR [39]. More works advocate that JNK is essential for inflammation and also plays a key role in IR [40]. Taken all of these together, the mutual action between oxidative stress, inflammation and JNK is closely.

Akt, a downstream target of PI3K, is triggered by the activation of insulin receptor in insulin signal pathway and facilitates the uptake of glucose with GLUT. Besides, the increased PI3K activity and activated Akt also can increase the insulin-stimulated glucose disposal [7]. Therefore, stopping JNK/PI3K/Akt pathway is an approach of stopping oxidative and inflammatory insult at the initiation of IR and T2DM. In this paper, oxidative stress and inflammation, induced by excessive concentration of glucose and insulin, were highly plausible causal mechanisms for increased expression of JNK and decreased of PI3K/Akt. Then the eventual outcome was the induced IR. But FC alleviated this signal pathway forcefully to keep insulin sensitivity, so improved IR was the effect of suppressing oxidative stress and inflammation. Based on the new strategies to address JNK, the long-standing IR can be prevented.

Conclusion

Oxidative stress and inflammation are main factors in IR and T2DM. Suppressing them may be an effective approach in preventing these disorders. Based on our results, FC from *Agrimonia Pilosa Ledeb* could ameliorate the glucose metabolism in IR model. Furthermore, it improved insulin sensitivity of adipocytes and adipose tissue. Then we found FC alleviated the disturbed oxidative homeostasis and also decreased the expression and secretion of inflammatory cytokines. What's more, JNK/PI3K/Akt pathway was also regulated by FC, suggesting that *Agrimonia Pilosa Ledeb* could act on this pathway to exert prevention on IR. So as an agent of anti-oxidative stress and anti-inflammation, FC from *Agrimonia Pilosa Ledeb* has a good prospect to be a health care product or natural drug for preventing IR.

Abbreviations

IR: insulin resistance; FC: flavonoid component; ROS: reactive oxidative species; SOD: superoxide dismutase; MDA: malondialdehyde; IRS-1: insulin receptor substrate-1; Nrf2: NF-E2-related factor 2; JNK: c-Jun N-terminal kinase; FBS: fetal bovine serum; BSA: bovine serum albumin; IBMX: 3-isobutylmethylxanthine; DEX: dexamethasone; 2-NBDG: 2-(N-[7-Nitrobenz-2-oxa-1, 3-diazol-4-yl)Amino]-2-Deoxyglucose; BCS: bovine calf serum; LFD, low-fat diet; HFD, high-fat diet; WAT, white adipose tissue.

Declarations

Ethics approval and consent to participate

All procedures were in accordance with the institutional guidelines for animal experimentation and approved by the Institutional Animal Care and Use Committee of Children's Hospital of Chongqing Medical University. This study was reported in accordance with ARRIVE guidelines.

Consent for publication

The authors declare no conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TWG contributed to the experiment design and data collection, performed tests, reviewed and approved the final manuscript as submitted. YP conceptualized and designed the study, directed and participated in the data analysis, interpreted data, edited the manuscript, and approved the final manuscript as submitted. LY, GC and JD contributed to the study design, participated establishing IR model and approved the final manuscript as submitted. LCZ contributed to the study design, participated establishing IR model, interpreted data, reviewed the manuscript, and approved the final manuscript as submitted. All authors read and approved the final manuscript.

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Statement

We have permission to collect *Agrimonia Pilosa Ledeb* for basic research.

References

1. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, Stein C, Basit A, Chan J, Mbanya JC. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract.* 2022;183:109119-.
2. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature.* 2006;440(7086):944–8.
3. Tangvarasittichai S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World J diabetes.* 2015;6(3):456.
4. Burgos-Morón. Abad-Jiménez, Maraón, Iannantuoni, Víctor: **Relationship Between Oxidative Stress, ER Stress, and Inflammation in Type 2 Diabetes: The Battle Continues.** *J Clin Med.* 2019;8(9):1385.
5. Glass CK, Olefsky JM. Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metabol.* 2012;15(5):635–45.
6. Passarelli M, Machado UF. AGEs-Induced and Endoplasmic Reticulum Stress/Inflammation-Mediated Regulation of GLUT4 Expression and Atherogenesis in Diabetes Mellitus. *Cells.* 2022;11(1):104-.
7. Kowalczyk A, Bourebaba N, Kornicka-Garbowska K, Turlej E, Marycz K, Bourebaba L. Hyoscyamus albus nortropane alkaloids reduce hyperglycemia and hyperinsulinemia induced in HepG2 cells through the regulation of SIRT1/NF-kB/JNK pathway. *Cell Communication and Signaling.* 2021;19(1):1–26.
8. Bennett BL, Satoh Y, Lewis AJ. JNK: a new therapeutic target for diabetes. *Curr Opin Pharmacol.* 2003;3(4):420–5.
9. Tomlinson C, Nahar L, Copland A, Kumarasamy Y, Mir-Babayev NF, Middleton M, Reid RG, Sarker SD. **Flavonol glycosides from the seeds of *Agrimonia eupatoria* (Rosaceae).** *Biochem Syst Ecol.* 2003;31(4):439–41.
10. Xu X, Qi X, Wang W, Chen G. Separation and determination of flavonoids in *Agrimonia pilosa* Ledeb. by capillary electrophoresis with electrochemical detection. *J Sep Sci.* 2005;28(7):647–52.
11. Kouno I, Baba N, Ohni Y, Kawano N. **Triterpenoids from *Agrimonia pilosa*.** *Phytochemistry.* 1988;27(1):297–9.
12. Guo T, Zhu L, Tan J, Zhou X, Xiao L, Liu X, Wang B. Promoting effect of triterpenoid compound from *Agrimonia pilosa* Ledeb on preadipocytes differentiation via up-regulation of PPAR γ expression. *Pharmacognosy magazine.* 2015;11(41):219.
13. Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: antioxidants or signalling molecules? *Free Radic Biol Med.* 2004;36(7):838–49.
14. Chen L, Teng H, Xie Z, Cao H, Cheang WS, Skalicka-Woniak K, Georgiev MI, Xiao J. **Modifications of dietary flavonoids towards improved bioactivity: An update on structure– activity relationship.** *CRC Critical Reviews in Food Technology.*
15. Gonzalez R, Ballester I, Lopez-Posadas R, Suarez M, Zarzuelo A, Martinez-Augustin O, Medina FSD. Effects of flavonoids and other polyphenols on inflammation. *Crit Rev Food Sci Nutr.*

