DA3-CH is a novel dual receptor agonist of glucagon like peptide-1 (GLP-1) and glucose dependent insulin stimulating polypeptide (GIP) [5]. The antioxidant effect of DA3-CH has been confirmed under different *in vitro* experimental conditions [6]. In myocardial cells stimulated by palmitate, GLP-1 can reduce the oxidative stress of cytoplasm and mitochondria, increase the expression of mitochondrial ATP synthase, reduce the leakage of creatine to extracellular media, and inhibit the oxidative damage of total DNA and mitochondrial DNA [7]. GIP can promote the formation of insulin vesicles and increase insulin release. In addition, it can also promote insulin synthesis and secretion through the phosphatidylinositol-3 kinase and protein kinase B pathways. However, there are few reports on the impact of DA3-CH on T2DM.

Adenylate activated protein kinase (AMPK) and acetyl CoA carboxylase (ACC) have been proved to be closely related with the occurrence, development, deterioration of Type 2 diabetes [8]. AMPK, known as a cellular energy regulator, is a key factor in regulating energy metabolism and participates in the endocrine metabolism processes of numerous target organs [9]. It can regulate glucose metabolism, lipid metabolism, and protein metabolism to maintain cellular energy homeostasis [10]. ACC plays a crucial role in the biosynthesis and metabolism of fatty acids and is a key regulator of fatty acid synthesis and oxidation pathways [11]. Therefore, regulating the expression of ACC often helps to improve fat deposition, and ACC can serve as a potential target for obesity and T2DM treatment [12]. However, if DA3-CH could improve rat type 2 diabetes through regulating AMPK/ACC signaling pathway has not been reported.

In this study, T2DM rat model was established successfully with high sugar and fat feed (67% normal food+10% lard+20% sucrose+2.5% cholesterol+ 0 5% sodium cholate) and streptomycin (STZ, 25 mg/kg) induction. Blood glucose, fat, and oxidative stress indicators were evaluated. We firstly demonstrate that DA3-CH alleviates T2DM through targeting AMPK/ ACC signaling pathway. The inhibition of apoptosis in the pancreatic tissues by DA3-CH is proved in this research. Our study might provide a novel therapeutic strategy for the prevention and treatment of T2DM through targeting DA3-CH and AMPK/ACC signaling pathway.

MATERIALS AND METHODS

Establishment of T2DM animal model

Specific pathogens free (SPF) grade male SD rats (200 ± 20) g purchased from Charles River were used in this research. The rats were raised on the condition of 23± 2° C, 35% ± 5% humidity, ventilation rate of 10-20 times/h. After adaptive feeding for 7 days, the rats were randomly divided into the following groups, control, T2DM, T2DM+DA3-CH, T2DM+Metformin (Met), and T2DM+DA3-CH+Com-C, with 10 rats in each group. The rats in the control group were fed with regular feed. The rest were given high sugar and fat feed (composition: 67% normal food+10% lard+20% sucrose+2.5% cholesterol+ 0 5% sodium cholate) for 4 weeks. Then, STZ (25 mg/kg) was injected intraperitoneally to create a model, and STZ (25 25 mg/kg) was injected again one week later. The control group received intraperitoneal injection of the same dose of normal saline. Three days after intraperitoneal injection, blood glucose was measured using a blood glucose meter. The blood glucose over 16.7 mmol/L was believed to successfully established T2DM model. The rats in the group T2DM+DA3-CH were treated with DA3-CH (10 nmol/kg) once a day through intra-peritoneal injection as described previously [13]. The rats in the group T2DM+Met were treated with Met (30 mg/kg) once a day through gavage. The rats in the group T2DM+DA3-CH+Com-C were treated with DA3-CH (10 nmol/kg) and Com-C (0.2 mg/kg) once a day through intraperitoneal injection. The rats in the control group and T2DM were treated with same amount normal saline through gavage. 12 weeks after feeding with high sugar and fat feed, the rats were sacrificed for detection.

