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**Research Article**

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**The Effects of Zinc Supplementation Combined with Metformin on Liver Lipid Metabolism in Mice with Type 2 Diabetes**

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**Abstract**

Hepatic complications, particularly non-alcoholic fatty liver disease, are highly prevalent in T2DM and are driven by dysregulated lipid metabolism and inflammation. Current therapies, including metformin, offer limited hepatic protection. This study investigated whether zinc supplementation synergizes with metformin can alleviate T2DM-associated liver damage. A T2DM mouse model was established using a high-fat high-sugar diet combined with low-dose streptozotocin, followed by a 6-week intervention with metformin, zinc sulfate, or their combination. The combined therapy most effectively restored hepatic zinc homeostasis and reduced serum liver injury markers, including ALT, AST, and ALP, while profoundly ameliorating hepatic steatosis, inflammatory infiltration, and glycogen loss. Mechanistically, co-treatment markedly suppressed lipogenesis by downregulating SREBP-1c, ACC, FASN expression and enzyme activities, while enhancing fatty acid oxidation through PPARα/CPT-1 activation and inhibiting lipid transport by reducing MTTP Protein and mRNA levels. Furthermore, the combination synergistically attenuated oxidative stress by lowering ROS and MDA levels and elevating antioxidant defenses, including SOD, GSH, and CAT activities, alongside metallothionein upregulation. As excessive ROS is a well-established trigger of NF-κB activation, this redox restoration translated into potent anti-inflammatory effects, in which the combination lowered the p-IκBα/IκBα ratio and rebalanced pro- and anti-inflammatory cytokines, surpassing the effect of zinc alone. These findings demonstrate that zinc and metformin synergistically reprogram hepatic lipid metabolism and reinforce antioxidant and anti-inflammatory defenses, offering a promising multi-targeted therapeutic strategy for T2DM-related liver injury.

**Keywords:** Type 2 diabetes mellitus; zinc; metformin; hepatic lipid metabolism; NF-κB pathway

## 1. Introduction

Type 2 diabetes mellitus (T2DM) disrupts glucose homeostasis and impairs hepatic lipid metabolism, triggering non-alcoholic fatty liver disease (NAFLD)[1, 2]. Population-based epidemiological studies have confirmed that patients with type 2 diabetes mellitus (T2DM) universally exhibit aberrant hepatic lipid profiles, and that hepatic lipid metabolic dysfunction becomes progressively more severe with increasing body weight and insulin resistance.[3]. Glucose intolerance and hepatic steatosis form a vicious cycle, whereby impaired intrahepatic lipid turnover exacerbates peripheral insulin resistance, while sustained hyperglycemia and hyperlipidemia further disrupt hepatic lipid homeostasis.[4]. Excessive hepatocellular lipid deposition is the hallmark of T2DM-related hepatic dysfunction[5]. Beyond lipid dysregulation, the diabetic microenvironment drives robust hepatic oxidative stress and persistent inflammatory cascades. Chronic high glucose and lipids boost reactive oxygen species (ROS), overwhelming hepatic antioxidant defenses. Excess ROS activates NF-κB and other pro-inflammatory pathways to secrete inflammatory cytokines; oxidative stress and inflammation reciprocally disrupt hepatic lipid synthesis and catabolism, aggravating intrahepatic lipid accumulation[6, 7].

Metformin serves as the first-line therapeutic agent for T2DM and effectively controls systemic glycemia[8, 9]. However, its regulatory efficacy against hepatic lipid metabolic abnormalities remains controversial and insufficient. Specifically, Metformin exerts mild hepatoprotective effects, yet its long-term

efficacy against diabetic liver injury remains inconsistent. It weakly regulates hepatic lipid metabolism and oxidative stress, two core triggers of NAFLD progression. Metformin alleviates steatosis and inflammation partially via upregulating hepatic SIRT1/UCP2[10], but its benefits diminish under severe insulin resistance and sustained oxidative stress. Although monotherapy lowers MDA and boosts antioxidant capacity to slightly improve dyslipidemia[11], it cannot fully reverse T2DM-related liver lesions[12].

Disrupted zinc homeostasis is a typical pathological feature of T2DM. Hypozincemia and tissue zinc depletion worsen metabolic disorders and multi-organ complications. Liver zinc levels can drop by up to 50% in diabetic animals and correlate with impaired insulin signaling and severe hepatic steatosis[13]. Zinc deficiency weakens hepatic redox defense. It causes oxidative stress, NF-κB activation and lipid disturbance. This accelerates the progression from simple steatosis to non-alcoholic steatohepatitis[14]. Clinical trials show that 50 mg/day zinc improves glycemic control and lipid profiles in newly diagnosed T2DM patients. It lowers fasting glucose, HbA1c and LDL cholesterol[15]. Zinc may exert antioxidant effects partly by inducing metallothionein[14]. However, whether zinc supplementation can restore hepatic zinc balance and relieve diabetic liver injury remains unclear. Met and zinc have mostly been studied separately for hepatic steatosis, oxidative stress and inflammation[16]. Metformin inhibits hepatic lipogenesis and promotes fatty acid oxidation through AMPK signaling[17]. The two treatments share some protective pathways but act on different molecular targets. Whether their combination gives synergistic liver protection is still unknown. No study has systematically compared zinc alone, metformin alone and combined therapy for hepatic histopathology, lipid metabolism, oxidative stress and NF-κB inflammatory signaling in T2DM mice.

Therefore, we established a T2DM mouse model by feeding a high-fat and high-sucrose diet and injecting low-dose STZ. Then we compared Zn, Met, Zn and Met combianation therapy. Hepatic histopathology, lipid metabolism, oxidative stress, and NF-κB inflammatory signaling were systematically assessed. We aimed to determine whether Zn supplementation restores hepatic zinc balance and whether combined therapy produces synergistic liver protection beyond either monotherapy. These findings may clarify the therapeutic potential of Zn and Met combination therapy for T2DM-associated NAFLD and provide experimental evidence for further clinical investigation.

## 2. Materials and methods

### 2.1 Experimental reagents

High-fat high-sucrose diet (D12492), standard chow, and animal bedding were purchased from Chengdu Dossy Experimental Animals Co., Ltd. STZ (S0130) was purchased from Sigma. MET hydrochloride (D9351) and $ZnSO_4 \cdot 7H_2O$(Z8090) were purchased from Beijing Solarbio Science & Technology Co., Ltd. ELISA kits (MM-0632M2, MM-0631M2, MM-1199M2, MM-0757M2, MM-0653M2) were purchased from Jiangsu Meimian Industrial Co., Ltd. Gene primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. after design via Primer-BLAST. Conventional laboratory consumables and residual reagents were preserved in our laboratory.

### 2.2 Experimental Animals

Eight-week-old specific pathogen-free (SPF) male ICR mice weighing 30 ± 2 g were obtained from Chengdu Dossy Experimental Animals Co., Ltd. Before the experiment, all mice were acclimated to a standard diet for one week. Then, 40 mice were assigned to the control group and continued on the normal diet, while the remaining 320 mice were fed a high-fat and high-sucrose diet with free access to water. The animal experimental protocol was approved by the Animal Welfare and Ethics Committee of Sichuan Agricultural University (Approval No. 20230436). Finally, 100 mice that had been successfully modeled for T2DM (T2DM group) and 25 healthy normal control mice (control group) were selected for the formal intervention trial.

