Profile of bile acids-FXR-FGF15 pathway in the glycolipid metabolism disorder of diabetic mice suffered with chronic stress

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Background. The development of diabetes and depression is related to imbalance of bile acids (BAs) synthesis and metabolism in rodents and humans. However, the role of BAs and their receptor FXR (farnesoid X receptor)/FGF15 (Fibroblast Growth Factor 15) signal pathway in diabetes and depression comorbidity remains largely unknown. This study was aimed to investigate the BAs-associated potential molecular mechanisms underlying glycolipid metabolism disorder in diabetic mice suffered with chronic stress. **Methods.** Type 2 Diabetes Mellitus (T2DM) mice model was first induced by high-fat diet and intraperitoneal injection of streptozotocin (STZ). Forty mice were randomly divided into two groups: the normal chow feeding group and the high-fat diet feeding group. After 2 weeks of feeding, the mice were therefore randomly divided into 4 groups: Control group, CUMS group, T2DM group, and T2DM+CUMS group. The T2DM group and T2DM+CUMS groups were intraperitoneal injection of STZ to induce T2DM model. The CUMS and T2DM+CUMS groups were exposed with chronic unpredictable mile stress (CUMS) to induce depression-like phenotype. Blood and tissue samples were obtained for relevant analysis and detection. **Results.** Compared with T2DM group mice, T2DM+CUMS group mice had higher blood glucose and lipid levels, insulin resistance, inflammation of the liver and pancreas, meanwhile further impaired liver function, and increased total bile acids. These changes were accompanied by inhibition of FXR signaling. Compared to those in T2DM group mice, chronic stress more inhibited FXR expression and its downstream target FGF15 in the ileum. **Conclusion.** FXR might play a role in diabetic glycolipid metabolism disorder aggravated by chronic stress. FXR and its downstream target FGF15 might be therapeutic targets for T2DM and depression comorbidity treatment.

Profile of Bile Acids-FXR-FGF15 Pathway in the Glycolipid Metabolism Disorder of Diabetic Mice Suffered with Chronic Stress

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Abstract

Background. The development of diabetes and depression is related to imbalance of bile acids (BAs) synthesis and metabolism in rodents and humans. However, the role of BAs and their receptor FXR (farnesoid X receptor)/FGF15 (Fibroblast Growth Factor 15) signal pathway in diabetes and depression comorbidity remains largely unknown. This study was aimed to investigate the BAs-associated potential molecular mechanisms underlying glycolipid metabolism disorder in diabetic mice suffered with chronic stress.

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Results. Compared with T2DM group mice, T2DM+CUMS group mice had higher blood glucose and lipid levels, insulin resistance, inflammation of the liver and pancreas, meanwhile further impaired liver function, and increased total bile acids. These changes were accompanied

by inhibition of FXR signaling. Compared to those in T2DM group mice, chronic stress more inhibited FXR expression and its downstream target FGF15 in the ileum.
Conclusion. FXR might play a role in diabetic glycolipid metabolism disorder aggravated by chronic stress. FXR and its downstream target FGF15 might be therapeutic targets for T2DM and depression comorbidity treatment.

Introduction

According to a report by the International Diabetes Federation (IDF), 10.5% (537 million) of the world will have diabetes in 2021 and it is expected to increase to 12.2% (783 million) by 2045 (Sun et al. 2022). Among them 30-40% of patients with diabetes will develop at least one complication within 10 years. Traditionally complications associated with diabetes include macrovascular disease as well as microvascular disease (Viigimaa et al. 2020). Currently, as people with diabetes live longer and longer, new complications emerge, including cancer, infections, psychological and mental disorders (Harding et al. 2019a). Patients with diabetes are at increased risk for major depression (Ali et al. 2006), anxiety (Fisher et al. 2008), and serious mental illnesses such as schizophrenia (Vancampfort et al. 2016). One in 4 patients with type 2 diabetes will develop major depression. Patients with diabetes usually have a higher risk of depression compared to those with normal blood glucose (Semenkovich et al. 2015). A systematic review concluded that the mean prevalence of depression in patients with type 2 diabetes was 28% (Harding et al. 2019b), compared with a global prevalence of depression of approximately 13% in the general population (Lim et al. 2018).

