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Therapy of empagliflozin plus metformin on T2DM mice shows no higher amelioration for glucose and lipid metabolism than empagliflozin monotherapy

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ABSTRACT

Aims: This study was designed to compare the effects of empagliflozin monotherapy and its combination with metformin on glucose and lipid modulations in T2DM mice.

Main methods: Nine-week-old male C57BLKS/J db/db mice (n = 32) were used as T2DM model, and their age-matched C57BLKS/J db/m mice (n = 8) were used as normal control. A total of 32 db/db mice were randomly divided into four groups (n = 8/group): the DMT1 group, treated with metformin (250 mg/kg/day); the DMT2 group, treated with metformin (250 mg/kg/day) plus empagliflozin (10 mg/kg/day); the DMT3 group, treated with empagliflozin (10 mg/kg/day); the T2DM control group (DM), received 0.5% Natrosol. The db/m mice received same administration as DM group.

Key findings: After four-week treatments, compared with T2DM control (DM), the empagliflozin or its combination with metformin dramatically increased the levels of plasma HDL-C (139.6% and 154.9%, respectively), with significant decrease in plasma TC (22.9% and 13.7%, respectively) and plasma TG (26% and 19.7%, respectively) and in hepatic TG (30.3% and 28.6%, respectively). The protein expressions of SREBP1c (75.3% and 54.0%, respectively) and APOC-III (51.2% and 50.2%, respectively) were reduced, while CPT1A (304.0% and 221.4%, respectively) and ApoA1 levels (90.0% and 85.3%, respectively) were enhanced. Although both interventions improve above-mentioned lipid homeostasis, there were no statistic differences between two groups ($p > 0.05$).

Significance: Our study demonstrated that current dose of combination therapy may have no higher amelioration than empagliflozin monotherapy for glucose and lipid metabolism in male T2DM mice when it followed a treatment shorter than that expected during clinical treatment.

1. Introduction

T2DM is a complicated metabolic dysregulation which is characterized by chronic hyperglycemia and varying degrees of insulin resistance [1,2]. Most traditional pharmacotherapy is difficult to achieve appropriate metabolic control which may attribute to the drug side effects and potential disease progression [3]. These antiglycemic drugs are sometimes accompanied by hypoglycemia and weight gain in T2DM patients when glycemia control takes effect [4]. SGLT2 inhibitors are a new class of antihyperglycemic drugs recently available for use in T2DM patients [5]. The mechanism of SGLT2 inhibitors is unique, which can maintain euglycemia by blocking the cotransport of renal

Abbreviations: T2DM, type 2 diabetes; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; AKT, protein kinase B; CPT1A, carnitine palmitoyltransferase A; SREBP1c, sterol regulatory element binding protein-1c; APOC-III, apolipoprotein CIII; ApoA1, apolipoprotein AI; PI3K, phosphoinositide 3-kinase; GSK3β, glycogen synthase kinase 3 beta; FOXO1, Forkhead box transcription factor O1; G6Pase, glucose 6-phosphatase; LDL-C, low density lipoprotein cholesterol; PEPCK, phosphoenolpyruvate carboxykinase

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sodium-glucose and excrete excess glucose through the urine rather than relying on insulin [6]. Recent studies have shown that SGLT2 inhibitors have the ability to increase the HDL-C level and to improve the hepatic steatosis in T2DM patients [7–9]. Metformin is usually proposed as the first-line oral hypoglycemic agent, and also been reported to alleviate T2DM-related hypertriglyceridaemia [10,11]. Some researchers suggest that combined therapy of empagliflozin plus metformin in certain clinical stage may have more potential to enhance glycemic control and reduce body weight than monotherapy [12,13]. Although the clinical effects of combined therapy are now firmly established, the mechanism that drugs affect both glucose and lipid homeostasis is still poorly understood. Therefore, the aim of the present study was to measure metabolic parameters in plasma and liver as well as related gene and protein expressions involved with lipid and glucose metabolism, elicited by the combination therapy of empagliflozin plus metformin or by empagliflozin monotherapy. Unexpectedly, our data showed that both combined intervention and empagliflozin monotherapy substantially improved glucose and lipid metabolism in T2DM mice, and that however, no better effects elicited by the combined intervention than by the monotherapy were observed.

2. Materials and methods

2.1. Experimental animals and treatments

The present study was conducted with the approval from the Animal Care and Use Committee of Anhui Medical University, in accordance with the International Guiding principles for Biomedical Research Involving Animals of CIOMS.

Six-week-old male C57BLKS/$J^{db/db}$ mice (n = 32) were used as T2DM model, and their age-matched male C57BLKS/$J^{db/m}$ mice (n = 8) were used as non-diabetes control, both of which were purchased from Changzhou Cavens, and those were originally from Jackson Laboratory, Bar Harbor USA. All animals were housed in a specific-pathogen-free (SPF) laboratory, and maintained within a temperature (20–26 °C) and relative humidity (50 ± 5%) controlled environment under a 12 h light/dark cycle, with ad libitum access to standard food (ingredients Protein: 27.38%; Fat:14.50%; Carbohydrate:58.12%) and water. The 24-hour food and water intake were measured every three days and took the average.

After 3 week acclimatization, a total of 32 db/db mice were randomly divided into four groups (n = 8/group): the DMT1 group, treated with metformin (250 mg/kg/day); the DMT2 group, treated with metformin (250 mg/kg/day) plus empagliflozin (10 mg/kg/day); the DMT3 group, treated with empagliflozin (10 mg/kg/day); the T2DM control group (DM), received 0.5% Natrosol (Sigma-Aldrich, USA). Meanwhile, a total of 8 db/m mice were used as the normal control group (NC), and its administration pattern were identical to DM group.

Metformin and empagliflozin (MCE, Shanghai, China) were respectively dissolved in 0.5% Natrosol and administered once per day for 4 weeks by gavage between 08:00 am and 09:00 am. The murine body weights and fasting plasma glucose (FPG) levels were measured each week.