### 2.3 T2DM mouse model establishment and experimental grouping

After 6 weeks, mice in the model group were fasted for 12 h and then injected intraperitoneally with STZ (30 mg/kg in citrate buffer) once daily for five consecutive days[18]， whereas the control group received an equivalent volume of citrate buffer. Two weeks after STZ administration, fasting blood glucose (FBG) was measured by tail-vein puncture after a 12-h fast. Mice with FBG ≥ 11.1 mmol/L were considered successfully modeled[19].

The successfully modeled mice were randomly divided into four groups (n = 25 per group): T2DM, MET, Zn, and Zn+MET. During the 6-week intervention, all mice were maintained on standard chow. The MET group received metformin at 250 mg/kg by oral gavage once daily (50 mg/mL in distilled water, 5 mL/kg)[20]. The Zn group received 0.02 mg/mL Zn in drinking water ad libitum, with fresh solution provided daily[21]. The Zn+MET group received both treatments accordingly. The model and control groups received an equal volume of distilled water. After 6 weeks, the mice were sacrificed and samples were collected.

### 2.4 Determination of Serum Biochemical Parameters, Hepatic Lipids, and Zinc Content

Blood samples were collected after 12 h of fasting. Serum was separated by centrifugation at 3,000 × g for 10 min at 4 °C and stored at −80 °C until analysis. Serum levels of ALT, AST, ALP, TP, ALB, GLB, T-Bil, TC, TG, HDL-C, LDL-C were measured using an automatic biochemical analyzer (Mindray Medical; BS-1000M) according to the manufacturer's instructions. GLB and the ALB/GLB ratio were calculated as GLB = TP − ALB and ALB/GLB, respectively.

Livers were excised, rinsed with ice-cold phosphate-buffered saline, blotted dry, and weighed. The liver index was calculated as (liver weight/body weight) × 100%. Hepatic TC and TG contents were determined using commercial ELISA kits according to the manufacturer's instructions, and the results were normalized to liver weigh. Hepatic zinc content was determined by inductively coupled plasma mass spectrometry (ICP-MS; [Platinum Elmer; PE NexION 300D]) after digestion; zinc concentrations were calculated from a standard curve and expressed as μg/g liver.

### 2.6 Liver Histopathological Examination

Liver tissues were fixed in 4% paraformaldehyde at 4 °C for 24 h. For H&E and PAS staining, tissues were dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned at 4 μm . Sections were dewaxed, rehydrated, and stained with H&E to evaluate hepatic architecture, inflammatory infiltration, and steatosis, or with PAS to assess glycogen distribution in hepatocytes. For Oil Red O staining, liver tissues were cryoprotected in 20–30% sucrose, embedded in OCT compound, and sectioned at 8–10 μm using a cryostat. Frozen sections were stained with Oil Red O to detect lipid droplet accumulation in hepatocytes. Stained sections were observed and imaged under a light microscope; representative images were captured for subsequent analysis.

### 2.7 Determination of Hepatic Lipid Metabolism-Related Enzyme Activities

Liver samples were homogenized in ice-cold and centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatants were collected. The activities of FASN, ACC, and CPT-1 were measured using commercial ELISA kits according to the manufacturer's instructions. Enzyme activities were normalized to total content.

### 2.8 RT-qPCR

After the intervention,liver from 5 mice in each group were collected. Total RNA was extracted using a magnetic bead-based RNA extraction kit. RNA purity and concentration were assessed using a micro-volume spectrophotometer, with an OD260/OD280 ratio of 1.8-2.1 considered acceptable. RT-qPCR was employed to detect the mRNA expression levels of fibrosis-related genes in renal tissues.

Primer Sequences

| Primer | forward primer (3'-5) | reverse prime (3'-5') |
|---|---|---|
| SREBP-1c | ACCACGGAGCCATGGATTG | CTCAGGAGAGTTGGCACCTG |
| ACC | ATGGGCTGCTTCTGTGACTC | GTTCATCCCTGGGGACCTTG |
| FASN | GCCCGTCAGTGTTCCCTATC | GGGGTTACTACCGGTCATCG |
| SCD1 | TTCCGCCACTCGCCTAC | GTAAGAACTGGAGATCTCTTGGA |
| PPARα | GATCAGATTCCGCCTGTCCG | GGTCTGGGTTGAGCTTTCCT |
| CPT-1 | GGAGAGCTTGCTACACGCA | GGAGATCGATGCCATCAGGG |
| ACOX1 | CTTGCTGAATCAGGGCACCA | TCGAAGATGAGTTCCGTGGC |
| MTTP | GCTGGAGAGAGTGGTGGTG | GGTCAGCAGAGGTGGTTC |
| SREBP-1c | ACCACGGAGCCATGGATTG | CTCAGGAGAGTTGGCACCTG |
| ACC | ATGGGCTGCTTCTGTGACTC | GTTCATCCCTGGGGACCTTG |
| p65 | GAGAGCACAGACCCCAACAA | GGTCACAGGGATAGGCACAG |
| IκBα | GCTGAAGAAGGAGCGGCTAT | TCGTAGGAGAAGGCGAGAGG |
| β-actin | CACTGTCGAGTCGCGTCC | TCATCCATGGCGAACTGGTG |

### 2.9 Western Blot

Liver tissue samples were lysed in RIPA lysis buffer on ice, followed by centrifugation at 4 °C, 12,000 × g for 5 min to obtain the protein supernatant. Protein concentrations were determined using a BCA kit. Loading buffer was added proportionally, and samples were denatured at high temperature and stored at −20 °C for subsequent use. Western blotting was performed following the previously established protocol of our research group, including SDS-PAGE, membrane transfer, immunoblotting, and signal development. The protein expression levels of hepatic lipid synthesis-, catabolism-, and transport-related proteins (SREBP-1c, ACC, FASN, CPT-1, PPARα, and MTTP) and NF-κB pathway-related factors (p65, IκBα, and phosphorylated IκBα [p-IκBα]) were determined.

### 2.10 Detection of oxidative stress-related markers in mouse liver

At the end of the 42-day intervention, liver tissues from five mice per group (n=5) were collected. Hepatic ROS levels were measured by flow cytometry using DCFH-DA-stained single-cell suspensions and expressed as mean fluorescence intensity. MDA and GSH contents, as well as CAT, SOD, and T-AOC activities, were measured using commercial kits according to the manufacturer's instructions and normalized to total protein; these indicators reflected hepatic lipid peroxidation and antioxidant capacity. Hepatic MT mRNA and protein expression were determined by RT-qPCR and Western blot as described in Sections 2.8 and 2.9, respectively.

### 2.11 Statistical Analysis

All experimental data were statistically analyzed using GraphPad Prism 10.5 software, and results were expressed as the mean ± standard deviation (Mean ± SD). Differences among multiple groups were analyzed by one-way ANOVA combined with t-tests. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant. Marking standards: Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the T2DM group, $P < 0.05$, $\#\#P < 0.01$; compared with the Zn+MET group, $\Delta P < 0.05$, $\Delta\Delta P < 0.01$.