Many factors contribute to the development of diabetes and depression comorbidity. These factors include not only common pathophysiologic factors, but also social and psychological factors, such as financial burden of treatment, fear of disability due to the disease, and strict dietary control or exercise requirements (Petrak et al. 2015). Additional, patients with diabetes who developed depression comorbidity often present poorer long-term glycemic control (Heckbert et al. 2010), non-compliance with medical treatment (Lin et al. 2006), and poorer metabolic control (Egede & Ellis 2010).

The diabetes and depression comorbidity has been recognized as an emerging global challenge (Fisher et al. 2012). Therefore, it is very important to study the pathogenesis of diabetes and depression comorbidity. Currently, the role of bile acids (BAs) in development of many diseases has become a hot topic. Bile acids are proved to be important signaling molecules that can act in various tissues of the body and participate in regulating the body's glucolipid metabolism and homeostasis (Perino & Schoonjans 2022). The bile acid receptor FXR (or NR1H4), and other ligand-activated nuclear receptors (pregnancy X receptor, vitamin D receptor and the membrane receptor TGR5) may all be involved in bile acid signaling (Sun et al. 2018).

Evidence documented that the development of either diabetes or depression is related to the bile acid signaling pathway. On the one side, there is an increased tendency of serum total bile acid

(TBA) levels in patients with type 2 diabetes compared to the normal population (Sonne et al. 2016).The serum bile acid concentrations were significantly higher in type 2 diabetic patients than those in the non-diabetic population and displayed a positive correlation with insulin resistance (Sun et al. 2016). The postprandial plasma concentrations of single bile acids and FGF19 were significantly increased in type 2 diabetic patients compared to non-type 2 diabetic patients (Sonne et al. 2016). Bile acids regulate glucose homeostasis by acting directly on FXR and TGR5 in the intestine, liver, and pancreas as well as by promoting FXR and inducing intestinal FGF15/19. FXR inhibits hepatic glycolysis and lipogenesis and reduces postprandial glucose utilization (Caron et al. 2013; Duran-Sandoval et al. 2005; Watanabe et al. 2004a). FXR and FGF15 play an important role in the development of diabetes (Yan et al. 2021).

On the other side in the last years it has become increasingly evident that bile acids affect brain function, during normal physiological and pathological conditions (Mertens et al. 2017). Although bile acids may be synthesized locally in the brain (Kiriyama & Nochi 2019), the majority of brain bile acids are taken up from the systemic circulation (Monteiro-Cardoso et al. 2021), which are mainly metabolites of the liver and intestinal microbiota. Therefore, bile acids in the systemic circulation can directly or indirectly affect central processes and thus be involved in the neuropathological processes of depression (Lirong et al. 2022).

In chronic stress-induced depressed rats the serum level of glycocholic acid (GCA) was elevated but that of bile acids (CA) was decreased compared to normal control rats (Zhu et al. 2020). In a clinical study of patients with Crohn's disease (CD), a positive correlation between GCA and both the Zung's Self-Assessment Scale for Anxiety (SAS) and the Self-Rating Scale for Depression (SDS) was observed (Feng et al. 2022). However, inconsistent result has also been reported, with one study showing a significant increase in blood cholic acids (CAs) in a chronic restraint-induced depression model mice (Zhang et al. 2010). Research found that FXR altered the bile acids blood concentration and the composition in the brain through FXR knockout mice, contributing to the regulation of homeostasis of multiple neurotransmitter systems in different brain regions and modulating neurobehavior (Huang et al. 2015). FXR expression was significantly downregulated in the medial prefrontal cortex of chronic social defeat stressed mice (Bao et al. 2021). These studies suggested a role for FXR in the development of depression and could be a potential and novel therapeutic target.