Measurement of blood glucose, HOMA-IR, body weight, food and water consumption

The animal weight, food consumption, and water consumption, were measured at a fixed time per week. The blood was collected at week 12. Before collecting blood, all animals ceased their feed supply after 22:00 the previous day, without cutting off water, and blood were collected at 9:00 am the next day. Blood glucose was measured with a glucose meter (Roche, US) through tail vein. HOMA-IR = (Fasting Plasma Glucose (mmol/L) × Fasting Serum Insulin

Hematoxylin-eosin (HE) staining

After sacrificing animals, the pancreas was isolated. The dehydrated sample was embedded with paraffin. After pre-cooling at 20° C for 30 minutes, the tissues were cut into 5 μm. After dewaxing, the tissues were immersed in hematoxylin solution (#G1140, Solarbio, China) for 5 minutes and washed with water until no staining solution flows out. The sections were incubated with PBS for 5 minutes for returning to blue. Then, the sections were stained with eosin

(#MB9898-3, Meilunbio, China) for 15 seconds. After decolorization with 95% ethanol for 10 seconds, tissues were washed with water. Tissues were immersed in xylene for transparency for 10 minutes. Neutral gum (#MB9899, Meilunbio) sealing was performed.

Tunel staining

Tunel staining kit (#C1098, Beyotime, China) was used in this study. The tissues were prepared as described in part 2.3. The tissues were incubated with protease K working solution (#ST537-2g, Beyotime) in a 37° C incubator for 30 minutes. After washing with PBS 3 times for 5 minutes each time, the tissues were incubated with 3% hydrogen peroxide (#16J29C, Boster, China) for 20 minutes. Incubation with biotin labeling solution at 37° C for 60 minutes, and Streptavidin HRP working solution at room temperature for 30 minutes were

multiple groups, and t-test was used to analyze the results of two groups. $p < 0.05$ indicate statistical differences.

Availability of data and material

The data and material used to support the findings of this study are included within the manuscript and Supplementary Files.

RESULTS

DA3-CH greatly improves T2DM symptoms

To investigate the regulatory role of DA3-CH in T2DM. The T2DM rat model was established successfully. Significant higher fasting blood glucose (Figure 1A), HOMA-IR (Figure 1B), food (Figure 1C) and water consumption (Figure 1D), but lower weight (Figure 1E) was observed in the group T2DM. However, both treatment with DA3-CH and Met could lessen T2DM symptoms by reducing fasting blood glucose (Figure 1A), HOMA-IR (Figure 1B), food (Figure 1C) and water consumption (Figure 1D), but increasing weight (Figure 1E). These findings suggest that DA3-CH presents protective effects on T2DM.

Significant tissue injury and apoptosis in pancreatic tissues of T2DM rats were inhibited by DA3-CH

HE, Tunel, and IHC staining were performed to investigate the influence of DA3-CH on pancreatic tissue injury, apoptosis level, and Bax expression *in vivo*. Increased tissue gap and disordered