## 3. Results

### 3.1 Zinc supplementation combined with metformin improves hepatic zinc homeostasis and liver function and histopathology injury in T2DM

The T2DM mouse model was successfully established via high-sugar high-fat diet plus low-dose intraperitoneal STZ injection[22]. Fig. 1A showed that hepatic zinc concentrations were markedly lower in the T2DM group versus the control group ($P < 0.01$), whereas MET, Zn, or Zn+MET group significantly increased hepatic zinc levels in T2DM group ($P < 0.05$ or $P < 0.01$), with the most prominent elevation in the Zn+MET group. As illustrated in Fig. 1B–I, the T2DM group displayed increased serum levels of ALT, AST, ALP, GLB and T-Bil, alongside reduced TP, ALB and ALB/GLB ratio relative to the control group ($P < 0.01$). All three interventions lowered serum levels of ALT and AST in the T2DM group; only the Zn+MET group significantly decreased ALP and elevated ALB serum levels ($P < 0.05$ or $P < 0.01$). The liver index (Fig. 1J) was markedly higher in the T2DM group than in the control group ($P < 0.01$). MET, Zn and Zn+MET groups all reduced the

liver index relative to the untreated T2DM mice ($P < 0.01$), and the Zn+MET group exhibited a lower liver index than either monotherapy ($P < 0.05$).

As shown in Fig. 1K–L, HE and PAS staining revealed severe diffuse hepatic steatosis, vacuolar degeneration, disorganized hepatic cords, inflammatory infiltration, hepatocellular necrosis and impaired glycogen deposition in the T2DM group. Single MET or Zn treatment partially alleviated hepatic steatosis, inflammation, necrosis and glycogen loss. The Zn+MET group further attenuated lipid vacuolation and inflammatory infiltration, nearly abolished hepatic inflammation, and produced denser, more evenly distributed PAS-positive glycogen granules compared with single-agent groups.

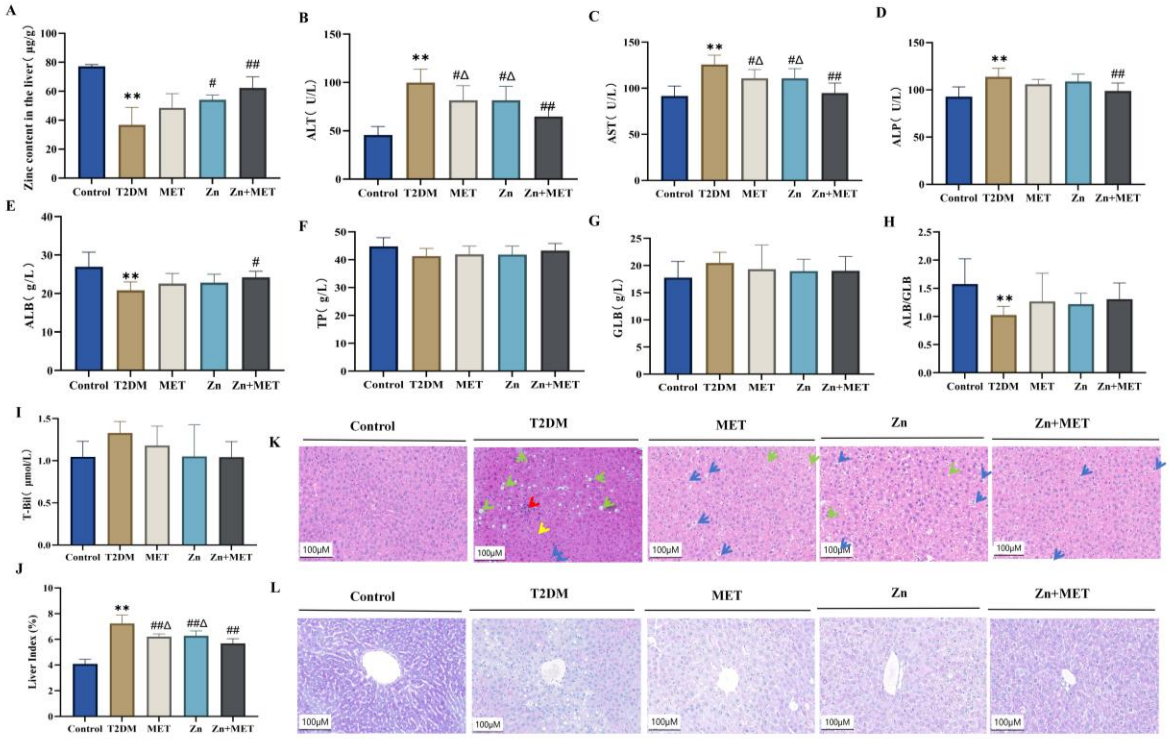



**Fig.1 hepatic zinc homeostasis and liver function injury in T2DM**

(A) Results of zinc content analysis in mouse livers; (B–I) Changes in serum ALT, AST, ALP, ALB, TP, GLB, ALB/GLB, and T-Bil levels in mice; (J) Mouse liver index; (K) HE staining of mouse liver; green arrows indicate fatty degeneration, blue arrows indicate vacuolar degeneration, red arrows indicate inflammatory cell infiltration, and yellow arrows indicate cellular necrosis; scale bar = 100 μm; (L) PAS-stained mouse liver sections; purple indicates glycogen-positive staining; scale bar = 100 μm. Compared to the control group, **P < 0.01; compared to the T2DM group, #P < 0.05, ##P < 0.01; compared to the Zn+MET group, respectively, ΔP < 0.05.

As shown in Fig. 2A–D, compared with the control group, the T2DM group exhibited elevated serum levels of TG, TC, LDL-C and HDL-C. MET or Zn group markedly reduced serum levels of ALT and AST activities ($P < 0.05$ or $P < 0.01$), whereas Zn+MET group further decreased serum ALP, GLB and T-Bil levels and increased ALB with restored TP content ($P < 0.05$ or $P < 0.01$). As illustrated in Fig. 2E–F, the T2DM

group exhibited marked increases in TG, TC and LDL-C levels, accompanied by reduced HDL-C levels ($P < 0.01$). Oil Red O staining (Fig. 2G) revealed extensive orange-red lipid droplet accumulation in hepatocytes of the T2DM group. Either MET or Zn monotherapy substantially diminished the number of cytoplasmic lipid droplets compared with T2DM group. The Zn+MET group showed the most striking mitigation of lipid deposition, with only sparse small lipid droplets visible within hepatocytes.