Notwithstanding, the profile of BAs and their receptor FXR/FGF15 signal pathway in diabetes and depression comorbidity remains largely unknown. This study was aimed to investigate the BAs-associated potential molecular mechanisms underlying glycolipid metabolism disorder aggravated with chronic stress in diabetic mice.

Materials & Methods

Animals

Male 5-week-old C57BL/6 mice (Animal Certificate No.: SCXK (Su) 2020-0009 from Jiangsu Huachuang Sino Pharma Tech Could., Ltd., Jiangsu, China) were used in this study. All of the animals were raised in a standardized animal room (temperature 22 ± 2 °C, lights on from 6 a.m.~ 6 p.m.), with free access to clean boiled water and rodent chow.

The high fed streptozotocin mice model

After two weeks of acclimation, the C57BL/6 mice were randomly assigned to two groups by random number table, one of which was fed with a chow diet (D12450J, 10% fat, 70% carbohydrate, 20% protein, purchased from Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd., Jiangsu, China), and the other a high-fat diet (D12492, 60% fat, 20% carbohydrate, and 20% protein, purchased from Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd., Jiangsu, China). After four weeks, the high-fat diet group received two-doses of streptozotocin (STZ, Sigma-Aldrich Co., St. Louis, Missouri, USA) intraperitoneally (80 mg/kg/d with an interval of two days) in 15 minutes of dissolution according to the adapted protocol(Furman 2021). The STZ powder was prepared immediately before use with a 0.1 mol/L, pH 4.5 citric acid-sodium citrate buffer (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China). Diabetes was verified 10 days after the last STZ administration by quantifying blood glucose levels by means of a Contour plus blood glucose monitoring system (Bayer, Leverkusen, Germany) through blood sample obtained from the tail vein. Mice with blood glucose concentrations higher than 13.9 mmol/L were considered to be diabetic and used to establish a chronic unpredictable mild stress (CUMS) model.

Chronic unpredictable mild stress protocol and experimental designs

Diabetic mice that met blood glucose requirements were included in the development of a depression model, which was used by CUMS to induce a depression-like phenotype in C57BL/6 mice. The CUMS procedure was adapted from the protocol (Nollet 2021). Briefly, it lasted for four weeks after the T2DM model was established, with diverse randomly assigned stressors: Damp sawdust for 24 hours; no sawdust for 24 hours; cage tilting for 24 hours; restraint stress for 2 hours; cycle disturbances; swimming at 4 °C for 5 minutes; cage vibration for 20 minutes; noise interference for 1.5 hours. During the stress procedure, two unexpected stressors were performed per day, and no single stressor was performed consecutively for two days. Appendix-table 1 shows the schedule of the stressors. Every mouse subjected to CUMS were housed separately in single cage, and others were housed in groups. After four weeks of CUMS, the mice were divided into four groups: Control, CUMS, T2DM, and T2DM+CUMS (n=10 per group). Fifty mice were utilized for this study and forty were included. Ten mice were excluded, including eight mice which did not meet the blood glucose requirements and another three mice were exclude because of technical failure during CUMS treatment.

Validation of CUMS mice model

Open field test

The mice (n=10 per group) were placed into the open-field chambers with a video capture system (50×50×40 cm^3, L×W×H) from the same position in turn, and Tracking master V3.0 software (Beijing Zhongshi Dichuang Technology Development Co., Ltd., Beijing, China) was opened to automatically record the mice's activities inside the chamber for 5 minutes. To avoid odor interference, the chambers were sprayed with 75% ethanol after each mouse test. Throughout the experiment, the light in the room was kept consistent and quiet.