2011;51(4):331–62.

16. Wedick NM, Pan A, Cassidy A, Rimm EB, Sampson L, Rosner B, Willett W, Hu FB, Sun Q, van Dam RM. **Dietary flavonoid intakes and risk of type 2 diabetes in US men and women.** *The American journal of clinical nutrition* 2012;ajcn. 028894.
17. Teng H, Chen L, Song H. The potential beneficial effects of phenolic compounds isolated from *A. pilosa* Ledeb on insulin-resistant hepatic HepG2 cells. *Food Funct.* 2016;7(10):4400–9.
18. Jang HH, Nam SY, Kim MJ, Kim JB, Choi JS, Kim HR, Lee YM. *Agrimonia pilosa* Ledeb. aqueous extract improves impaired glucose tolerance in high-fat diet-fed rats by decreasing the inflammatory response. *BMC Complement Altern Med.* 2017;17(1):1–8.
19. Jang H-H, Bae JH, Kim M-J, Park MY, Kim HR, Lee Y-M. *Agrimonia pilosa* Ledeb. ameliorates hyperglycemia and hepatic steatosis in ovariectomized rats fed a high-fat diet. *Nutrients.* 2020;12(6):1631.
20. Liu X, Zhu L, Tan J, Zhou X, Xiao L, Yang X, Wang B. Glucosidase inhibitory activity and antioxidant activity of flavonoid compound and triterpenoid compound from *Agrimonia Pilosa* Ledeb. *BMC Complement Altern Med.* 2014;14(1):12.
21. Nugent C, Prins JB, Whitehead JP, Savage D, Wentworth JM, Chatterjee VK, O’Rahilly S. Potentiation of glucose uptake in 3T3-L1 adipocytes by PPAR γ agonists is maintained in cells expressing a PPAR γ dominant-negative mutant: evidence for selectivity in the downstream responses to PPAR γ activation. *Mol Endocrinol.* 2001;15(10):1729–38.
22. Nelson BA, Robinson KA, Buse MG. High glucose and glucosamine induce insulin resistance via different mechanisms in 3T3-L1 adipocytes. *Diabetes.* 2000;49(6):981–91.
23. Wang L, Xu ML, Rasmussen SK, Wang M-H. **Vomifoliol 9- O- α -arabinofuranosyl (1 $\rightarrow$ 6)- β -d-glucopyranoside from the leaves of *Diospyros Kaki* stimulates the glucose uptake in HepG2 and 3T3-L1 cells.** *Carbohydr Res.* 2011;346(10):1212–6.
24. Ahmed B, Sultana R, Greene MW. Adipose tissue and insulin resistance in obese. *Biomed Pharmacother.* 2021;137:111315.
25. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature.* 2002;420(6913):333–6.
26. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest.* 2004;114(12):1752–61.
27. Gillum MP, Kotas ME, Erion DM, Kursawe R, Chatterjee P, Nead KT, Muike ES, Hsiao JJ, Frederick DW, Yonemitsu S. SirT1 regulates adipose tissue inflammation. *Diabetes.* 2011;60(12):3235–45.
28. Cataldi S, Costa V, Ciccodicola A, Aprile M: **PPAR γ and Diabetes: Beyond the Genome and Towards Personalized Medicine.** 2021.
29. Jain N, Bhansali S, Kurpad AV, Hawkins M, Sharma A, Kaur S, Rastogi A, Bhansali A. Effect of a Dual PPAR α/γ agonist on Insulin Sensitivity in Patients of Type 2 Diabetes with Hypertriglyceridemia- Randomized double-blind placebo-controlled trial. *Sci Rep.* 2019;9(1):1–9.

30. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α -and obesity-induced insulin resistance. *Science*. 1996;271(5249):665–70.
31. Abel ED, Peroni O, Kim JK, Kim Y-B, Boss O. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature*. 2001;409(6821):729–33.
32. Cheng X, Siow RC, Mann GE. Impaired redox signaling and antioxidant gene expression in endothelial cells in diabetes: a role for mitochondria and the nuclear factor-E2-related factor 2-Kelch-like ECH-associated protein 1 defense pathway. *Antioxid Redox Signal*. 2011;14(3):469–87.
33. Napetschnig J, Wu H. Molecular basis of NF- κ B signaling. *Annual Rev Biophys*. 2013;42:443.
34. Xu C, Dza B, Yla B, Wwa B, Wba B, Jiao G. **NLRP3 inflammasome and IL-1 β pathway in type 2 diabetes and atherosclerosis: Friend or foe? - ScienceDirect.**
35. Xiao B, Ai H, Wu Q, Qiang F, Wei D. Insulin Resistance Is Associated with Interleukin 1 β (IL-1 β) in Non-Diabetic Hemodialysis Patients. *Med Sci Monit Int Med J Experimental Clin Res*. 2018;24:897–902.
36. Stephens JM, Lee J, Pilch PF. Tumor necrosis factor- α -induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. *J Biol Chem*. 1997;272(2):971–6.
37. Peraldi P, Xu M, Spiegelman BM. Thiazolidinediones block tumor necrosis factor- α -induced inhibition of insulin signaling. *J Clin Invest*. 1997;100(7):1863.
38. Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor- α , overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem*. 2003;278(46):45777–84.
39. Kaneto H, Matsuoka T-a, Nakatani Y, Kawamori D, Miyatsuka T, Matsuhisa M, Yamasaki Y. Oxidative stress, ER stress, and the JNK pathway in type 2 diabetes. *J Mol Med*. 2005;83(6):429–39.
40. Han MS, Jung DY, Morel C, Lakhani SA, Kim JK, Flavell RA, Davis RJ. JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. *Science*. 2013;339(6116):218–22.