2.2. Blood and tissue sample collection

At the end of the research, after 8 h fasting, mice were euthanized via 10% chloral hydrate anesthesia and killed by cervical dislocation. Whole blood was collected from the orbital venous plexus, and allowed it to clot by leaving it undisturbed at room temperature. Serum samples were obtained by centrifuging at 2000–3000 rpm at −4 °C for 20 min, then stored at −80 °C. After sacrifice, the liver tissues were weighed and then rinsed with cold phosphate-buffered saline (PBS). Two-thirds of livers per mice were frozen in liquid nitrogen immediately and kept at −80 °C for Immunoblotting and Biochemical analysis. The remaining livers were preserved in RNAlater for gene expression test.

2.3. Biochemical assays

The levels of plasma total cholesterol (TC; Roche, USA), triglyceride (TG; Roche, USA), low-density lipoprotein cholesterol (LDL-C; Roche, USA) in serum were detected by autoanalyzer according to the manufacturers' instructions. The levels of Hemoglobin A1c (HbA1c; Cusabio biotech Co. Ltd., Wuhan, China), high density lipoprotein cholesterol (HDL-C; Colorful Gene Biotech Co. Ltd., Wuhan, China) in serum were measured by ELISA kits. The concentrations of hepatic TG and TC (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) were analyzed by colorimetric methods according to the provided protocols.

2.4. Protein extraction and Western blotting

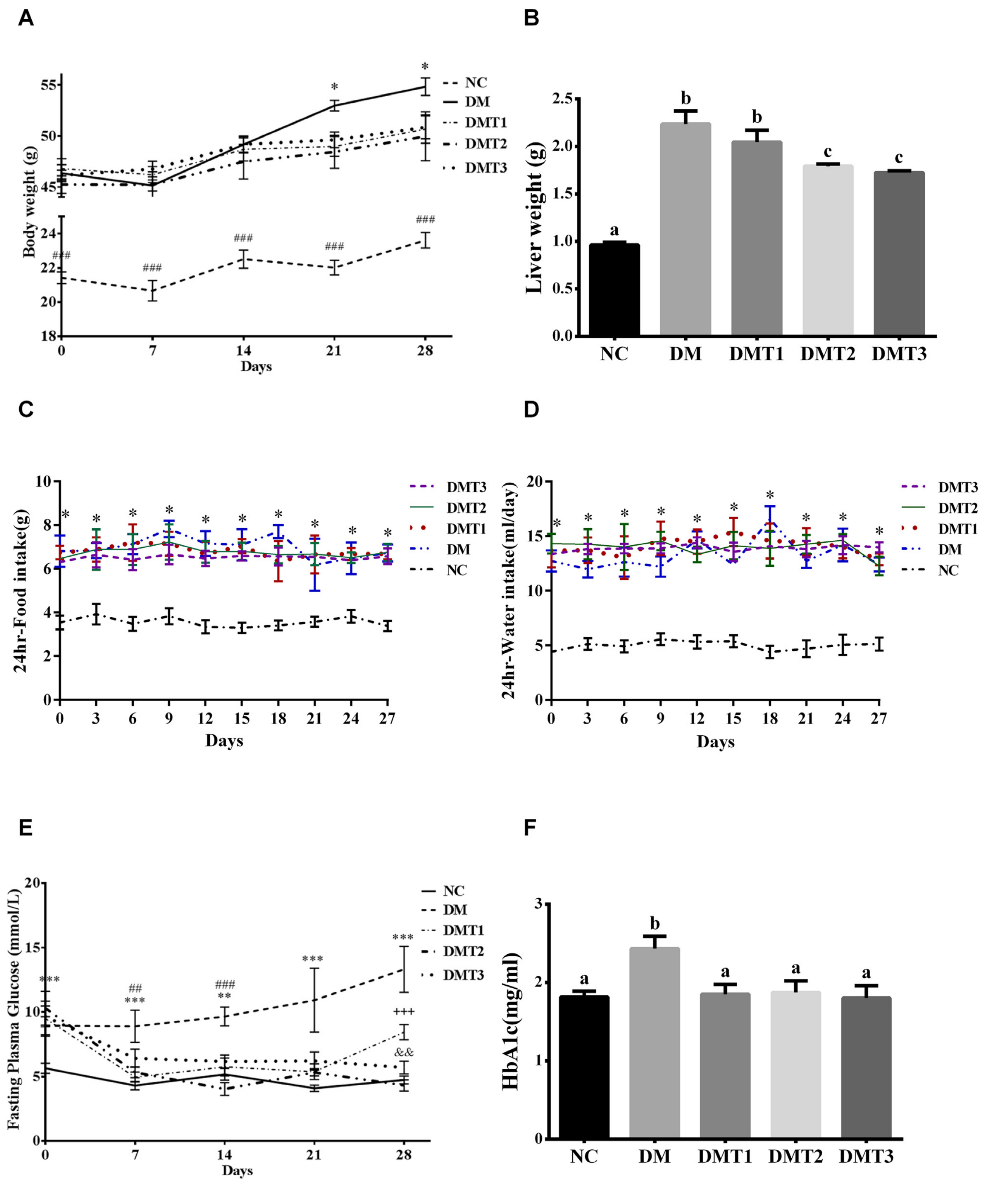
One part of the collected liver tissues was homogenized in ice-cold RIPA lysis-buffer containing 2% phosphatase inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF). The supernatants were obtained from tissue homogenates by centrifuging at 15,000 rpm at 4 °C for 15 min. Total protein concentrations were determined by bicinchoninic acid (BCA) kits. The equal amount of proteins for each lane was aliquoted, denatured and then frozen at −80 °C before running the gel. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with skim milk for 1.5 h, the PVDF membranes were incubated with appropriate primary antibody at 4 °C overnight, washed four times with Tris-buffered saline-T (TBST) buffer for 8 min/each, and then incubated with secondary antibodies at room temperature for 1–1.5 h. Targeted proteins were detected using enhanced chemifluorescence reagent and the density of the bands was quantified by scanning densitometry using Image Software. To be specific, the membranes were incubated with antiserum against AKT (1:1000, #4691S, Cell Signaling Technology Inc., USA), FoxO1 (1:1000, #2880P, Cell Signaling Technology Inc., USA), PEPCK (1:1000, #12940S, Cell Signaling Technology Inc., USA), G6Pase (1:1000, #RJ2284392, Thermo Scientific, USA), phosphor-AKT T308 (1:1000, #13038P, Cell Signaling Technology Inc., USA), phosphor-AKT S473 (1:1000, #4060P, Cell Signaling Technology Inc., USA), phosphor-FoxO1 (1:1000, #9464P, Cell Signaling Technology Inc., USA), SREBP2 (1:1000, #ab155017, Abcam, USA), SREBP1c (1:1000, #ab3259, Abcam, USA), phosphor-PI3K (1:1000, #ab182651, Abcam, USA), PI3K (1:1000, #4249T, Cell Signaling Technology Inc., USA), CPT1A (1:1000, #ab128568, Abcam, USA), GSK3β (1:1000, #12456P, Cell Signaling Technology Inc., USA), phosphor-GSK3β (1:1000, #5558P, Cell Signaling Technology Inc., USA), ApoA1 (1:1000, #ab52945, Abcam, USA), ApoB100 (1:1000, #ab20737, Abcam, USA), ApoC-III (1:1000, #ab55984). Each sample was analyzed by three independent tests involving different gels.