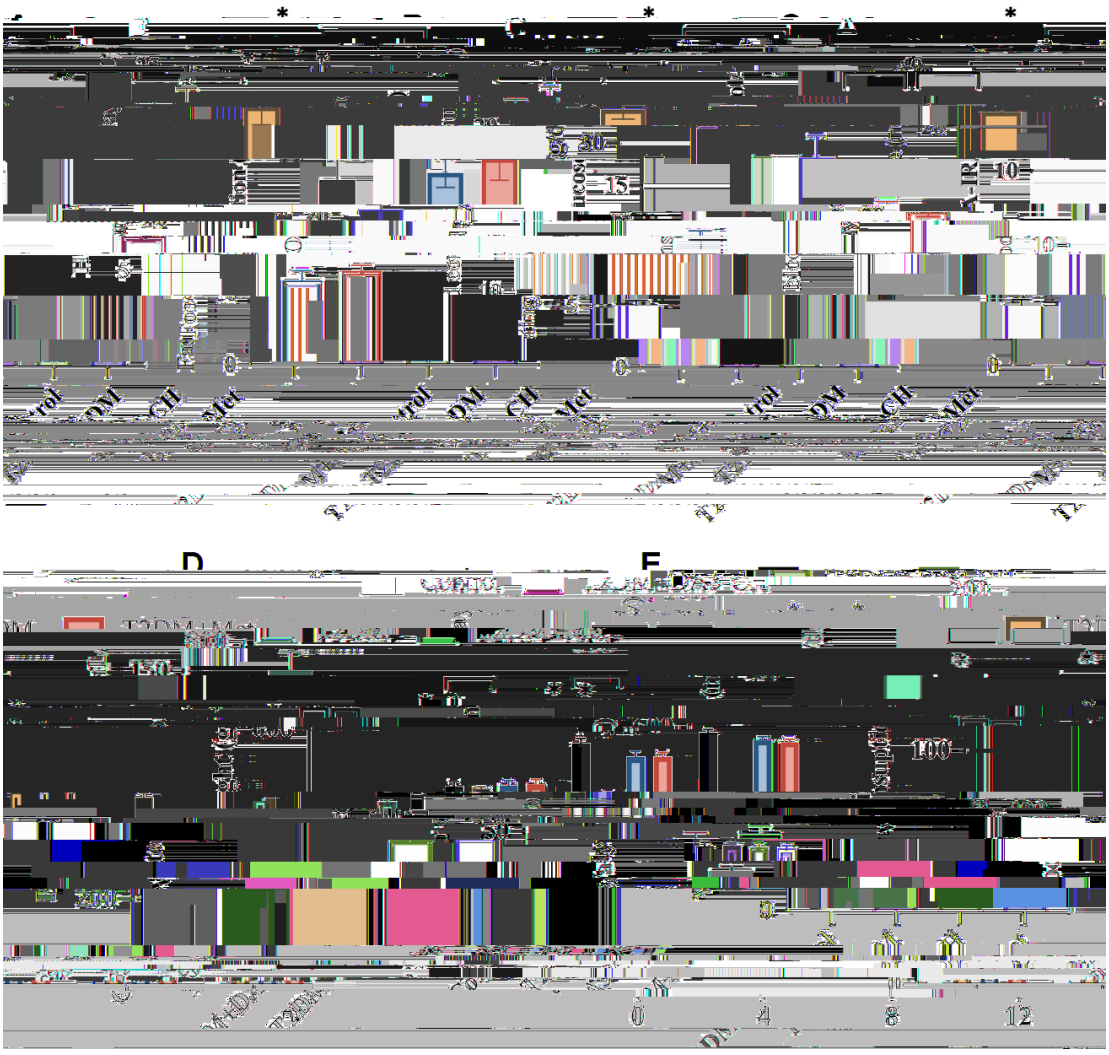

Figure 1. DA3-CH greatly improves T2DM symptoms. **A** **B** **C** **D** **E**

arrangement in the group T2DM were improved by DA3-CH and Met (Figure 2A). In addition, remarkable increase of apoptosis in pancreatic tissue (Figure 2B, 2C) and higher expression of Bax (Figure 2D, 2E) in T2DM rats were suppressed by DA3-CH, indicating that DA3-CH could suppress the pancreatic tissue injury and apoptosis *in vivo*.

DA3-CH markedly inhibited the blood fat levels and oxidative stress condition of T2DM rats

Meanwhile, we found that DA3-CH could improve the blood fat level by reducing total cholesterol (Figure 3A), triglyceride (Figure 3B), but increasing high density lipoprotein (Figure 3C). In addition, DA3-CH greatly inhibited the oxidative stress condition by promoting GSH-PX (Figure 3D), SOD (Figure