Figures

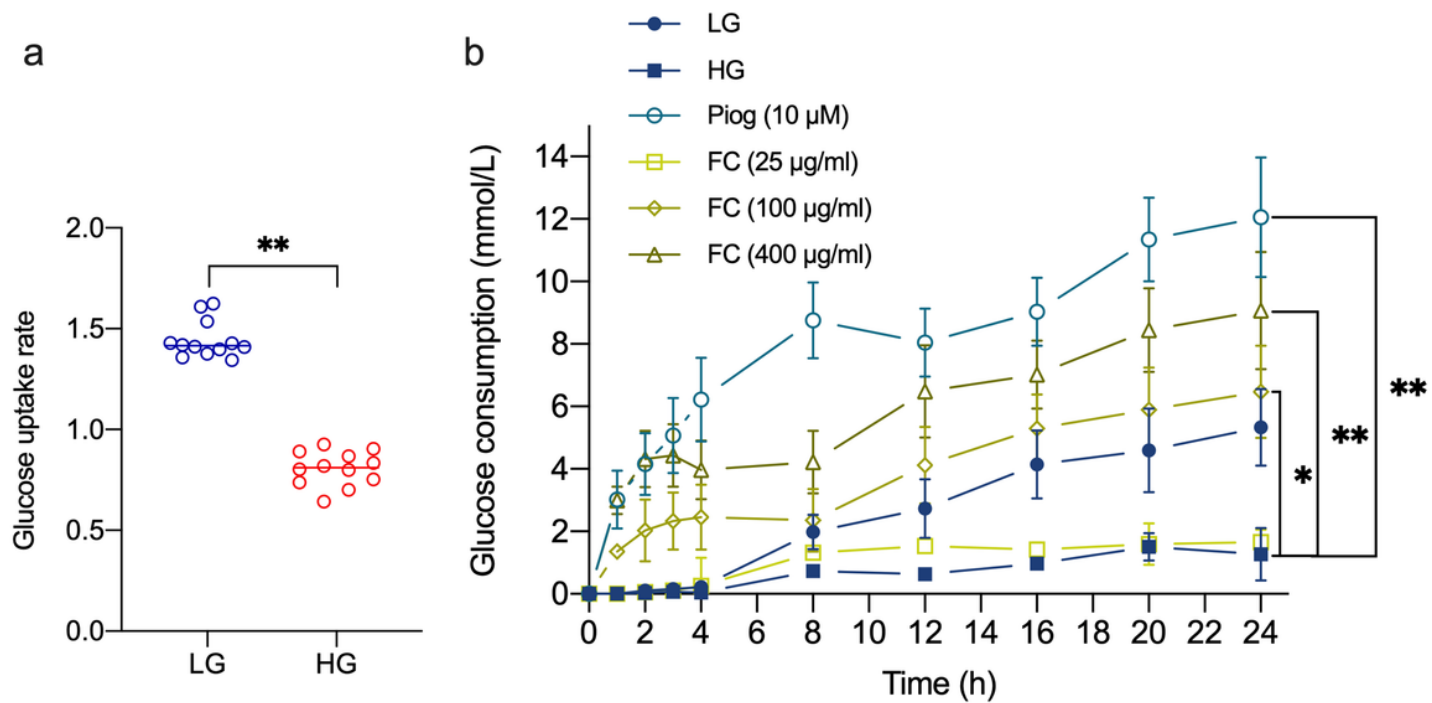


Figure 1

A, The 2-NBDG uptake rate in 3T3-L1 adipocytes. Insulin-stimulated glucose uptake was assayed with 2-NBDG. B, FC improved the glucose consumption of 3T3-L1 adipocytes with IR. Data are expressed as mean $\pm$ SEM (n=12). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$. Piog, pioglitazone; FC, flavonoid component.