2.5. RNA extraction and real-time PCR

Total RNA was extracted and purified from liver tissues of mice using the Trizol reagent (TOYOBO, Japan) according to the manufacturer's instructions. 1 μg total RNA was reverse transcribed into cDNA in a final volume (26 μL) by using SYBR Green qPCR Reagent Kit (TOYOBO, Japan). The house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for internal mRNA reference. Gene expression was calculated by $2^{-\Delta\Delta Ct}$ method based on Ct values with TaqMan® software (Roche, Switzerland), normalized against the GAPDH.

2.6. Statistical analysis

All data were analyzed using SPSS 16.0 software (IBM Corporation, Armonk, USA), and presented as the mean ± SEM. Analyses of



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differences among groups were performed using one-way or two-way ANOVA, followed by Student-Newman-Keuls (SNK) multiple range test or Tukey's multiple comparison test. A value of $p < 0.05$ was considered to be statistically significant. Graphics were constructed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

3. Results

3.1. Combined therapy of empagliflozin plus metformin displayed no higher improvements than empagliflozin monotherapy for the body weights, liver weights and plasma glucose levels in T2DM mice

The influence of different drugs on the body weight of db/db mice

Fig. 1. Combined intervention of empagliflozin plus metformin indicated no higher improvements than empagliflozin monotherapy for the body weights, liver weights and plasma glucose levels in T2DM mice.
(A) Murine body weights in various groups with or without treatments. Like single intervention by empagliflozin or metformin, empagliflozin plus metformin exerted the same degree of alleviation for the body weights of diabetic mice after two weeks of treatment, which are, however, still sharply higher than those in normal control group. $^{\#\#\#}p < 0.001$ vs. NC group; $^{*}p < 0.05$, DMT1 group vs. DM group.
(B) Murine liver weights in different groups with or without treatments. Combined intervention of empagliflozin plus metformin significantly improved the murine diabetic liver weights, which was similar to the effect by empagliflozin single therapy. Each bar with different letters (a, b, c) represents statistic difference.
(C) The 24 h-food intake in various groups with or without treatments. Like T2DM control group, all treatment groups showed the same degree of food intake for 4 weeks, but which were significantly higher than that in normal control group. The food intake was measured every three days. $^{*}p < 0.05$ vs. NC group.
(D) The 24 h-water intake in various groups with or without treatments. Like T2DM control group, all treatment groups exerted the same degree of water intake for 4 weeks, but which were significantly higher than that in normal control group. The water intake was measured every three days. $^{*}p < 0.05$ vs. NC group.
(E) Fasting plasma glucose (FPG) levels showed various changes with or without treatments. Like single intervention by empagliflozin, the combined therapy elicited the same degree of inhibition for the hyperglycemia in diabetic mice after four weeks of treatment, which was very close to that in normal control group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.001$ vs. NC group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, DMT3 group vs. DM group; $^{+++}p < 0.001$, DMT1 group vs. DM group; $^{\&\&}p < 0.01$, DMT1 group vs. DMT3 group.
(F) Plasma HbA1c levels in diverse groups with or without treatments. Three different interventions demonstrated the same degree of dramatic amelioration for HbA1c in diabetic mice. Results are presented as the mean ± SEM of three independent experiments. Each bar with different letters (a, b, c) is significantly different, $p < 0.05$, (ANOVA, analyzed by Tukey or SNK). NC: normal control group, DM: T2DM (db/db) control group, DMT1: db/db mice treated with metformin group, DMT2: db/db mice treated with empagliflozin plus metformin group, DMT3: db/db mice treated with empagliflozin group.

was shown in Fig. 1A. The T2DM mice did clearly have higher body weight relative to NC group ($p < 0.001$), nonetheless, three drugs could significantly and equally lower the all murine body weights after two weeks of treatments ($p < 0.05$). Meanwhile, DM group showed incremental liver weights compared with NC group, whereas they were identically attenuated by empagliflozin or combined therapy (Fig. 1B, $p < 0.05$). On the contrary, metformin monotherapy had no similar effect ($p > 0.05$). Moreover, all therapies showed no alleviation on food or water intake in db/db mice, which were, however, significantly higher than that in NC group (Fig. 1C and D, $p < 0.05$). It suggested that the loss of liver weight in db/db mice treated with empagliflozin may not be related to food or water intake. As compared with normal control, T2DM mice were characterized by fasting hyperglycemia (Fig. 1E, $p < 0.001$). It should be pointed out that all interventions corrected the FPG levels of db/db mice after one-week of treatment ($p < 0.01$). Moreover, the effect of the improvement was more obvious after two or three weeks of treatment ($p < 0.01$). At the end of this study, the results showed that the FPG levels of the mice treated with metformin were much lower than that of DM group ($p < 0.01$), while the reduction degrees were higher in DMT2 and DMT3 groups, which were close to that in NC group ($p > 0.05$). In addition, we also analyzed HbA1C levels in plasma which were significantly increased in DM group, but they were markedly decreased by all therapies (Fig. 1F, $p < 0.05$).

3.2. Empagliflozin or its combination with metformin vastly and equally increased plasma HDL-C levels, and decreased hepatic triglycerides in T2DM mice

In order to assess the effects of different drug interventions on lipid metabolism under T2DM conditions, we determined the levels of TC, TG, HDL-C, LDL-C in murine plasma and the contents of TC and TG in the livers from db/db mice. Upon comparison with NC group, plasma TC, TG and LDL-C levels as well as hepatic TG level were significantly higher, whereas the plasma HDL-C levels were quite opposite in DM group (Fig. 2A–D and F, $p < 0.05$). We also noticed that the hepatic TC levels remained the same in all groups (Fig. 2E, $p > 0.05$).