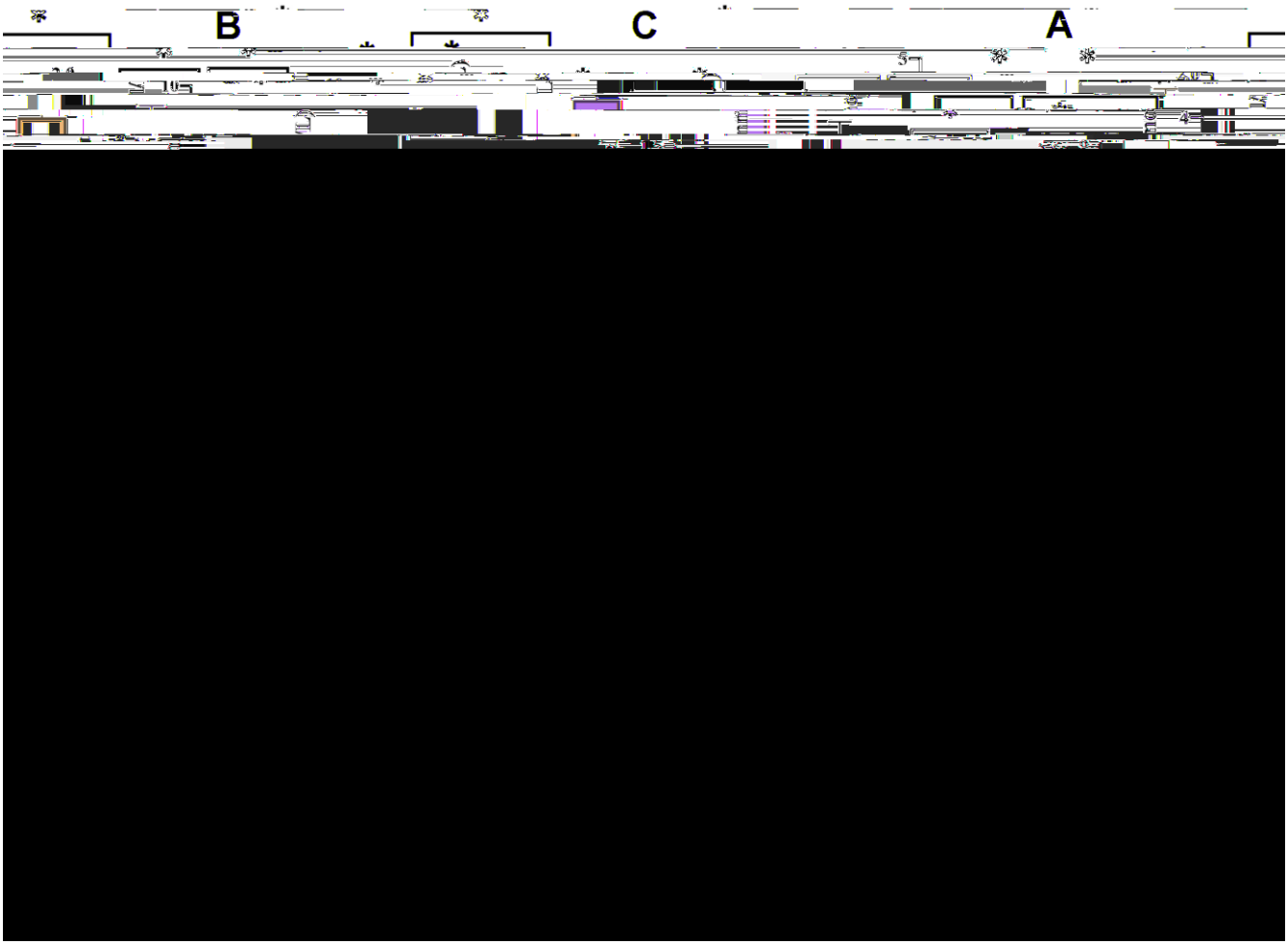

Figure 3. DA3-CH markedly inhibited the blood fat levels and oxidative stress condition of T2DM rats. A B C D E F

Figure 4. The inactivation of AMPK/ACC signaling pathway in T2DM rats was promoted by DA3-CH. A B C

DISCUSSION

GLP-1 is secreted by L cells in the lower segment of the small intestine, also known as incretin [14]. Its main biological function is to stimulate glucose mediated insulin synthesis, secretion, and inhibit glucagon -1 can increase the proliferation [15]. DA3-CH is the agonist of GLP-1.

Studies have shown that DA3-CH has a significant protective effect on cerebral ischemia-reperfusion injury with diabetes, which can improve neurological defects, reduce the area of cerebral infarction, and reduce the expression of inflammatory factors in the acute phase of cerebral ischemia-reperfusion injury [13, 16]. However, the effect of DA3-CH on the function of pancreatic tissue in T2DM rats has not investigate the inhibition effect of DA3-CH on T2DM. In this research, we firstly demonstrated that DA3-CH alleviated the pancreatic tissue injury and apoptosis of T2DM rats.

The activation of AMPK can affect the biological functions of cells, such as cell proliferation, apoptosis, growth, autophagy, and mitochondrial regulation [17]. At the same time, AMPK is also involved in obesity Metabolic syndrome and other metabolic diseases play an important role in the prevention and treatment of tumors, and can serve as potential drug targets, providing new ideas and prospects for drug research and development [18, 19].

ACC plays an important role in metabolic diseases. After treatment with ACC inhibitor, increased fatty acid oxidation, decreased fat triglycerides, increased insulin sensitivity, and reversed liver steatosis were observed in high-fat fed rats [20, 21]. However, the role of AMPK/ACC in T2DM is seldom reported. We found that AMPK/ACC signaling pathway was inhibited in T2DM, but was activated by DA3-CH and Met treatment.