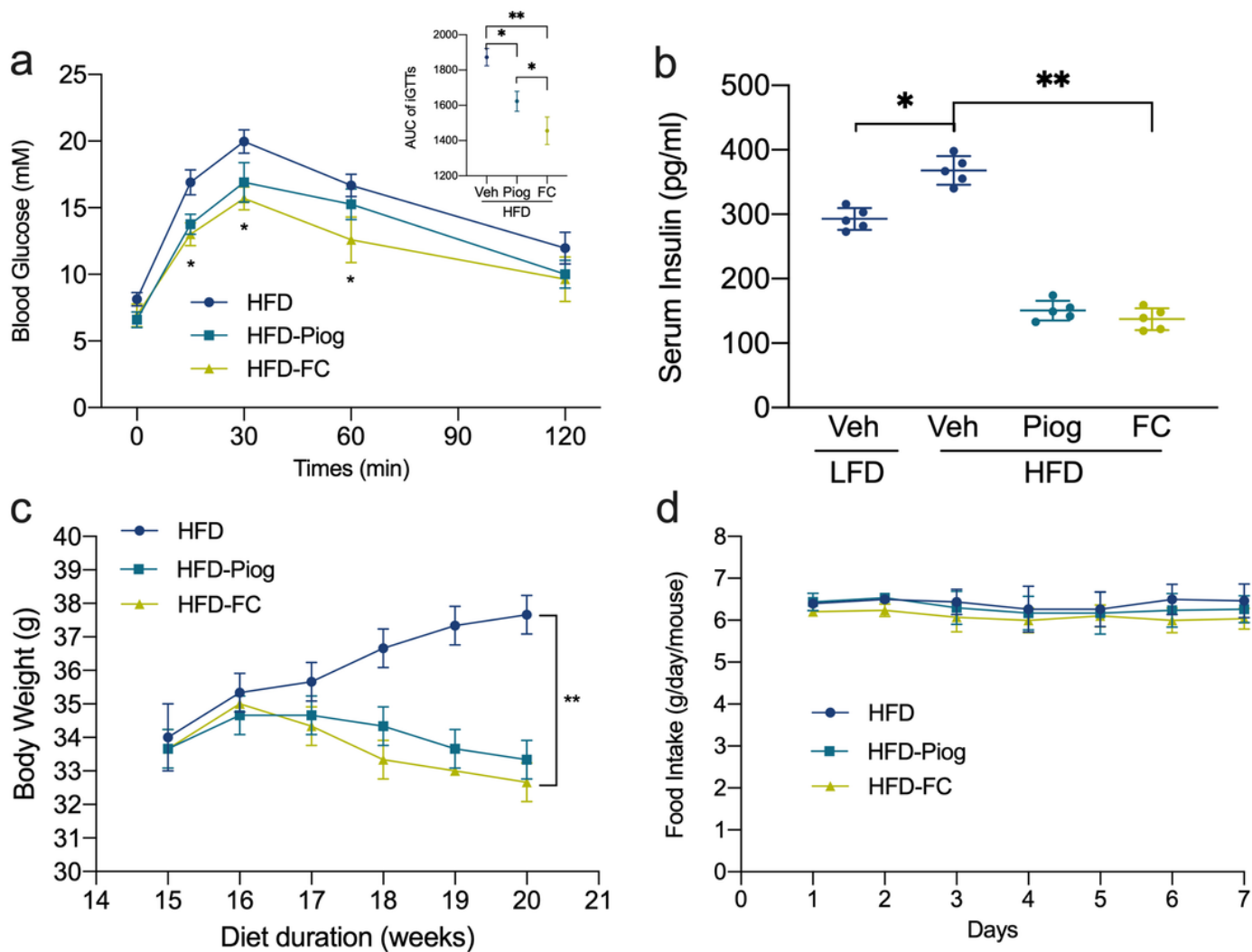


Figure 2

FC recovered the disturbed glucose metabolism and insulin resistance. iGTTs (A) were performed after starvation overnight after 20 weeks and AUC of iGTTs was calculated (A, the inserted figure). The fed serum insulin (B) after 20 weeks, body weight (C) from the 15th week to 20th week and food intake (D) in the last week were examined. Data are expressed as mean $\pm$ SEM ($n=6$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$. HFD, high fat diet; LFD, low fat diet; iGTTs, intra-peritoneal glucose tolerance tests; AUC, area under curve.