The empagliflozin alone and the combined treatment resulted in the lowered plasma TC levels and hepatic TG levels compared to those in DM and DMT1 groups (Fig. 2A and F, $p < 0.05$), while plasma HDL-C levels were vastly enhanced (Fig. 2C, $p < 0.05$). However, Fig. 2D displayed that all interventions had no impact on plasma LDL-C ($p > 0.05$). In comparison with DM group, all interventions showed a significant inhibition on plasma TG levels (Fig. 2B, $p < 0.05$). Furthermore, as shown in Fig. 2C and F, the intervention impacts in DMT2 and DMT3 groups were markedly better than those in DMT1 group, but there was no significant difference between DMT2 and DMT3 therapies ($p > 0.05$), suggesting the similar intervention effects for lipid metabolism in T2DM mice induced by empagliflozin and its combination with metformin.

3.3. The empagliflozin and its combination with metformin significantly and equally inhibited the hepatic fatty acid synthesis, promoted its degradation and enhanced the apoA1 expression in T2DM mice

To further explore whether the changes of gene transcription and protein synthesis caused by the three therapies were associated with lipid homeostasis in the livers from db/db mice, we respectively examined the murine hepatic SREBP1c, SREBP2 and CPT1A protein levels. Our data clearly demonstrated that the $SREBP_1C$ protein levels were raised drastically in DM group, and that treatment with empagliflozin or the combination of empagliflozin plus metformin substantially reduced SREBP1c levels, although the inhibitions elicited by metformin monotherapy or combined intervention were more obvious and closer to NC group than others (Fig. 3A, $p < 0.05$). In addition, the hepatic CPT1A levels were completely suppressed by the T2DM in DM group, and the empagliflozin or its combination with metformin dramatically increased the CPT1A levels in diabetic murine livers (Fig. 3B, $p < 0.05$), but metformin single intervention did not improve this protein level (Fig. 3B, $p > 0.05$). Given these effects, we can see that the empagliflozin or its combination with metformin significantly lowered the diabetic murine liver weights (Fig. 1B, $p < 0.05$), while metformin intervention did not improve the fatty livers in T2DM mice (Fig. 1B, $p > 0.05$). Additionally, it is worth mentioning that the expression of SREBP2 was not affected by all drug therapies, which was consistent with hepatic TC data (Figs. 2E and 3C). On the contrary, the APOC-III protein levels have been dramatically curbed by all interventions, which match up with plasma TG data (Figs. 2B and 3E).

In addition, we detected the mRNA and protein expressions of apoA1 and apoB100 in the murine livers by western blotting and quantitative RT-PCR, respectively. We discovered that the mRNA and protein levels of apoA1 have dropped dramatically in diabetic livers in DM group compared with NC group, whereas empagliflozin or combined intervention of empagliflozin plus metformin tremendously reversed the decline, but there was no statistical difference between the two intervened groups (Fig. 3D and E, $p < 0.05$). In contrast, none of three interventions or diabetes itself could change the hepatic apoB100 expression compared to the healthy control mice (Fig. 3D and F, $p < 0.05$).