Tail suspension test

The mice (n=10 per group) were attached to the hook with medical tape at 3/4 of the tail and suspended in an inverted suspension position. The suspended mouse was about 30 cm from the chamber's bottom, with a camera placed at the level of the suspension device. Tracking master V3.0 software (Beijing Zhongshi Dichuang Technology Development Co., Ltd., Beijing, China) was launched, and the immobility time of the mice in the last 4 minutes of 6 minutes was automatically recorded.

Forced swimming test

The mice (n=10 per group) were placed in a cylindrical bucket with a diameter of 10 cm and a height of 25 cm, and the water level in the bucket was such that the mice could stretch their entire bodies without their tails touching the bottom of the bucket (the water temperature was 23~25°C, and the water level was 15 cm high). Tracking master V3.0 software (Beijing Zhongshi Dichuang Technology Development Co., Ltd., Beijing, China) was initiated, and the mice’s immobility time in the last 4 minutes within 5 minutes was recorded (the mice were considered immobile when they were floating on the water surface and their limbs were not moving or their limbs were slightly paddling).

Sucrose preference test

During the experiment, each animal (n=10 per group) would be given two identical bottles: one containing clean boiled water and the other containing a 1% sucrose solution. Following the 15h test, liquid consumption was measured. Sucrose preference proportion = sucrose solution consumption / (sucrose solution consumption + boiled water consumption) ×100%.

Sacrifice and samples collection

Mice were sacrificed under anesthesia with 4% chloral hydrate after the behavioral tests were completed. Blood was collected, coagulated at room temperature for 30 minutes, and centrifuged at 3000 rpm for 15 minutes.

The supernatant serum was separated and divided into several vials before being stored at -80°C. Livers and ilea were dissected and immediately frozen in liquid nitrogen after cardiac perfusion. The liver was weighed before being cut into two parts, one fixed with cold 4% paraformaldehyde and the other flash frozen and stored at -80 °C, while the pancreas was fixed with cold 4% paraformaldehyde. Serum samples would be tested for serum lipids, liver function, and insulin levels. Western blotting was performed on frozen livers and ilea. Immunohistochemistry was performed on fixed livers and pancreas.

Serum lipids and liver function

Serum levels of glutamic oxaloacetic transaminase (ALT), glutamic alanine transaminase (AST), total cholesterol (CHO), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), non-esterified fatty acid (NEFA), and blood glucose (GLU) were measured by a Beckman Coulter biochemical analysis system (AU5800).

Enzyme-linked immunosorbent assays

Glycated hemoglobin A1c(GHbA1c) concentrations in lysate for red blood cell and serum insulin levels were measured by Mouse Glycated Hemoglobin A1c(GHbA1c) ELISA Kit (#CSB-E08141m, CUSABIO, Wuhan, China, https://www.cusabio.com/) and Mouse Insulin ELISA kit (#KE10089, Proteintech Group, Inc., Wuhan, China) according to the manufacturer's instructions.

Western blotting

RIPA was used to extract total proteins from liver and ileum samples (n=3 per group), and the extracts were used to detect FXR, SHP, and FGF15 protein expression. Protein concentration was determined using the BCA method (Yoche Biotechnology, Shanghai, China), and proteins were denatured sequentially using gel electrophoresis and wet membrane transfer. After 2 hours of blocking at room temperature, diluted primary antibodies of FXR (1:1000, Cell Signaling Technology, Inc., Boston, USA), FGF15 (1:1000, Santa Cruz Biotechnology, Inc., California, USA), SHP (1:500, ABclonal Technology Co., Ltd., Wuhan, China), GAPDH (1:10,000, ABclonal Technology Co., Ltd., Wuhan, China), and β-actin (1:1000, ImmunoWay Biotechnology, Plano, TX, USA) were added for overnight incubation at 4°C. After recovering the primary antibodies, the membrane was washed three times with TBST (Sangon Biotech, Shanghai, China) for 10 minutes each, and the secondary antibodies (1:5,000, ImmunoWay Biotechnology, Plano, TX, USA) were added to incubate for 1 hour at room temperature. The ECL reagent was used for image development after washing the membrane, and the protein bands were analyzed using Image J software.