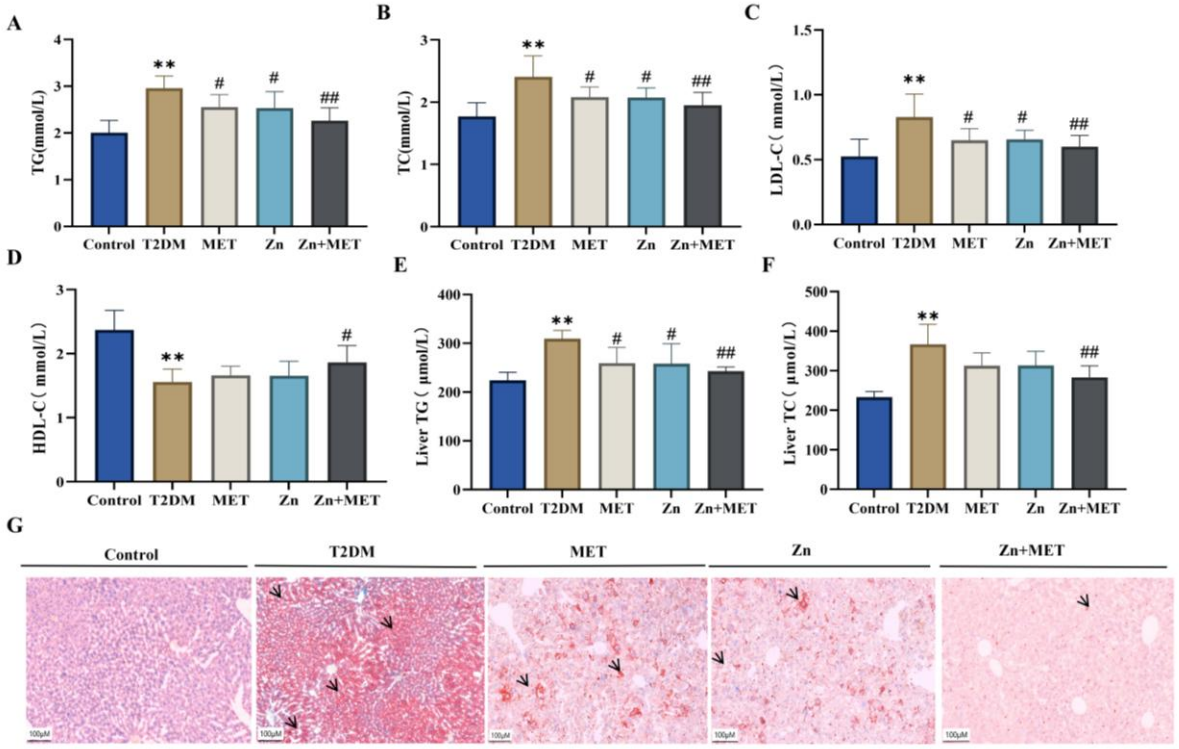



**Fig.2 hepatic zinc homeostasis and liver function injury in T2DM**

(A–D) Changes in serum TG, TC, LDL-C and HDL-C levels in mice; (E–F) TG and TC levels as well as Oil Red O staining in mouse livers; red lipid droplets indicate lipid-positive staining; scale bar = 100 μm. Compared to the Control group, $^{**}P < 0.01$; Compared to the T2DM group, #P < 0.05, ##P < 0.01.

### 3.2 Zinc supplementation combined with metformin intervenes in hepatic lipid synthesis and lipolysis in T2DM

As shown in Fig. 3A–K, compared with the control group, the T2DM group exhibited markedly elevated protein and mRNA levels of lipogenic mediators SREBP-1c, ACC, and FASN, while the protein and mRNA levels of fatty acid oxidation regulators PPARα and CPT-1 were drastically reduced ($P < 0.01$). Either MET or Zn group suppressed SREBP-1c, ACC, and FASN while simultaneously upregulating PPARα and CPT-1 in the T2DM group ($P < 0.05$ or $P < 0.01$). The Zn+MET group fully reversed the dysregulation of these lipid metabolism-related proteins and genes, with superior efficacy relative to single-agent interventions ($P < 0.05$ or $P < 0.01$).

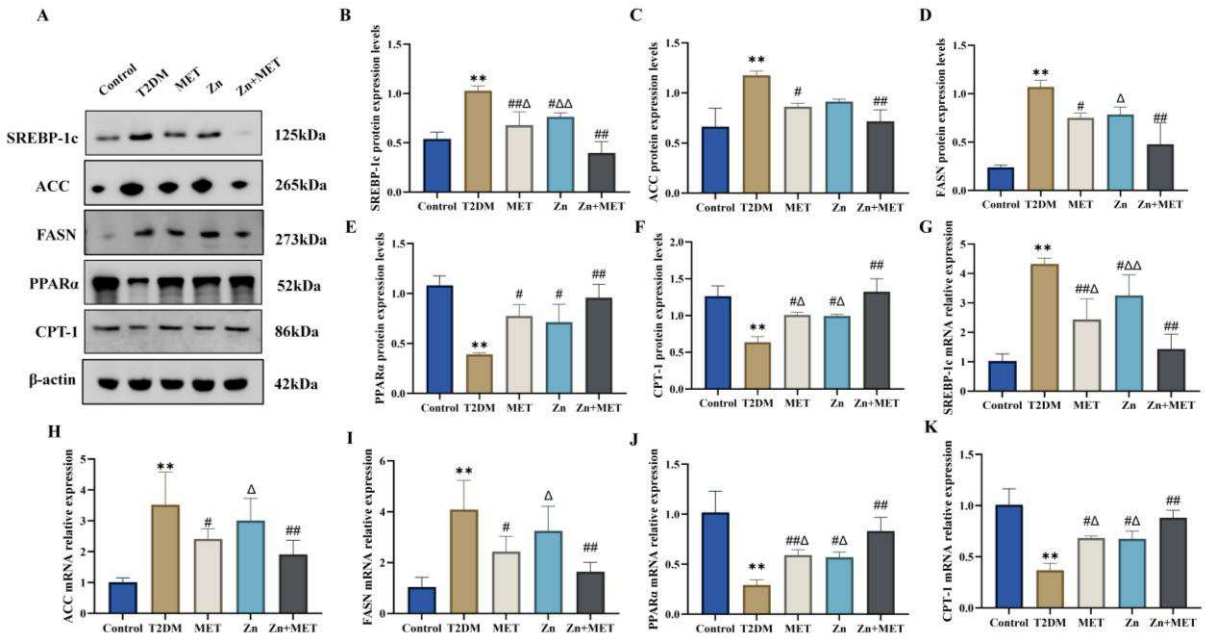



**Fig. 3 hepatic zinc homeostasis and liver function injury in T2DM**

(A) Western blot bands of lipid synthesis-related and lipolysis-related proteins in mouse liver; (B–F) Relative protein expression levels of hepatic SREBP-1c, ACC, FASN, PPARα, and CPT-1 in mice; (G–K) Relative mRNA expression levels of hepatic SREBP-1c, ACC, FASN, PPARα, and CPT-1 in mouse liver. Compared to the control group, **P < 0.01; compared to the T2DM group, #P < 0.05, ##P < 0.01; compared to the Zn+MET group, respectively, ΔP < 0.05, ΔΔP < 0.05.

### 3.3 Zinc supplementation combined with metformin regulates hepatic lipid metabolism-related enzyme activities and lipid transport in T2DM

As shown in Fig. 4A-C, compared with the control group, T2DM group displayed markedly elevated hepatic FASN and ACC lipogenic enzyme activities and reduced CPT-1 fatty acid oxidation activity ($P < 0.01$). MET group suppressed FASN and ACC and restored CPT-1 activity ($P < 0.05$), Zn group alone only increased CPT-1 activity ($P < 0.01$), and the Zn+MET group fully reversed all three enzyme alterations ($P < 0.01$), outperforming monotherapies ($P < 0.05$). MTTP protein and mRNA levels (Fig. 4D-E) were markedly upregulated in T2DM group relative to control group ($P < 0.01$). MET group decreased MTTP protein and mRNA levels ($P < 0.05$ or $P < 0.01$), Zn group only reduced MTTP mRNA level ($P < 0.05$), and the Zn+MET group strongly inhibited MTTP overexpression at both transcriptional and translational levels ($P < 0.01$), with greater suppression than individual treatments ($P < 0.05$ or $P < 0.01$).