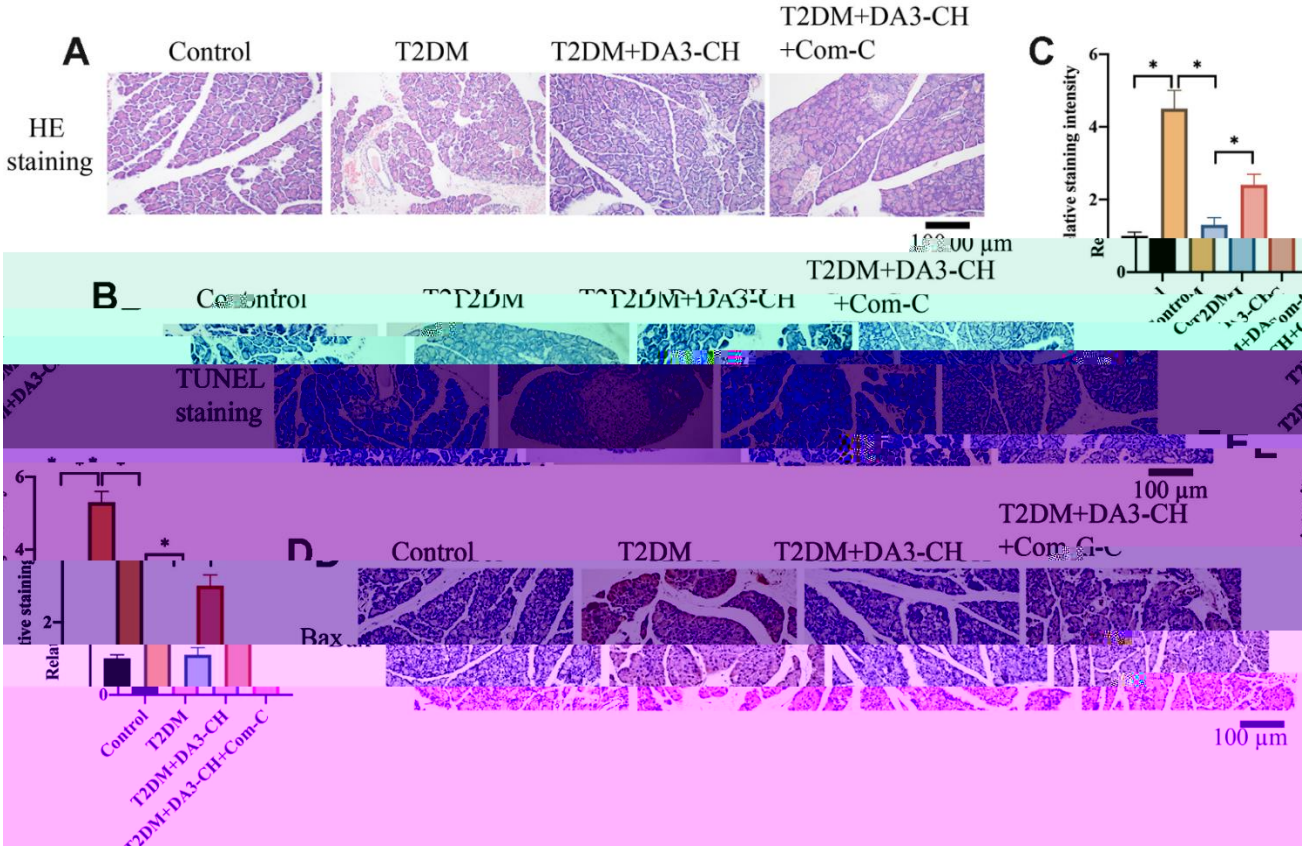

Figure 5. Inactivation of AMPK/ACC signaling pathway by Com-C significantly reversed the influence of DA3-CH on pancreatic injury. A B C D E

CONCLUSIONS

In summary, we proved that DA3-CH could greatly improve T2DM symptoms by reducing blood glucose, blood fat, pancreatic tissue injury, apoptosis, and oxidative stress condition. However, the influence of DA3-CH was significantly reversed by Com-C, the inhibitor of AMPK/ACC signaling pathway. DA3-CH might improve T2DM through targeting AMPK/ACC signaling pathway.