Total RNA extraction and quantification by qPCR

TRIzol reagent (Thermo fisher scientific, MA, USA) was used to extract total RNA from mouse liver and ileum tissues (n=3 per group). The products were then reverse transcribed using the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (AG11728, Accurate Biotechnology, Hunan, Co., Ltd., Hunan, China). qPCR with reverse transcription was performed using the SYBR Green Premix Pro Taq HS qPCR Kit (AG11718, Accurate Biotechnology, Hunan, Co., Ltd., Hunan, China) on Applied Biosystems Quant Studio 6 Flex apparatus (Thermo fisher scientific, MA, USA), and the Ct values were calculated using the 2-ΔΔCt method to analyze the relative gene expression level. Appendix-table 2 shows the forward and reverse sequences of the qPCR primers that were designed and synthesized (Sangon Biotech, Shanghai, China). All the

procedures were carried out in accordance with the manufacturer's instructions. The target gene values were normalized to ACTB and the relative expression levels were displayed as fold changes relative to control group.

Histopathological Analysis

Dehydrated liver specimens (n=3-4 per group) were embedded in paraffin, cut into 4~6 μm sections, and stained with H&E for morphological examination. Oil Red O staining revealed lipid deposition in the liver. Frozen liver sections were air-dried at room temperature for 2 hours before being fixed for 5 minutes with 4% paraformaldehyde. To remove excess water, the sections were washed and rinsed in 60% isopropanol. The sections were then incubated in Oil Red O reagents for 10 minutes before being removed from the solution with 60% isopropanol. Hematoxylin was used to stain the nuclei for 5 minutes before washing them with water.

Statistical analysis

Graphpad 9.3.0 statistical software was used for the analysis. The experimental data were presented as mean ± standard error (SEM). All data were analyzed with unpaired two-tailed Student's t-test (for data from two groups) and one-way ANOVA followed by Tukey's multiple comparison post hoc test (for data from more than two groups). A difference of $P<0.05$ indicates a statistically significant difference.

Results

Type 2 diabetic mice with depression-like phenotype comorbidity model validation

Mice in the experimental group were subjected to 4 weeks of high-fat chow intervention and STZ via intraperitoneal injection after acclimation and randomization to create a T2DM model. The diabetic model mice that met the glycemic requirements were then included in the 4-week CUMS process to establish a depression-like phenotype (Figure 1A).

Behavioral analysis was used to verify whether the depression-like phenotype was successfully induced in diabetic mice. In the open field test, the distance and duration of activity in the central area were significantly shorter in the CUMS group than in the Control group. However, the T2DM+CUMS group showed shorter distance and duration of activity in the central area and less total traveled distance compared to the T2DM group (Figure 1B). In the tail suspension and forced swimming tests, CUMS mice presented a longer immobility time when compared to the Control group. Mice in the T2DM+CUMS group also exhibited significantly longer immobility time for tail suspension and forced swimming compared to the T2DM group (Figure 1C, D). In the sucrose preference test, CUMS or T2DM mice showed a lower percentage of sucrose preference compared to the Control group, while T2DM+CUMS showed a lower level of sucrose preference compared to CUMS (Figure 1E).