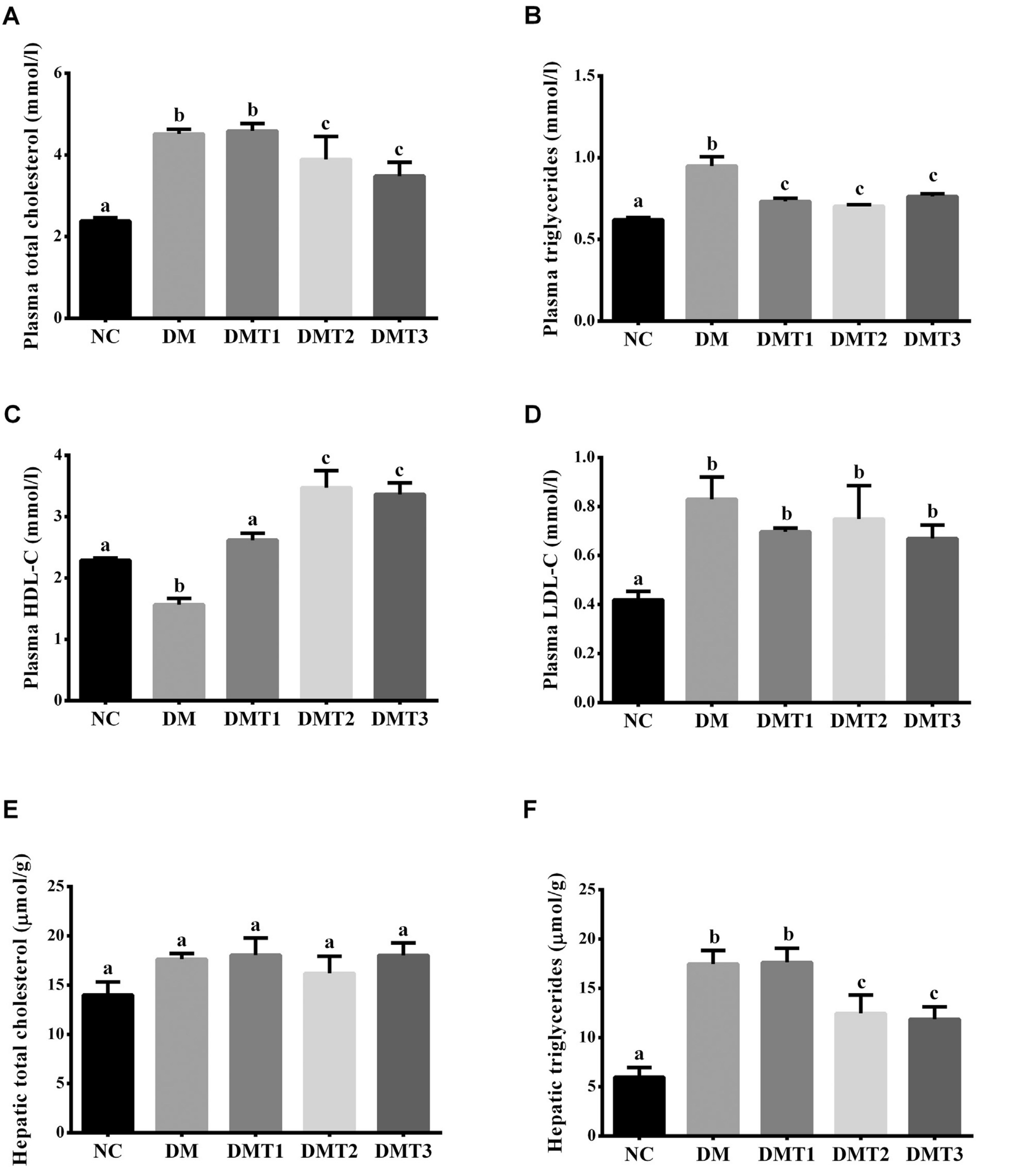

Fig. 2. After 4-week treatment, combined intervention of empagliflozin plus metformin sharply ameliorated the lipid metabolism in db/db mice, which was similar to the effect by empagliflozin single therapy.
(A) Plasma total cholesterol (TC) levels of five different groups. (B) Plasma triglycerides (TG) levels of five different groups. (C) Plasma HDL-C levels of five different groups. (D) Plasma LDL-C levels of five different groups. (E) The murine liver TC contents in five different groups. (F) The murine liver TG contents in five different groups. Results are presented as the mean ± SEM. Each bar with different letters (a, b, c) represents statistic difference, $P < 0.05$ (ANOVA, analyzed by Tukey or SNK). NC: normal control group, DM: T2DM (db/db) control group, DMT1: db/db mice treated with metformin group, DMT2: db/db mice treated with empagliflozin plus metformin group, DMT3: db/db mice treated with empagliflozin group.

3.4. Three drug therapies respectively normalized the hepatic insulin sensitivity in db/db mice

To explore whether empagliflozin or its combination with metformin can improve insulin resistance in T2DM mice, we investigated the phosphorylation levels of several components of the hepatic insulin–PI3K–AKT signaling pathway. We found that although T2DM sharply inhibited the PI3K phosphorylation levels, the PI3K activity was normalized respectively by the three drug treatments compared with healthy control group (Fig. 4A, $p < 0.05$). Furthermore, the ratios of p-