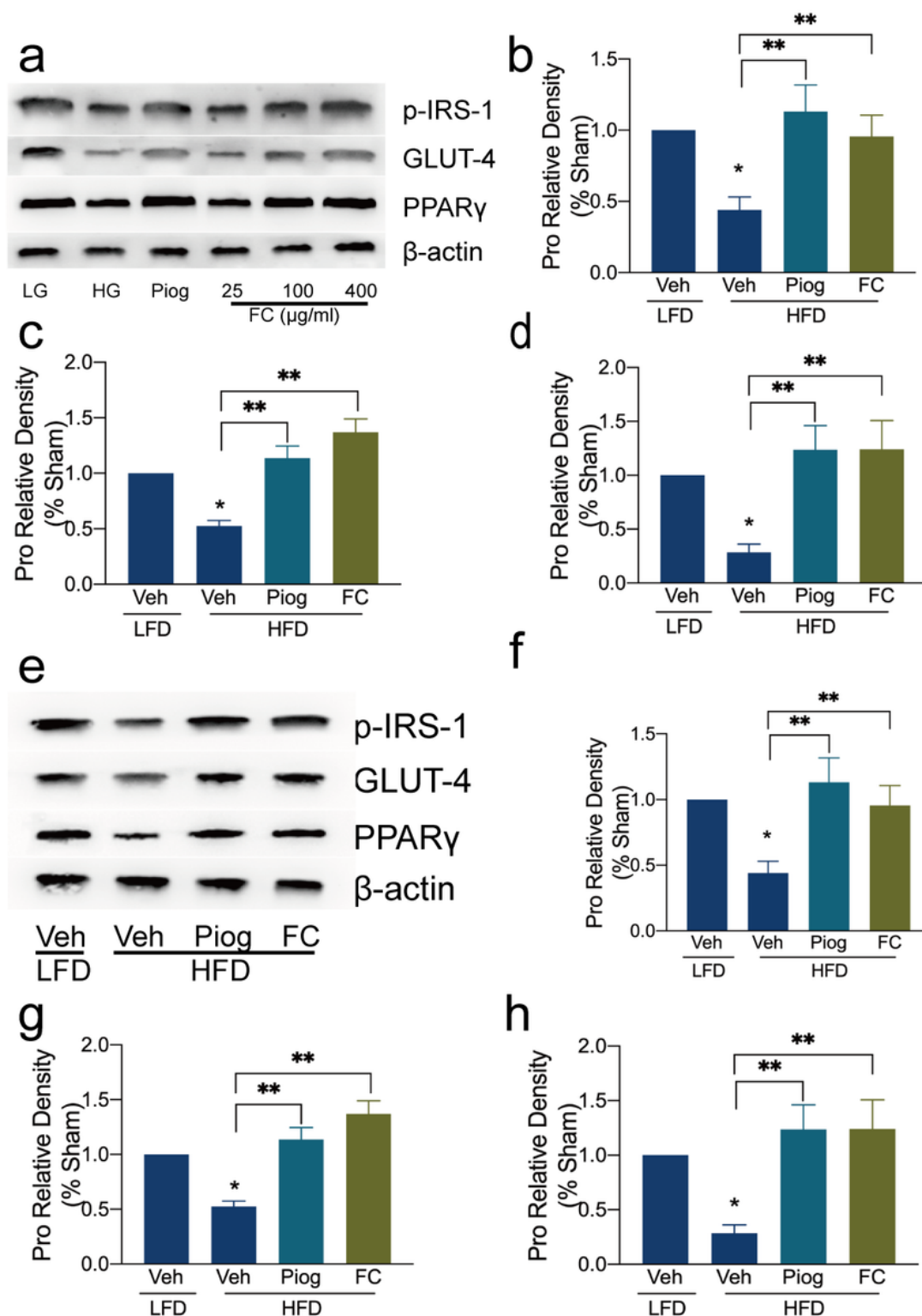


Figure 3

Effect of FC on the impaired insulin signal pathway in vitro and in vivo. A, B, E and F referred to the tyrosine phosphorylation of IRS-1 in 3T3-L1 adipocytes or C57BL/6 mice IR model. A, C, E and G referred to the expression of GLUT-4. A, D, E and H referred to the expression of PPAR γ . The column graphs were the statistical results of band intensities and the intensities were quantified by Quantity One. Data are expressed as mean $\pm$ SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