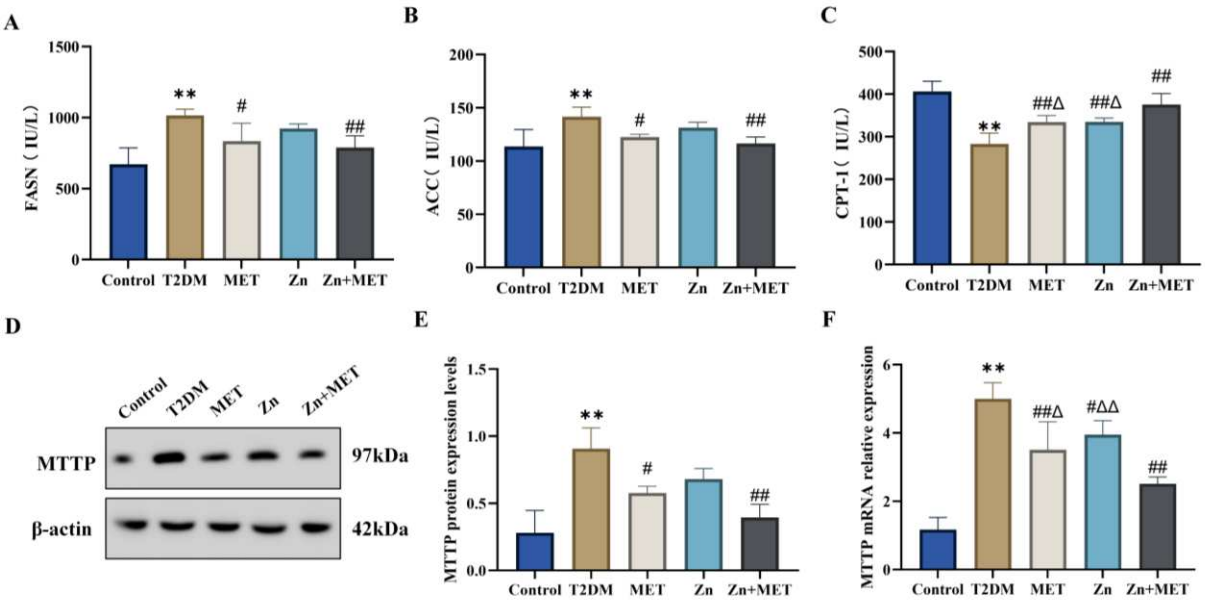



**Fig. 4 hepatic zinc homeostasis and liver function injury in T2DM**

(A–C) Results of FASN, ACC, and CPT-1 activity assays in mouse liver; (D) Western blot bands for lipid transport-associated factors; (E) Relative expression levels of MTTP in mouse liver; (F) Relative mRNA expression levels of MTTP in mouse liver. Compared to the control group, **P < 0.01; compared to the T2DM group, #P < 0.05, ##P < 0.01; compared to the Zn+MET group, respectively, ΔP < 0.05, ΔΔP < 0.05.

### 3.4 Zinc supplementation combined with metformin alleviates hepatic oxidative stress injury in T2DM

As shown in Fig. 5A–G, hepatic ROS and MDA increased while T-AOC, SOD, GSH and CAT decreased in T2DM group relative to control group (P < 0.01). MET, Zn and Zn+MET groups reduced ROS and MDA and restored T-AOC, SOD, GSH and CAT antioxidant indicators (P < 0.05 or P < 0.01)，the Zn+MET group exhibited greater ROS and MDA reduction and stronger recovery of antioxidant biomarkers (P < 0.05 or P < 0.01). Fig. 5H–J revealed downregulated MT protein and mRNA levels in T2DM group versus control group. Zn and Zn+MET groups significantly elevated MT protein and mRNA levels (P < 0.05), while MET group showed no obvious effect. Notably, the Zn+MET group exhibited superior antioxidant effects over monotherapies (P < 0.05 or P < 0.01).

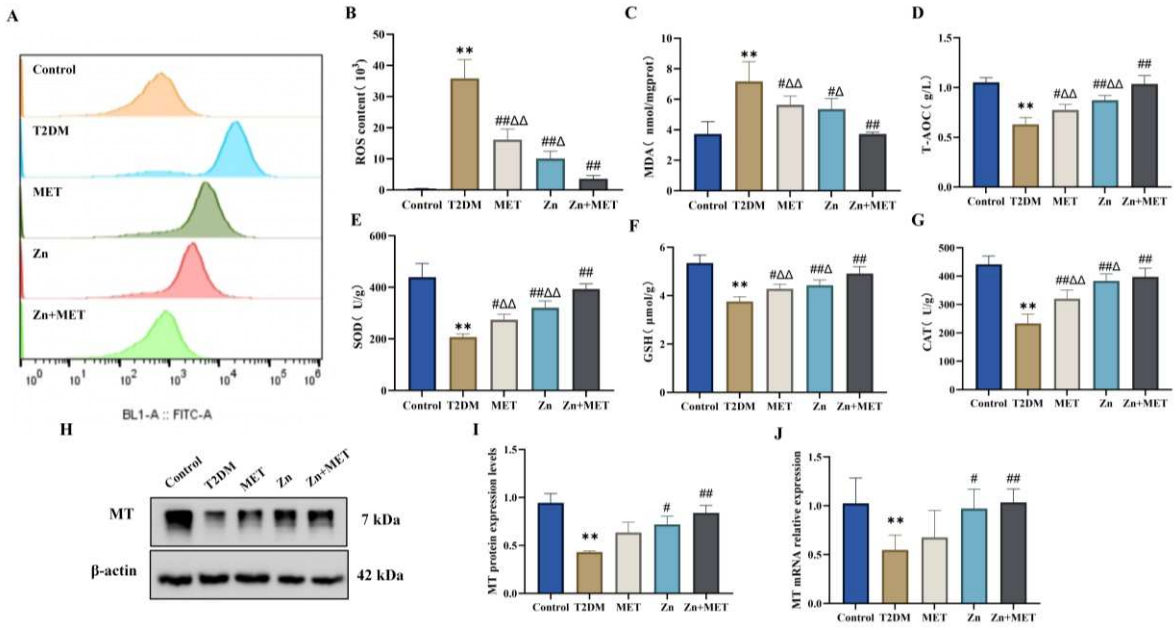



**Fig. 5 hepatic zinc homeostasis and liver function injury in T2DM**

(A–B) Flow cytometry detection of ROS fluorescence intensity and quantitative statistics in mouse liver; (C–G) Determination of MDA content, T-AOC level, SOD activity, GSH content, and CAT activity in mouse liver; (H–J) Western blot bands of MT protein, relative MT mRNA expression, and MT protein expression levels in mouse liver. Compared to the control group, **P < 0.01; compared to the T2DM group, #P < 0.05, P < 0.01; compared to the Zn+MET group, respectively, ΔP < 0.05, ΔΔP < 0.05.

### 3.5 Zinc supplementation combined with metformin improves hepatic inflammatory injury in T2DM by regulating the hepatic NF-κB pathway

In Fig. 6A-G, compared with the control group, the T2DM group showed no significant alterations in hepatic p65 protein and mRNA levels. By contrast, IκBα mRNA and protein levels were markedly downregulated, whereas p-IκBα abundance and the p-IκBα/IκBα ratio increased substantially (P < 0.01). Compared with the T2DM group, MET, Zn, and Zn+MET groups all significantly upregulated IκBα mRNA levels (P < 0.05 or P < 0.01). Reductions in p-IκBα levels and the p-IκBα/IκBα ratio were only observed in the MET and Zn+MET groups (P < 0.05), while the Zn group showed no obvious change. Fig. 6H–L revealed that compared with the control group, the T2DM group had elevated levels of hepatic pro-inflammatory cytokines of IL-1β, IL-6, TNF-α and decreased levels of anti-inflammatory cytokines of IL-4, IL-10 (P < 0.01). All three interventions reduced levels of IL-1β, IL-6 and TNF-α (P < 0.05 or P < 0.01), and the MET and Zn+MET groups significantly increased IL-4 and IL-10 levels (P < 0.05 or P < 0.01). Notably, the Zn+MET group exerted stronger anti-inflammatory effects than either the Zn group or the MET group (P < 0.05).