Type 2 diabetic mice with depression-like phenotype in glycolipid metabolism

At the end of the CUMS protocol, metabolic measurements in vivo, including body weight, fasting blood glucose and serum lipids (CHO, TG, LDL, HDL, NEFA), were performed to assess the effect of T2DM and depression comorbidity on glycolipid metabolism. All stressed mice had significantly lower body weight gains and unstable blood glucose levels (Figure 2A, C). There was no significant difference in body weight among the four groups of mice, but the body weight of mice in the T2DM group was slightly higher than that in the T2DM+CUMS group (Figure 2B). Fasting blood glucose was measured after the CUMS model was established, and it was discovered that T2DM mice had higher blood glucose levels than Control mice. However, there was no significant difference between the mice in the T2DM+CUMS group and the mice in the T2DM group (Figure 2D). When serum lipids were compared, it was discovered that there were lipid disorders among these groups. T2DM was linked to higher levels of serum CHO, LDL, HDL and NEFA (Figure 2E, G-I). Serum CHO, TG, LDL, and NEFA levels were significantly higher in T2DM+CUMS mice than in T2DM mice (Figure 2E-H). In terms of the GHbA1c test, T2DM+CUMS mice had higher GHbA1c levels than CUMS mice, but there was no significant difference when compared to T2DM mice (Figure 2J).

Insulin-related indicators and histopathological analysis of the pancreas in type 2 diabetic mice with depression-like phenotype

As stated, serum glucose levels in CUMS and T2DM mice were higher than that in Control. Furthermore, the T2DM+CUMS group had higher serum glucose levels than the CUMS or T2DM group (Figure 3A). What's more, in the insulin test, it was easily discovered that in fasting conditions, CUMS mice showed a significant increase in fasting insulin (Figure 3B). Meanwhile, T2DM+CUMS group showed slightly increase than T2DM group (Figure 3B). Although not statistically significant, the HOMA-IR index increased in stressed mice, particularly in the CUMS and T2DM groups when compared to Control mice. T2DM+CUMS mice also had a higher HOMA-IR index than T2DM mice (Figure 3C). For the HOMA-β index, the T2DM group had a lower HOMA-β index than the Control group, but there was no significant difference in HOMA-β index of T2DM+CUMS when compared to T2DM (Figure 3D).
The pancreatic histomorphology revealed that the islets of the Control group mice were round or oval cell clusters of various sizes, complete and regular structures, clear edges, and scattered among the pancreatic vesicles, with more cell clusters and abundant β-cells in the islets, uniform in size, full and tightly arranged, whereas the islets of the CUMS group mice were swollen and deformed, with light cytoplasmic staining or vacuolation. The number of islet cell clusters decreased in the T2DM group, along with shrunken islet area, irregular morphology, unclear islet margin, disorganized structure, swollen and deformed islet cells, light or vacuolated cytoplasmic staining, and obvious nuclear consolidation or nuclear loss. Besides that, some of the exocrine gland vesicles were deep inside the islet, whereas in the T2DM+CUMS group, the islet area was severely shrunk, the morphology was irregular, the islet cells were swollen and deformed, and the cytoplasmic staining was light or vacuolated when compared to T2DM mice (Figure 3E).

Liver function, total bile acids and histopathological analysis of the liver in type 2 diabetic mice with depression-like phenotype

We found that comorbidity aggravated liver steatosis, lobular inflammation by liver pathological analysis (Figure 4A-C). In Control group, hepatic lobules and hepatic cords were irregularly arranged, hepatocytes were unequal in size, hepatocytes were cloudy and swollen, and there was a small amount of inflammatory cell infiltration in the confluent area. Hepatic lobules and cords were irregularly arranged in the CUMS group, hepatocytes were unequal in size, hepatocytes were cloudy and swollen with partial loss of nuclei, and inflammatory cells were infiltrated in the confluent area, indicating moderate lesions. Hepatic lobules and cords were irregularly arranged in T2DM mice, hepatocytes were unequal in size, hepatocytes were degenerated, and balloon-like changes were visible, indicating mild to moderate steatosis. Furthermore, the T2DM+CUMS group had unequally arranged lobules and hepatic cords, as well as hepatocytes that were degenerated and ballooned, indicating moderate to severe steatosis (see Figure 4A). Then, we investigated the effect of comorbidity on liver steatosis and inflammation, but there was no significant difference in inflammation and steatosis scores in the liver of the T2DM+CUMS group compared to the T2DM group. In addition, comorbidity increased lipid accumulation in the liver as assessed by Oil red O staining (Figure 4D, E). Moreover, comorbidity increased the liver/body weight of mice in CUMS group or T2DM group (Figure 4F). We then measured ALT and AST levels in the liver, and comorbidity increased both levels (Figure 4G, H). Total bile acid was determined in four groups, it was higher in T2DM+CUMS group than T2DM group (Figure 4I). Taken together, our data suggest that comorbidity worsens liver function, hepatic steatosis and inflammation, as well as increases total bile acids in T2DM.