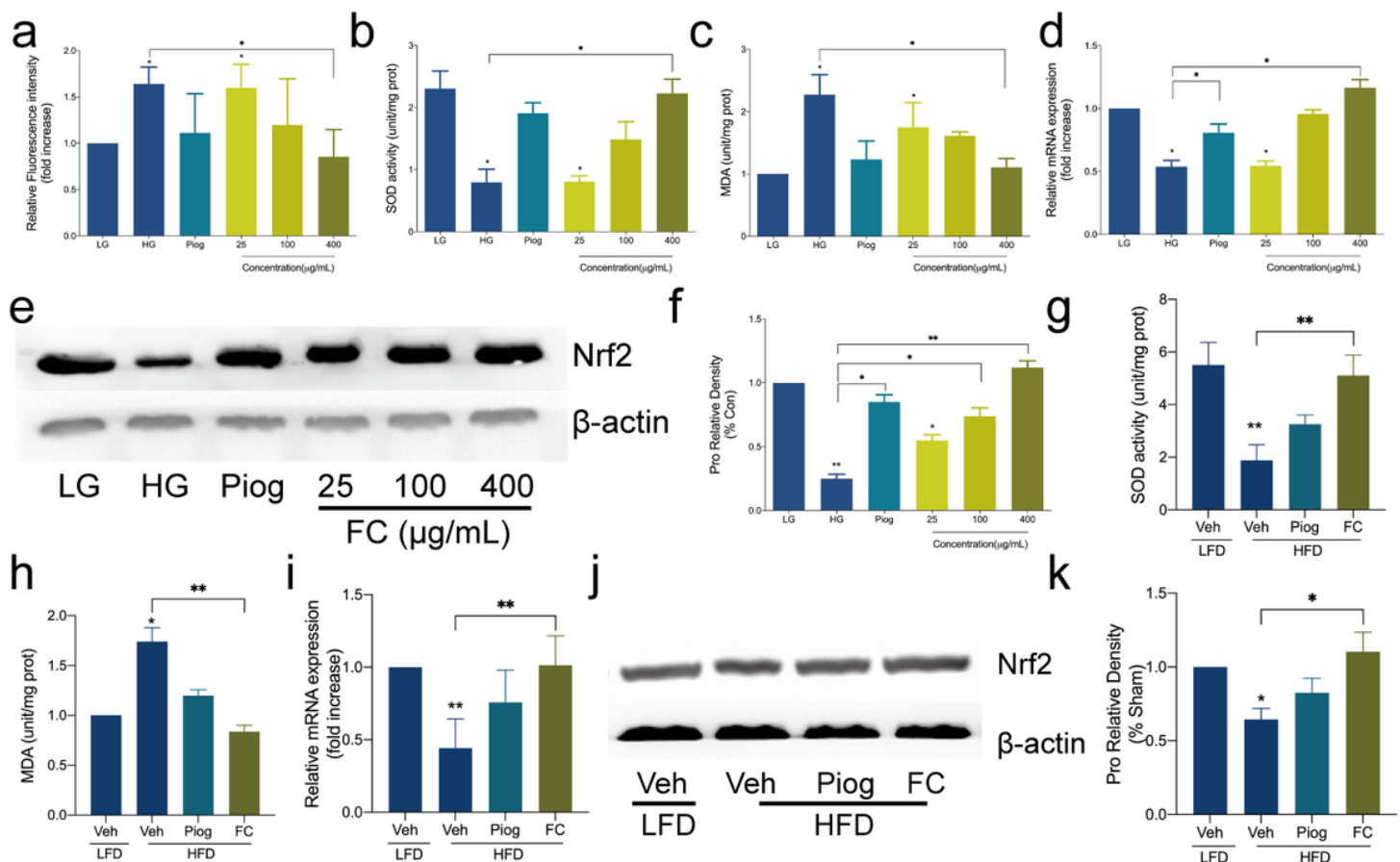


Figure 4

Effect of FC on the oxidative stress in vitro and in vivo. A referred to intracellular ROS. B and G referred to the activity of SOD. C and H referred to the content of MDA. D and I referred to the gene expression of Nrf2. E, F, J and K referred to the protein expression of Nrf2 in 3T3-L1 adipocytes or C57BL/6 mice IR model. Data are expressed as mean $\pm$ SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$. SOD, superoxide dismutase; MDA, malondialdehyde.

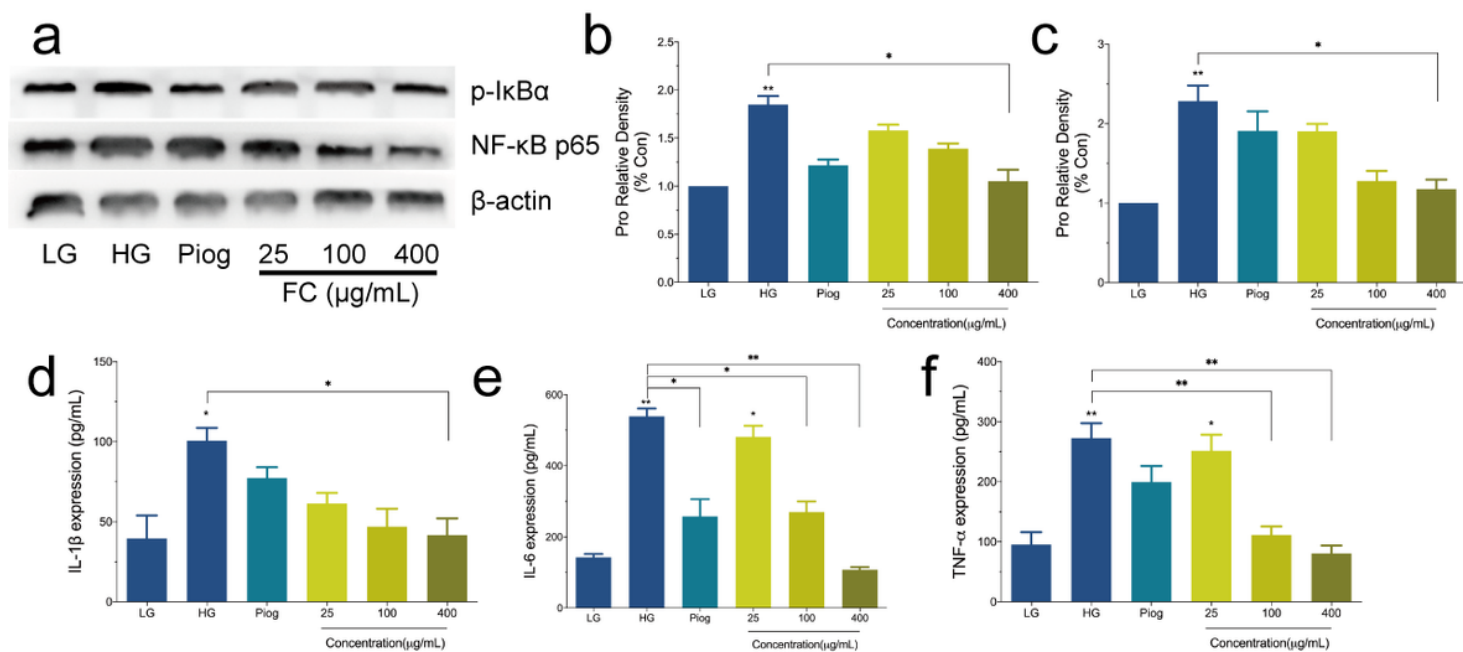


Figure 5

Effect of FC on the inflammatory stress in 3T3-L1 adipocytes IR model. A and B referred to the threonine phosphorylation of IκBα. A and C referred to the expression of NF-κB p65. D, E and F referred to the expression and secretion of IL-1β, IL-6 and TNF-α. Data are expressed as mean ± SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