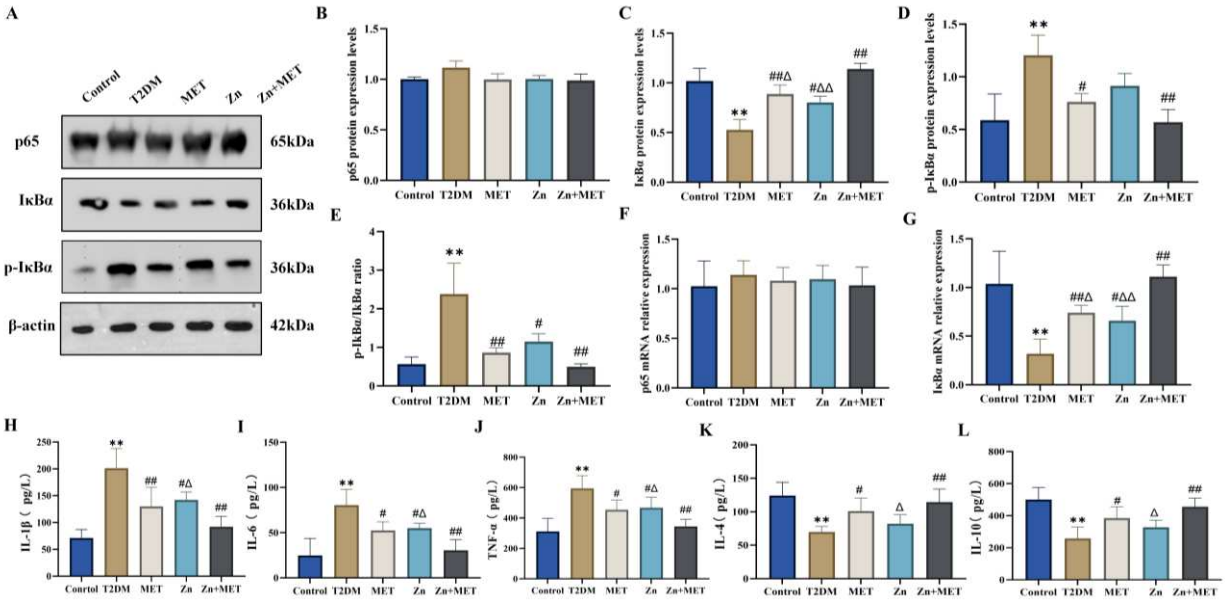



**Fig-6 hepatic zinc homeostasis and liver function injury in T2DM**

(A) Western blot bands of factors related to the NF-κB pathway; (B–E) Relative expression levels of p65, IκBα, p-IκBα, and the p-IκBα/IκBα ratio in mouse liver; (F–G) Relative mRNA expression levels of p65 and IκBα in mouse liver; (H–L) Detection results of hepatic inflammatory cytokines including IL-1β, IL-6, TNF-α, IL-4, and IL-10 in mice. Compared to the control group, **P < 0.01; compared to the T2DM group, P < 0.05, ##P < 0.01; compared to the Zn+MET group, respectively, ΔP < 0.05, ΔΔP < 0.05.

**4. Discussion**

The maintenance of hepatic zinc homeostasis relies on a finely tuned equilibrium between zinc uptake, intracellular sequestration and efflux. In T2DM, sustained hyperglycemia disrupts this balance and depletes hepatic zinc stores[23, 24] [25]. Hepatic zinc deficiency is therefore a primary pathological feature of diabetic liver injury rather than a secondary incidental change. Consistent with previous reports[26, 27] , we observed significantly reduced hepatic zinc concentrations in T2DM mice, supporting the notion that hepatic zinc depletion is an intrinsic characteristic of diabetes-associated hepatopathy

Metformin, the first-line oral T2DM drug, improves insulin sensitivity and facilitates hepatic zinc accumulation, whereas zinc supplementation directly restores disrupted trace-element signaling in diabetic hepatocytes[28] [29, 30]. When administered separately, MET or Zn group partially ameliorated liver dysfunction, hepatic steatosis and inflammatory infiltration. However, the Zn+MET group achieved superior improvements in liver biochemistry, liver index and hepatic histopathology than either MET or Zn group, including reduced lipid vacuolation, attenuated inflammatory cell infiltration and recovered glycogen deposition. This synergistic hepatoprotection indicates that targeting disrupted zinc homeostasis represents a viable combinational strategy for T2DM-related liver damage.

Hepatic steatosis in T2DM arises from disrupted crosstalk between hepatic lipogenesis and mitochondrial fatty-acid β-oxidation[31]. The transcription factor SREBP-1c drives the transcription of lipogenic ACC and

FASN[32], while PPARα and its downstream target CPT1 govern fatty-acid oxidation[33]. In this study, the Zn+MET group reversed the dysregulated lipid regulatory network by suppressing SREBP-1c, ACC and FASN at protein and mRNA levels while upregulating PPARα and CPT1, with reduced lipogenic enzyme activity and restored CPT1 activity. Furthermore, this combination blocked MTTP overexpression at both transcriptional and translational levels. T2DM-induced MTTP overexpression represents a compensatory response to excessive intracellular lipid burden[34]; reduced MTTP after combined intervention reflects overall amelioration of hepatic lipid overload rather than enhanced lipid retention. MET or Zn group only modulates a single lipid pathway and cannot fully reverse steatosis. By concurrently suppressing lipogenesis and enhancing fatty-acid catabolism, the Zn+MET group produces stronger lipid-regulating effects than monotherapy.

Oxidative stress acts as a core mediator of diabetic hepatic injury. T2DM group displayed elevated ROS and MDA alongside depletion of antioxidant molecules including T-AOC, SOD, GSH and CAT. The Zn+MET group restored hepatic redox balance and exhibited stronger antioxidant capacity than MET or Zn group, an effect closely linked to MT induction. Zinc serves as a cofactor for antioxidant enzymes and can activate MT to scavenge free radicals and limit lipid peroxidation[35, 36]. Notably, Notably, Zn, but not metformin, significantly induced hepatic MT expression, whereas metformin is reported to limit ROS generation through AMPK-dependent signaling[37]. The combination may therefore rebuild antioxidant defense mainly through Zn/MT-mediated scavenging while simultaneously reducing ROS production through metformin-associated AMPK signaling.

The NF-κB signaling cascade serves as a central driver of chronic hepatic inflammation in T2DM. Total p65 expression remained unchanged between control and T2DM groups. Diabetes reduced IκBα abundance and increased the p-IκBα/IκBα ratio, triggering a pro-inflammatory phenotype with higher IL-1β, IL-6, TNF-α and lower anti-inflammatory IL-4 and IL-10. MET or Zn+MET group upregulated IκBα and reduced the p-IκBα/IκBα ratio, whereas Zn group failed to inhibit IκBα phosphorylation. All three groups reduced pro-inflammatory cytokines, while only MET and Zn+MET group rescued anti-inflammatory cytokine levels. This differential response suggests Metformin is required to potentiate zinc's anti-inflammatory actions. Metformin blocks NF-κB protein activation by reducing IκBα phosphorylation[38], while Zn exerts auxiliary antioxidative protection by scavenging upstream ROS without directly modifying IκBα phosphorylation[39]. The Zn+MET group rebalances hepatic cytokine profiles and suppresses NF-κB-mediated inflammation more effectively than single-agent treatment.