T2DM and depression-like phenotype regulated by FXR/SHP/FGF15

Compared with the T2DM group, the hepatic FXR mRNA level was slightly lower in the T2DM+CUMS group, but the SHP mRNA level was slightly higher in the liver (Figure 5A, B). T2DM+CUMS group had lower protein level of hepatic FXR than T2DM group. However, in the liver, an increasing tendency in the protein level of SHP were found (Figure 5C).
The level of ileal FXR mRNA expression was slightly lower in T2DM+CUMS mice than in T2DM mice. In the T2DM+CUMS group, ileal FGF15 mRNA expression was significantly lower than in the T2DM group (Figure 5D, E). When compared to the T2DM group, ileal FXR protein level was significantly lower in the T2DM+CUMS group. Furthermore, it showed that a lower ileal FGF15 protein level in T2DM+CUMS group (Figure 5F).

Discussion

The comorbidity of mental and physical diseases is a major challenge in the world of health care. The comorbidity of depression and diabetes is a typical example of mental/physical comorbidity, with the prevalence of which is increasing and likely to continue to increase due to increasing life expectancy and various other causes. Patients with diabetes are prone to have depression during the disease progression, and they tend to have the following characteristics: female

gender, younger and/or older age, individuals living alone, poor social support, low socioeconomic status, poor sleep and lack of physical exercises and diet (Agardh et al. 2011; Bădescu et al. 2016; Sartorius 2018). Besides, a systematic review indicated that the prevalence rate of depression is nearly more than twice as high in people with type 2 diabetes (19.1%, range 6.5-33% vs. 10.7%, range 3.8-19.4%) compared to those without (Roy & Lloyd 2012). And the growing evidence also showed that depression and type 2 diabetes share a common biological origin, in particular an overactivation of innate immunity leading to a cytokine-mediated inflammatory response with possible abnormalities through the regulation of the hypothalamic-pituitary-adrenal axis(Moulton et al. 2015). Moreover, the development of diabetes and depression is associated with dysregulation of bile acid synthesis and metabolism in rodents or humans (Feng et al. 2022; Lirong et al. 2022; Sonne et al. 2016; Sun et al. 2016; Zhu et al. 2020). As an important component of the liver-gut-brain axis, studies have also confirmed the key role of the bile acid nuclear receptor FXR in diabetes or depression (Bao et al. 2021; Caron et al. 2013; Duran-Sandoval et al. 2005; Huang et al. 2015; Watanabe et al. 2004a; Yan et al. 2021). In summary, there exists a strong association between diabetes and depression.

Although studies indicated that bile acid nuclear receptor FXR might play an important role in diabetes or depression, there are fewer in-depth studies on the liver-gut axis FXR and its target genes SHP and FGF15 regulating the comorbidity of diabetes and depression. Therefore, we set out to elucidate the role of FXR and its target genes SHP and FGF15 in the development of diabetic mice aggravated by chronic stress.

The C57BL/6 mouse strain is highly sensitive to the metabolic effects of high-fat feeding, and it is a simple model to obtain(Luo J 1998). In addition, the use of STZ restimulation to reduce the number of β cells and further mimic T2DM has been investigated in the past(Gilbert et al. 2011; Podrini et al. 2013; Shao et al. 2014). In this study, we successfully established a T2DM mice model using 4-week high-fed diet combined with STZ (i.p.). T2DM mice were continued to the establishment of CUMS after being tested for fasting blood glucose levels. Behavioral tests validated the CUMS model, and the T2DM mice with depression-like phenotype mice model was well established. In sucrose preference test, it showed that T2DM mice got lower sucrose preference rate when comparing with Control, which indicated that T2DM mice developed a depression-like phenotype during the course of the disease, just as reported(Hai-Na et al. 2020). In addition, it was found that CUMS exacerbated the depression-like phenotype in diabetic mice. The diabetic mice developed a depression-like phenotype in the development of the disease, which further worsened when CUMS was continued to be given.