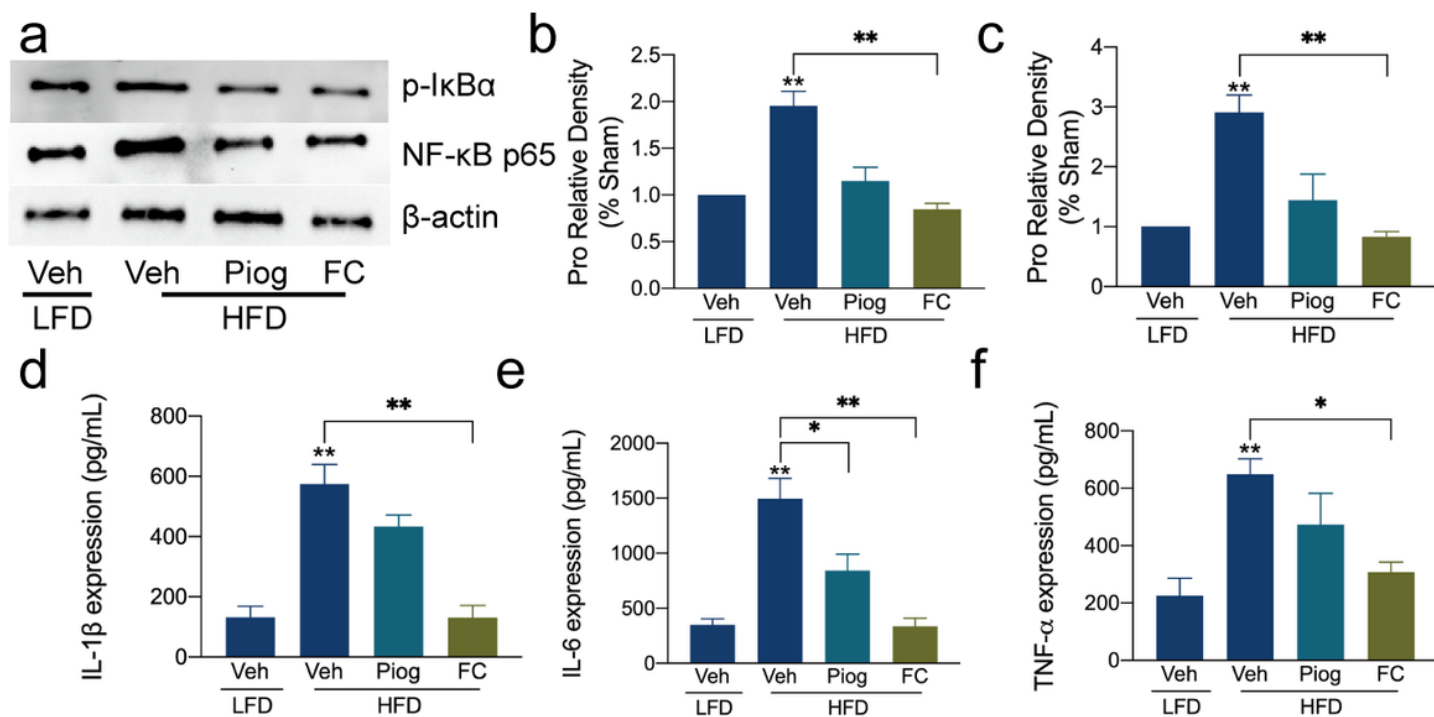


Figure 6

Effect of FC on the inflammatory stress in C57BL/6 mice IR model. A and B referred to the threonine phosphorylation of IκBα. A and C referred to the expression of NF-κB p65. D, E and F referred to the expression and secretion of IL-1β, IL-6 and TNF-α. Data are expressed as mean ± SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