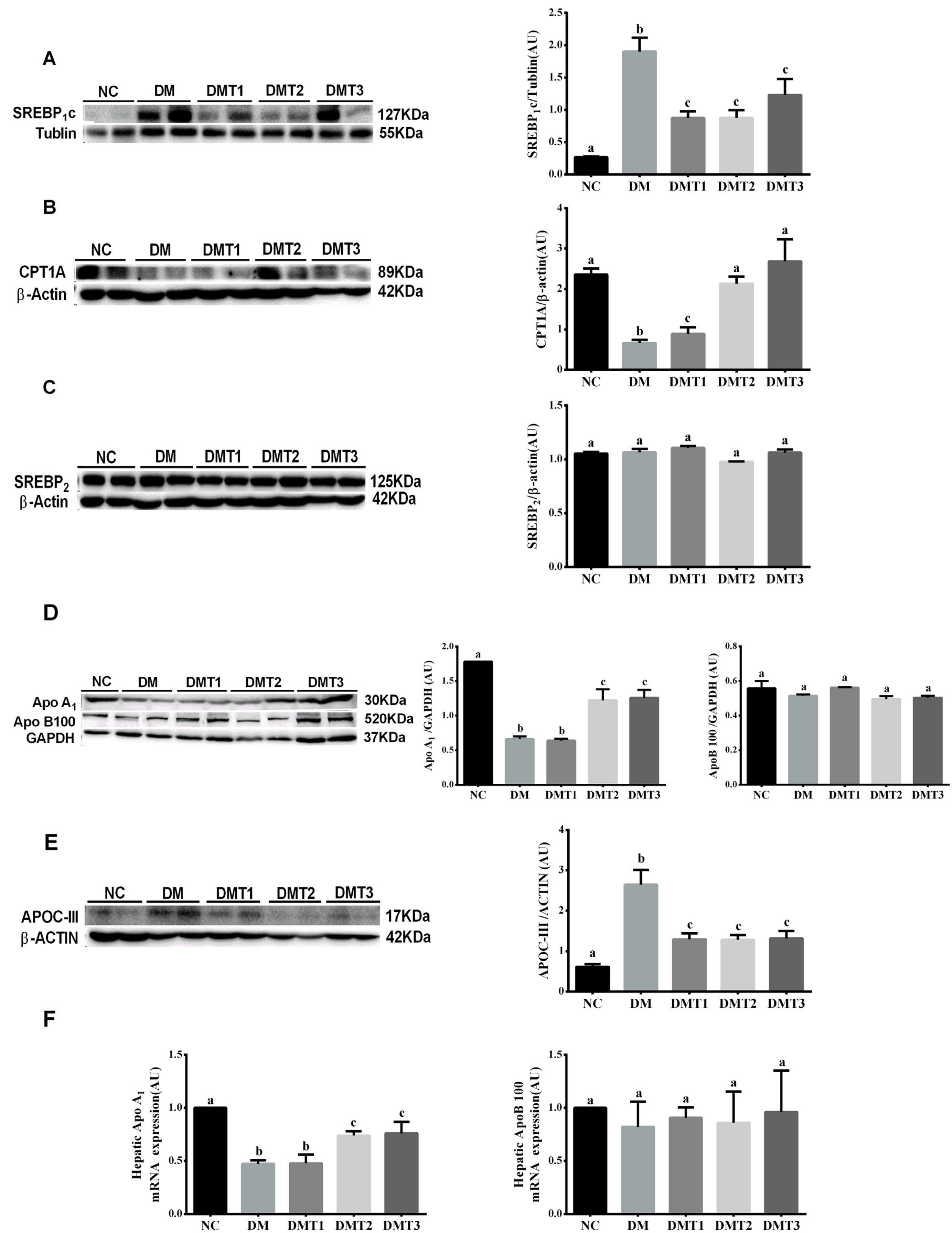

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Fig. 3. Like single intervention by empagliflozin, combined intervention of empagliflozin plus metformin elicited the same degree of improvement for the hepatic lipogenesis in diabetic mice. (A) The protein levels of SREBP1 under various interventions were determined by immunoblotting and quantified by tubulin. (B) The protein levels of CPT1A under various interventions were determined by immunoblotting and quantified by β-actin. (C) The protein levels of SREBP2 under various interventions were determined by immunoblotting and quantified by β-actin. (D) ApoA1 and ApoB100 expression levels in the liver of db/db mice were detected by immunoblotting and quantified by GAPDH respectively. (E) The protein levels of APOC-III under various interventions were determined by immunoblotting and quantified by β-actin. (F) The hepatic apoA1 and apoB100 mRNA expression levels were examined by quantitative RT-PCR. Results are presented as the mean ± SEM of three independent experiments. Each bar with different letters (a, b, c) represents statistic difference, $p < 0.05$ (ANOVA, analyzed by Tukey or SNK). NC: normal control group, DM: T2DM control group, DMT1: db/db mice treated with metformin group, DMT2: db/db mice treated with empagliflozin plus metformin group, DMT3: db/db mice treated with empagliflozin group.

PI3K to total PI3K indicated the even greater increase in PI3K activity elicited by the empagliflozin monotherapy compared to that in the NC group (Fig. 4A, $p < 0.05$).

Next, we examined the PI3K downstream targets AKT (Thr308, Ser473) and FoxO1. As expected, we observed that the ratios of p-AKT (S473) and p-AKT (T308) to total AKT were remarkably lower in DM group than those in the NC group, and that the ratios of p-AKT (T308) to total AKT were drastically enhanced by three respective drug therapies (Fig. 4B, $p < 0.05$). Intriguingly, the elevated phosphorylation levels of p-AKT (T308) induced by combined therapy of empagliflozin plus metformin were even significantly lower than those by empagliflozin or metformin monotherapy (Fig. 4B, $p < 0.05$). Meanwhile, the ratios of p-AKT (S473) to total AKT were totally and equally normalized by all three respective treatments (Fig. 4C).

Subsequently, we explored the FoxO1 phosphorylation by the three treatments. As shown in Fig. 4D, although T2DM enhanced the total protein levels of FoxO1, the ratios of p-FoxO1 to FoxO1 were significantly reduced in DM group, indicating the remarkably elevated FoXO1 activity in diabetic murine livers. However, empagliflozin alone or its combination with metformin normalized the FoxO1 activity (Fig. 4D, $p < 0.05$), demonstrating the similar correction effects for insulin sensitivity in T2DM mice exerted by the monotherapy and the combined therapy.

3.5. *Empagliflozin monotherapy exhibited most effective inhibition of hepatic gluconeogenesis in db/db mice*

In order to explore whether the three respective interventions are effective in inhibiting abnormal gluconeogenesis in T2DM mice, we examined murine hepatic PEPCK and G6Pase levels by Western blotting. Fig. 5A demonstrates that the protein expression levels of PEPCK were sharply increased in diabetic livers in DM group compared with the NC group ($p < 0.05$). In contrast, the treatment with metformin or combined drugs of empagliflozin plus metformin dramatically suppressed the PEPCK elevation in T2DM mice (Fig. 5A, $p < 0.05$). Moreover, the empagliflozin monotherapy even totally normalized the PEPCK levels compared with that in healthy control mice (Fig. 5A). Likewise, the diabetes itself sharply increased the hepatic G6Pase levels. The metformin or its combination with empagliflozin completely normalized the G6Pase levels (Fig. 5B, $p < 0.05$). In particular, empagliflozin monotherapy even best inhibited the hepatic G6Pase levels compared with the two other interventions (Fig. 5B, $p < 0.05$). We also detected the activity of GSK3β, which plays a vital role in glycogen synthesis. Compared to normal control, the ratios of p-GSK3β to GSK3β were remarkably lessened in DM group, suggesting the enhanced GSK3β activity or decreased glycogen synthesis in diabetic murine livers. However, the decreases were normalized by empagliflozin or combined therapy. But metformin monotherapy did not show the same degree of the inhibition for GSK3β activity, although the significant increase in phosphorylated GSK3β was also found by its treatment (Fig. 5C). Owing to these effective inhibitions, the hyperglycemia in T2DM mice was remarkably improved by the three respective drug therapies starting from one-week treatment (Fig. 1C). Similarly, the blood HbA1C was totally normalized by three interventions as well (Fig. 1D).

4. Discussion

The T2DM patients are frequently accompanied with lipid dysregulation. This metabolic abnormality is owing to multiple mechanisms. On the one hand, the insensitivity of insulin weakens its antilipolytic ability in adipose tissue to curb the release of fatty acid into plasma. On the other hand, hyperinsulinemia promotes up-regulation of the transcription factors related to de novo lipogenesis, leading to the lipid accumulation in the diabetic livers [14]. In the present study, our findings suggest that all the three interventions substantially mitigated the insulin resistance in db/db mice, and that these effects were realized by regulating the critical proteins involved in the insulin signaling, such as PI3K, AKT, and FoxO1. This is understandable for metformin function in livers which was supported by published literature [15,16], but the empagliflozin's function in liver has not been totally clear. Previous studies demonstrated that in male ZDF rats or db/db mice subjected to a euglycemic–hyperinsulinemic clamp, treatment with empagliflozin might completely recover insulin sensitivity at the whole-body level and in insulin-sensitive tissues [17,18]. According to our current data, we assume that improvements in insulin sensitivity in diabetic liver by empagliflozin resulted from a sustained reduction in hyperglycemia and decreases in glucose toxicity. On the other hand, the empagliflozin could augment the glucose sensitivity in beta cells, thereby improving beta cell function in T2DM patients [19], presumably, the similar impacts elicited by empagliflozin may exert on mice.

Another unexpected result from the present study is that the combination therapy of metformin plus empagliflozin did not show the added effects than empagliflozin single therapy. The explanation for this may be due to the different functional mechanisms exerted by empagliflozin and metformin, respectively. The previous studies indicated that metformin might improve hepatic insulin sensitivity via inhibiting the glycogenolysis and gluconeogenesis, but this usually occurs without influencing hepatic fat contents under T2DM condition [20], which is consistent with our current results. But how the empagliflozin improves the hepatic insulin signaling is an unresolved question so far. Our current data may shed a light into this question.