Single-drug intervention often targets only one pathological node and may trigger compensatory activation of alternative pathogenic pathways, leading to limited therapeutic outcomes[40]. In contrast, The Zn+MET group concurrently restores hepatic zinc homeostasis, ameliorates lipid disorders, enhances antioxidant defense and blocks NF-κB-driven inflammation. Metformin and zinc have each been investigated individually in diabetes research; however, few prior works systematically characterize their synergistic effects on the integrated hepatic pathological cascade in T2DM. In summary, Zn combined with metformin exerts synergistic hepatoprotective effects by restoring hepatic zinc homeostasis, remodeling lipid metabolism, strengthening

antioxidant capacity and inhibiting NF-κB-mediated inflammatory signaling. This combination may represent a promising multi-target therapeutic approach for T2DM-associated hepatic injury.

**References**


1. Targher G, Lonardo A, Byrne CD (2018) Nonalcoholic fatty liver disease and chronic vascular complications of diabetes mellitus. Nat Rev Endocrinol 14:99-114. https://doi.org/10.1038/nrendo.2017.173
2. Zhang W, Cheng Q, Yin L, Liu Y, Chen L, Jiang Z, Jiang X, Qian S, Li B, Wu M *et al* (2024) Jujuboside A through YY1/CYP2E1 signaling alleviated type 2 diabetes-associated fatty liver disease by ameliorating hepatic lipid accumulation, inflammation, and oxidative stress. Chem Biol Interact 400:111157. https://doi.org/10.1016/j.cbi.2024.111157
3. Ghislain J, Poitout V (2021) Targeting lipid GPCRs to treat type 2 diabetes mellitus - progress and challenges. Nat Rev Endocrinol 17:162-175. https://doi.org/10.1038/s41574-020-00459-w
4. Shu Y, Shen W, Feng W, Pan M, Xu X, Zheng S, Jin H (2025) Targeting HMGCS2: Ketogenesis Suppression Accelerates NAFLD Progression in T2DM Comorbidity, While Cynaroside Ameliorates NASH in Concomitant T2DM. Biomolecules 15. https://doi.org/10.3390/biom15081181
5. Barroso E, Jurado-Aguilar J, Wahli W, Palomer X, Vázquez-Carrera M (2024) Increased hepatic gluconeogenesis and type 2 diabetes mellitus. Trends Endocrinol Metab 35:1062-1077. https://doi.org/10.1016/j.tem.2024.05.006
6. Lin R, Xu L, Zhou Y, Fan Y, Xie H, Li W, Hu T, Liu C (2026) Integrated Bulk and Single-Cell RNA-Seq Analysis Reveals Transcriptional Activation of PTGS2 by FOS in Progression From T2DM to T2DM-Associated NAFLD. J Cell Mol Med 30:e71182. https://doi.org/10.1111/jcmm.71182
7. Stefan N, Yki-Järvinen H, Neuschwander-Tetri BA (2025) Metabolic dysfunction-associated steatotic liver disease: heterogeneous pathomechanisms and effectiveness of metabolism-based treatment. Lancet Diabetes Endocrinol 13:134-148. https://doi.org/10.1016/s2213-8587(24)00318-8
8. Inzucchi SE, Lipska KJ, Mayo H, Bailey CJ, McGuire DK (2014) Metformin in patients with type 2 diabetes and kidney disease: a systematic review. Jama 312:2668-2675. https://doi.org/10.1001/jama.2014.15298
9. Aroda V R, Ratner R E (2018) Metformin and Type 2 Diabetes Prevention. Diabetes Spectr 31:336-342. https://doi.org/doi:10.2337/ds18-0020
10. Xu J, Li N, Wang J, Zhang C, Ding S, Jiao Y, Zhang J (2013) Effect of metformin on the expression of SIRT1 and UCP2 in rat liver of type 2 diabetes mellitus and nonalcoholic fatty liver. Zhong Nan Da Xue Xue Bao Yi Xue Ban 38:882-887. https://doi.org/10.3969/j.issn.1672-7347.2013.09.003
11. Misra S, Deb T, Devi S, Singh A, Goel R (2026) Metformin modulates antioxidant and anti-inflammatory biomarkers in newly diagnosed patients with type 2 diabetes mellitus: an observational study. J Basic Clin Physiol Pharmacol 37:213-222. https://doi.org/10.1515/jbcpp-2025-0201
12. Foretz M, Guigas B, Viollet B (2019) Understanding the glucoregulatory mechanisms of metformin in type 2 diabetes mellitus. Nat Rev Endocrinol 15:569-589. https://doi.org/10.1038/s41574-019-0242-2

13. Bahadoran Z (2026) Rethinking Zinc Requirements in Diabetes Mellitus: Comprehensive Review of Experimental and Clinical Studies Evidence. Biol Trace Elem Res 204:3896-3912. https://doi.org/10.1007/s12011-025-04939-w
14. Kateel R, Kashyap NN, Reddy SK, Shetty S, Kumari MK, Ullal SD, Holla SN, Aroor A, Hill MA, Belle VS *et al* (2025) Chronic β-carotene, magnesium, and zinc supplementation together with metformin attenuates diabetes-related complications in aged rats. Clin Nutr 50:183-197. https://doi.org/10.1016/j.clnu.2025.05.004
15. Martens T, Beck RW, Bailey R, Ruedy KJ, Calhoun P, Peters AL, Pop-Busui R, Philis-Tsimikas A, Bao S, Umpierrez G *et al* (2021) Effect of Continuous Glucose Monitoring on Glycemic Control in Patients With Type 2 Diabetes Treated With Basal Insulin: A Randomized Clinical Trial. Jama 325:2262-2272. https://doi.org/10.1001/jama.2021.7444
16. Luo Y, Sun S, Zhang Y, Liu S, Zeng H, Li JE, Huang J, Fang L, Yang S, Yu P *et al* (2024) Effects of Oltipraz on the Glycolipid Metabolism and the Nrf2/HO-1 Pathway in Type 2 Diabetic Mice. Drug Des Devel Ther 18:5685-5700. https://doi.org/10.2147/dddt.S485729
17. Hao DD, Zhang L, Liu SL, Sun ZJ, Si RG, Zhang FN, Yang LX, Jia XL, Li TZ, Ai L *et al* (2025) Acteoside inhibits hepatic lipid accumulation and oxidative stress in type 2 diabetic mice via the Nrf2/HO-1 Pathway. J Toxicol Environ Health A 88:616-625. https://doi.org/10.1080/15287394.2025.2473558
18. Zhang Y, Wang S, Yang Y, Zhao S, You J, Wang J, Cai J, Wang H, Wang J, Zhang W *et al* (2023) Scarless wound healing programmed by core-shell microneedles. Nat Commun 14:3431. https://doi.org/10.1038/s41467-023-39129-6
19. Li C, Ni S, Sun H, Zhu S, Feng Y, Yang X, Huang Q, Jiang S, Tang N (2023) Effects of PM 2.5 and high-fat diet interaction on blood glucose metabolism in adolescent male Wistar rats: A serum metabolomics analysis based on ultra-high performance liquid chromatography/mass spectrometry. Ecotoxicology and environmental safety 262:115200. https://doi.org/10.1016/j.ecoenv.2023.115200
20. Duc Nguyen H, Hoang NMH, Jo WH, Ham JR, Lee MK, Kim MS (2022) Associations among the TREM-1 Pathway, Tau Hyperphosphorylation, Prolactin Expression, and Metformin in Diabetes Mice. Neuroimmunomodulation 29:359-368. https://doi.org/10.1159/000521013
21. Li S, Wang K, Xu G, Ji J, Qin C (2016) The intervention effect of zinc supplementation on irbesartan treatment for atherosclerosis of ApoE-/- mice. Pak J Pharm Sci 29:1907-1912.
22. Zhang J, Jiang J, Li H, Deng J, Dong W, Deng H (2025) MAM-Mediated Mitochondrial Ca 2+ Overload and Endoplasmic Reticulum Stress Aggravates Synaptic Plasticity Impairment in Diabetic Mice. Brain Sci 15. https://doi.org/10.3390/brainsci15111157
23. Martinez-Morata I, Dennehy K, Chepurny O, Delibasi T, Schilling K, Navas-Acien A, El Muayed M (2025) Type 2 diabetes mellitus and zinc dysregulation: From basic and epidemiological evidence to interventions. J Trace Elem Med Biol 91:127733. https://doi.org/10.1016/j.jtemb.2025.127733
24. Ye Z, Cheng M, Fan L, Ma J, Zhang Y, Gu P, Xie Y, You X, Zhou M, Wang B *et al* (2023) Plasma microRNA expression profiles associated with zinc exposure and type 2 diabetes mellitus: Exploring potential role of miR-144-3p in zinc-induced insulin resistance. Environ Int 172:107807. https://doi.org/10.1016/j.envint.2023.107807