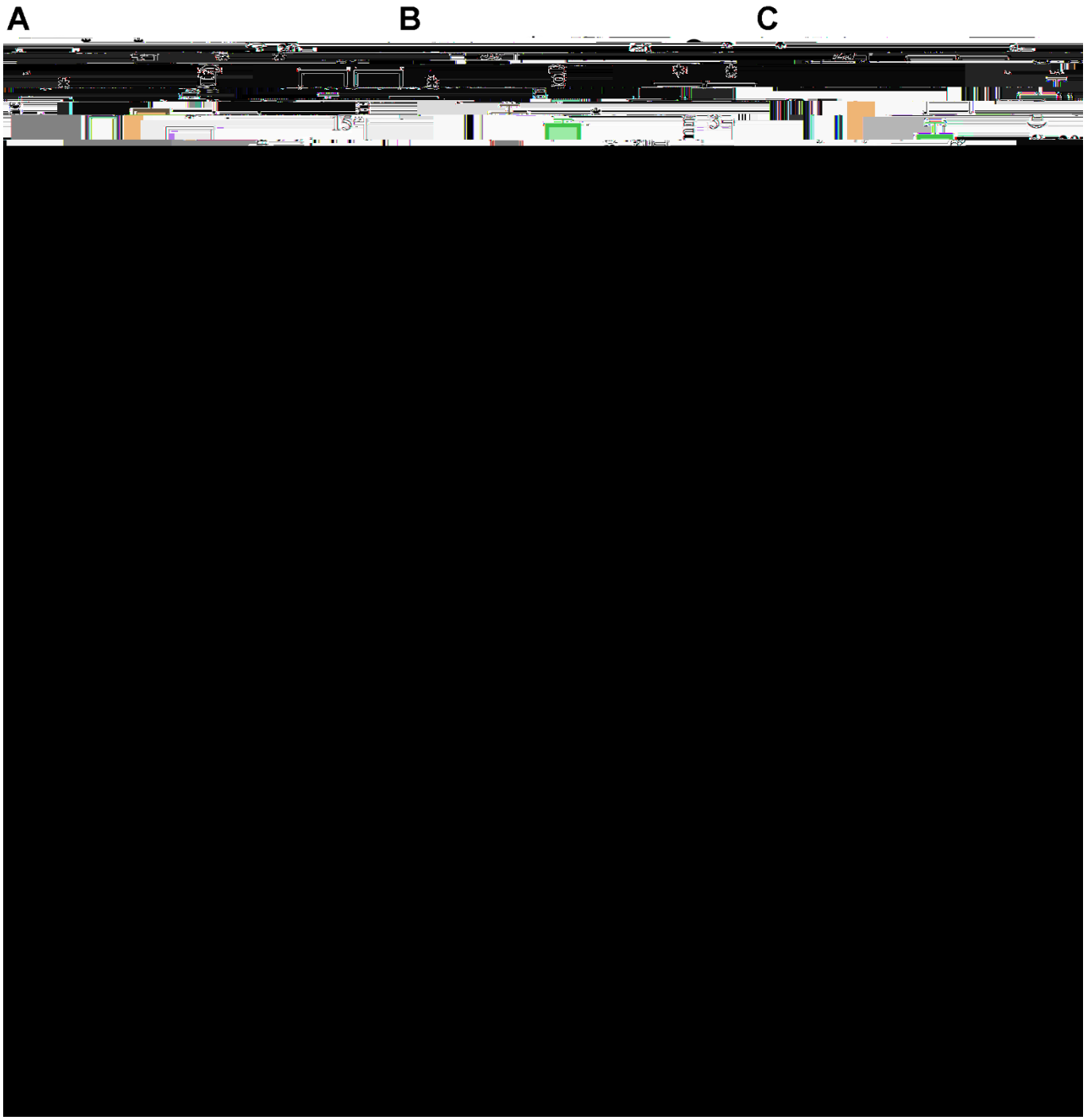

Figure 6. Inactivation of AMPK/ACC signaling pathway by Com-C significantly reversed the influence of DA3-CH on fat levels and oxidative stress condition. A B C D E F

Abbreviations

T2DM: Type 2 diabetes mellitus; GLP-1: glucagon like peptide-1; GIP: glucose dependent insulin stimulating polypeptide; AMPK: Adenylate activated protein kinase; ACC: acetyl CoA carboxylase; STZ: streptomycin; SPF: Specific pathogens free; Met: Metformin.

AUTHOR CONTRIBUTIONS

HX and JX conceived and designed the experiments; JX, PC, QZ, SC, XD performed the experiments; HX wrote the paper.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL STATEMENT

The animal experimental protocol was approved by Medical Ethics Committee of Fuzhou Second Hospital (IACUC FJABR2022026020).

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REFERENCES