Hepatic gluconeogenesis plays a key role in maintaining glucose homeostasis during fasting under normal physiology, which is inappropriately activated by T2DM [21,22]. Previous studies suggested that the long-term empagliflozin treatment might attenuate the diabetes-induced increase in liver gluconeogenesis [23], but there is no study focusing on the effect of combined therapy of empagliflozin plus metformin. PEPCK and G6Pase are involved in the rate-limiting steps to control gluconeogenesis. In this study, we demonstrated that the two key protein levels were dramatically repressed by the three respective treatments in T2DM mice, and that empagliflozin single intervention most effectively improved the levels (Fig. 4A and B). The reason may be probably that empagliflozin might recover the insulin sensitivity in diabetic livers [24], thereby reducing the nuclear localization of FoxO1 to inhibit gluconeogenesis. Meanwhile, lipids may regulate hepatic gluconeogenesis by acting as metabolic substrates [25]. Under the double influence of insulin signaling and lipid, empagliflozin may function better than metformin monotherapy, which is consistent with our data. Given that the concrete mechanisms of metformin or empagliflozin to improving gluconeogenesis have not been totally

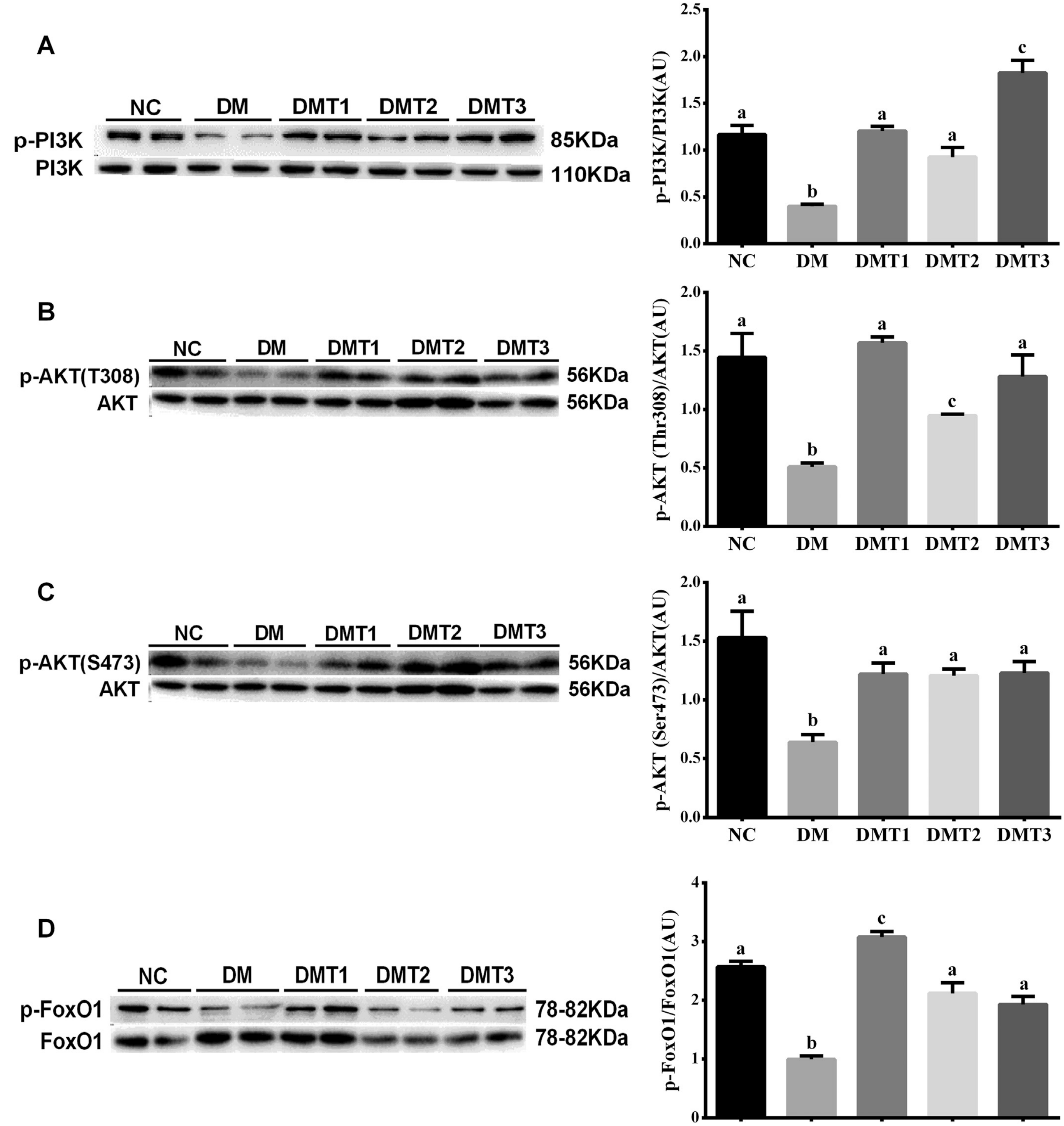

Fig. 4. Combined therapy of empagliflozin plus metformin showed no higher amelioration for hepatic insulin signaling in T2DM mice than empagliflozin single intervention. (A) Displayed are the protein levels of PI3K and p-PI3K. The phosphorylated PI3K was quantified by total PI3K. (B) The protein levels of AKT and p-AKT T308 were revealed by immunoblotting, and the AKT Thr308 phosphorylation was quantified by total AKT. (C) The protein levels of AKT and p-AKT S473 are indicated by immunoblotting, and AKT Ser473 phosphorylation was quantified by total AKT. (D) The protein levels of FoxO1 and p-FoxO1 are demonstrated by immunoblotting, and the phosphorylated FoxO1 was quantified by total FoxO1. Results are presented as the mean ± SEM of three independent experiments. Each bar with different letters (a, b, c) represents statistic difference, $p < 0.05$ (ANOVA, analyzed by Tukey or SNK). NC: normal control group, DM: T2DM control group, DMT1: db/db mice treated with metformin group, DMT2: db/db mice treated with empagliflozin plus metformin group, DMT3: db/db mice treated with empagliflozin group.