25. Bandeira VDS, Pires LV, Hashimoto LL, Alencar LL, Almondes KGS, Lottenberg SA, Cozzolino SMF (2017) Association of reduced zinc status with poor glycemic control in individuals with type 2 diabetes mellitus. J Trace Elem Med Biol 44:132-136. https://doi.org/10.1016/j.jtemb.2017.07.004
26. Akgun-Unal N, Unal O, Altun G, Gulbahce-Mutlu E, Akkoca A, Ayyildiz M (2026) The Effect of Zinc Sulfate Treatment on Diabetic Cardiomyopathy in an Aged Female Rat Model of Type 2 Diabetes. Nutrients 18. https://doi.org/10.3390/nu18122005
27. Ammar HI, Shamseldeen AM, Shoukry HS, Ashour H, Kamar SS, Rashed LA, Fadel M, Srivastava A, Dhingra S (2021) Metformin impairs homing ability and efficacy of mesenchymal stem cells for cardiac repair in streptozotocin-induced diabetic cardiomyopathy in rats. Am J Physiol Heart Circ Physiol 320:H1290-H1302. https://doi.org/10.1152/ajpheart.00317.2020
28. Geraldes P, Hiraoka-Yamamoto J, Matsumoto M, Clermont A, Leitges M, Marette A, Aiello LP, Kern TS, King GL (2009) Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy. Nat Med 15:1298-1306. https://doi.org/10.1038/nm.2052
29. Yang Z, Liu H, Wei J, Liu R, Zhang J, Sun M, Shen C, Liu J, Men K, Chen Y *et al* (2024) Bisphenol mixtures, metal mixtures and type 2 diabetes mellitus: Insights from metabolite profiling. Environ Int 190:108921. https://doi.org/10.1016/j.envint.2024.108921
30. Li H, Zhang M, Ma J, Li W, Liu X, Li Y, Ma J, Yang D, Tie Y, Bai H *et al* (2025) Zinc Combined with Metformin Corrects Zinc Homeostasis and Improves Steroid Synthesis and Semen Quality in Male Type 2 Diabetic Mice by Activating PI3K/AKT/mTOR Pathway. Biol Trace Elem Res 203:4659-4670. https://doi.org/10.1007/s12011-025-04518-z
31. Souza-Tavares H, Miranda CS, Vasques-Monteiro IML, Sandoval C, Santana-Oliveira DA, Silva-Veiga FM, Fernandes-da-Silva A, Souza-Mello V (2023) Peroxisome proliferator-activated receptors as targets to treat metabolic diseases: Focus on the adipose tissue, liver, and pancreas. World journal of gastroenterology 29:4136-4155. https://doi.org/10.3748/wjg.v29.i26.4136
32. Hayashi Y, Kinoshita K, Lu TM, Tseng HY, Maki K, Kimura S, Yano E, Saeki A, Yasukochi A, Minami K *et al* (2026) Inhibitor of DNA binding/differentiation 4 deficiency impairs hepatic fatty acid synthesis and is associated with epigenomic alterations in chromatin accessibility. Molecular metabolism 111:102416. https://doi.org/10.1016/j.molmet.2026.102416
33. Chen C, Bu F, Gao Y, Wang Y, Zhang H, Gao J, Yuan J, Wang Z, Zhong Y, Jiang Q *et al* (2026) Perfluorooctanoic acid-induced exacerbation of myocardial ischemia-reperfusion injury: Roles of peroxisome proliferation activated receptor alpha-related fatty acid metabolism. Ecotoxicology and environmental safety 323:120602. https://doi.org/10.1016/j.ecoenv.2026.120602
34. Hernández-Martín M, Garcimartín A, Bocanegra A, Redondo-Castillejo R, Quevedo-Torremocha C, Macho-González A, García Fernández RA, Bastida S, Benedí J, Sánchez-Muniz FJ *et al* (2024) Silicon as a Functional Meat Ingredient Improves Jejunal and Hepatic Cholesterol Homeostasis in a Late-Stage Type 2 Diabetes Mellitus Rat Model. Foods (Basel, Switzerland) 13. https://doi.org/10.3390/foods13121794

35. Xu M, Rui D, Yan Y, Xu S, Niu Q, Feng G, Wang Y, Li S, Jing M (2017) Oxidative Damage Induced by Arsenic in Mice or Rats: A Systematic Review and Meta-Analysis. Biol Trace Elem Res 176:154-175. https://doi.org/10.1007/s12011-016-0810-4
36. Jaikumkao K, Thongnak L, Htun KT, Pengrattanachot N, Phengpol N, Sutthasupha P, Promsan S, Montha N, Sriburee S, Kothan S *et al* (2024) Dapagliflozin and metformin in combination ameliorates diabetic nephropathy by suppressing oxidative stress, inflammation, and apoptosis and activating autophagy in diabetic rats. Biochim Biophys Acta Mol Basis Dis 1870:166912. https://doi.org/10.1016/j.bbadis.2023.166912
37. Nassif RM, Chalhoub E, Chedid P, Hurtado-Nedelec M, Raya E, Dang PM, Marie JC, El-Benna J (2022) Metformin Inhibits ROS Production by Human M2 Macrophages via the Activation of AMPK. Biomedicines 10. https://doi.org/10.3390/biomedicines10020319
38. Hattori Y, Suzuki K, Hattori S, Kasai K (2006) Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension (Dallas, Tex : 1979) 47:1183-1188. https://doi.org/10.1161/01.Hyp.0000221429.94591.72
39. Jarosz M, Olbert M, Wyszogrodzka G, Młyniec K, Librowski T (2017) Antioxidant and anti-inflammatory effects of zinc. Zinc-dependent NF-κB signaling. Inflammopharmacology 25:11-24. https://doi.org/10.1007/s10787-017-0309-4
40. Zimmermann GR, Lehár J, Keith CT (2007) Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug discovery today 12:34-42. https://doi.org/10.1016/j.drudis.2006.11.008

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### Author Contributions

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### Data Availability

The datasets generated and analyzed in this study are available from the corresponding author upon reasonable request. All animal experimental procedures were approved by the Animal Welfare and Ethics Committee of Sichuan Agricultural University (Approval No. 20230436).

### Consent to participate

Informed consent was obtained from all individual participants included in the study.

### Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in all Figures.