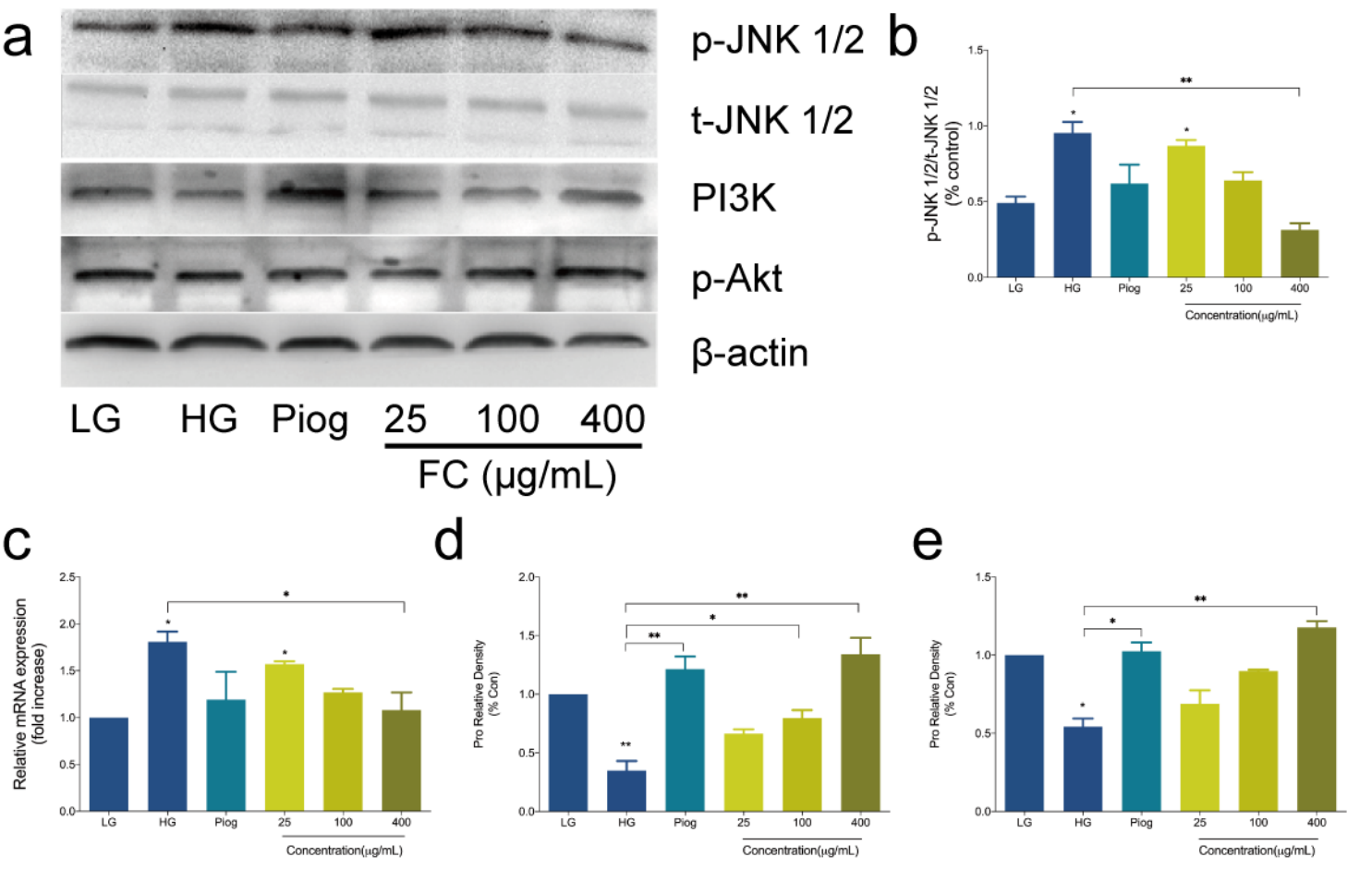


Figure 7

Empact of FC on JNK/PI3K/Akt pathway triggered by insulin resistance in adipocytes. A and B referred to the threonine phosphorylation of JNK. A and C referred to the gene expression of JNK. A and D referred to the expression of PI3K. A and E referred to the tyrosine phosphorylation of Akt. Data are expressed as mean ± SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

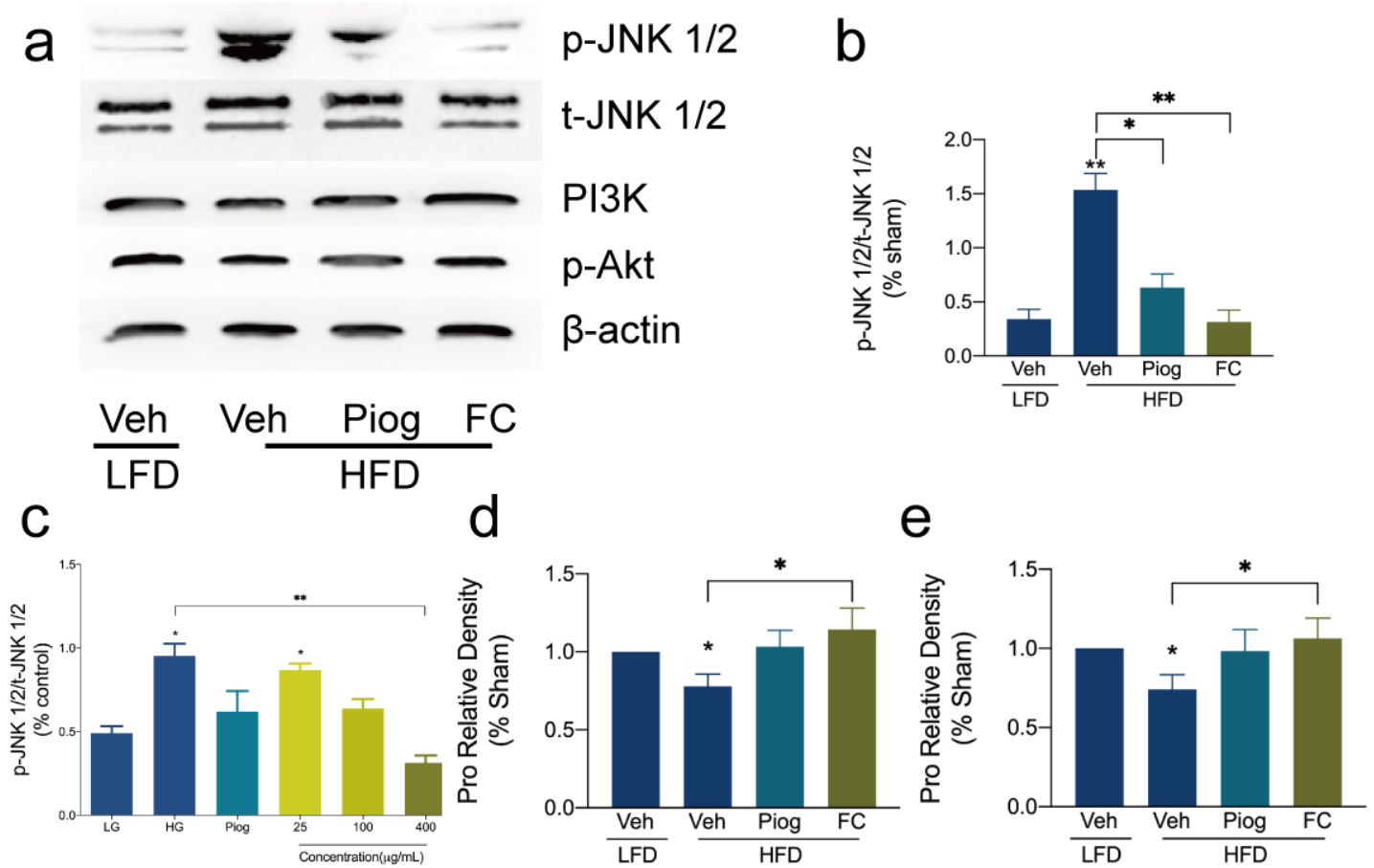


Figure 8

Effect of FC on the signal pathway in mice. A and B referred to the threonine phosphorylation of JNK. A and C referred to the gene expression of JNK. A and D referred to the expression of PI3K. A and E referred to the tyrosine phosphorylation of Akt. Data are expressed as mean $\pm$ SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

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