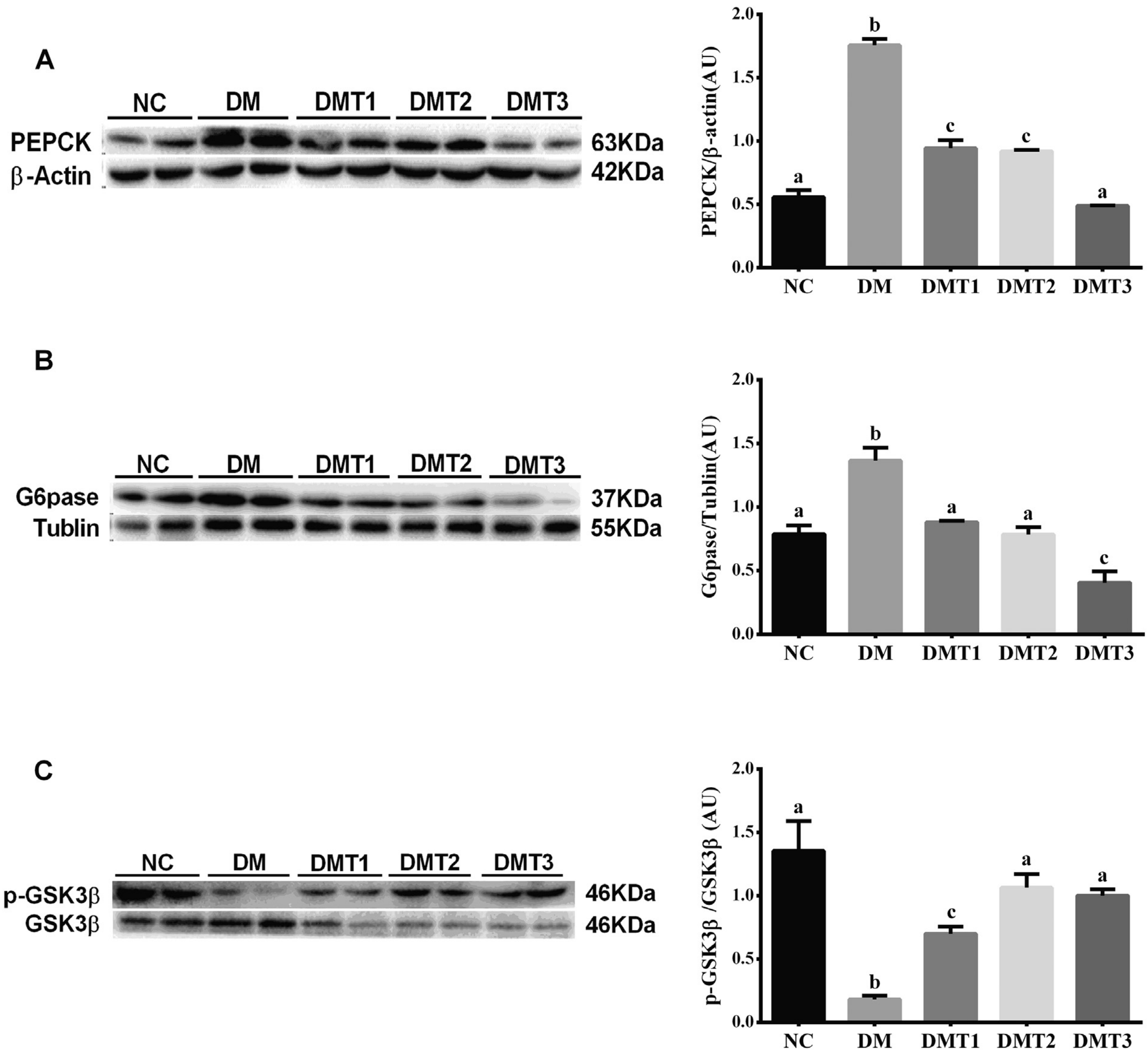

Fig. 5. Combined treatment of empagliflozin plus metformin demonstrated no better amelioration for gluconeogenesis or glycogen synthesis than empagliflozin single intervention in T2DM mice.

(A) The protein expression levels of PEPCK in NC, DM, DMT1, DMT2 and DMT3 groups were assayed by immunoblotting and quantified by actin levels. (B) The protein expression levels of G6pase in NC, DM, DMT1, DMT2 and DMT3 groups were detected by immunoblotting and quantified by tubulin levels. (C) The levels of phosphorylated GSK3β in NC, DM, DMT1, DMT2 and DMT3 groups were assayed by immunoblotting and quantified by total GSK3β. Each bar with different letters (a, b, c) represents statistic difference, $p < 0.05$ (ANOVA, analyzed by Tukey or SNK). NC: normal control group, DM: T2DM control group, DMT1: db/db mice treated with metformin group, DMT2: db/db mice treated with empagliflozin plus metformin group, DMT3: db/db mice treated with empagliflozin group.

understood [26], the combined intervention of empagliflozin plus metformin may have no additive effect, and further studies are required to determine which drug plays a major role. Our current data revealed that empagliflozin or combined therapy elicited the same degree of inhibition of GSK3β activity or promotion of GS activity, which is consistent with our data that the FPG levels in T2DM mice were both normalized by the two therapies after four-week treatment.

There are several limitations by our current study that should be stated. Firstly, the sample size of male mice in each group should be enlarged to confirm the effects of different treatments on T2DM mice. Secondly, our intervention only lasted for a relatively short length of time (four weeks) that did not simulate the long-term effects for human patients. Thirdly, current research involved only one dosing level tested in animals, which may result in limited interpretation for our data. Fourthly, the testing only one age (9 weeks) of db/db mice may be deficient of other effects by combined therapy. Hence further study may be required to explore extensive other effects elicited by either monotherapy or combined intervention of the two drugs.

In conclusion, combination therapy of empagliflozin plus metformin may have no synergic effects, and show no higher amelioration for the glucose and lipid homeostasis than empagliflozin single intervention in T2DM mice, at least based on the short to medium term drug treatment. Our current results may provide novel reference for clinical intervention.

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Author contributions

Chen K.Y. and Jiang Z.X. designed the study, provided research fund, supervised the research and reviewed/revised the manuscript. Wang Z.G. and Zhou J. performed the experiments, analyzed and interpreted data, and wrote the draft of manuscript. Lu M.R. and Liang Y.W. validate the data. All authors read and approved the manuscript for publication.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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