We discovered a bidirectional predisposing risk between T2DM and depression in a 4-week CUMS mouse model. We took serum samples from mice to look for relevant biochemical indicators. Indeed, chronic stress increased CHO, TG, HDL, LDL, and NEFA levels of diabetic mice, while diabetes also increased CHO, TG, LDL, HDL and NEFA levels in stressed mice. It

is generally accepted that diabetic mice disturb serum lipid profile with higher TC, TG and LDL-C levels and lower HDL-C level (Manickam et al. 2022). However, the lipid profile of diabetic mice was not unanimously reflected in the literature. Interestingly, in our study, we found that diabetic mice show elevated HDL, which in line with some studies (Ivanovic et al. 2015; Qin et al. 2018).

Insulin resistance and β cell failure are hallmarks of T2DM(Defronzo 2009). Insulin resistance is defined as impaired peripheral tissue response to insulin stimulation, resulting in elevated peripheral insulin levels(Polidori et al. 2022). It has a bidirectional causal interaction with depression. Insulin resistance raises the risk of depression and worsens it, and depression raises the risk of insulin resistance and worsens it(Watson et al. 2018). A meta-analysis study found that insulin levels and the HOMA-IR index were higher in patients with acute depression(Fernandes et al. 2022). In our study, stressed mice in the CUMS or T2DM+CUMS groups developed insulin resistance. The main physiological function of β-cells is insulin synthesis and secretion. When pancreatic β-cells are dysfunctional and insulin secretion is lacking, blood glucose levels rise dramatically and diabetes develops gradually. Normal pancreatic β-cells are highly sensitive to changes in blood glucose levels, and a decrease in the number of pancreatic β-cells, whether direct or indirect, can directly lead to impaired insulin secretion and β-cell function(Rorsman & Ashcroft 2018). T2DM+CUMS group comorbid lower HOMA-β index were observed in our studies, as our data indicated. Moreover, the number and area of pancreatic islet β-cells were reduced. The gold standard for assessing the concentration of glycemic control is GHbA1c, which reflects the average blood glucose level over the previous 8-12 weeks. The GHbA1c result revealed that it did not raise the concentration of GHbA1c in the presence of comorbid CUMS.

Notably, the diabetes and depression comorbidity mice showed decreased levels in HDL and GHbA1c compared to the diabetic mice, although the differences were not significant. We considered that the lower levels of HDL and GHbA1c might influence by some metabolic enzymes associated with lipid accumulation, and the CUMS exposure might play a role in these mechanisms. However, further researches are needed to verify these.
According to the results of the liver pathology, liver function, liver weight/body weight ratio and total bile acid level, the T2DM+CUMS group had a worsening degree of lesions such as steatosis and inflammatory cell infiltration compared to T2DM or CUMS, and also increased liver/body weight, ALT or AST, total bile acid level, indicating a disturbance of lipid metabolism after the comorbidity of depression and T2DM.

Bile acids are relatively strong detergents, and they are potentially toxic to cells. Therefore, the cellular levels of bile acids must be strictly controlled. It was found that FXR is a major regulator of bile acid homeostasis and may have a role in protecting cells from bile acid toxicity. FXR is a nuclear receptor superfamily ligand-activated member that is primarily expressed in the liver and

ileum and plays an important role in the development of metabolic diseases(Matsubara et al. 2013). FXR activation in the liver has been shown to protect against the development of hepatic steatosis. Hepatic FXR deficiency exacerbates hepatic steatosis in a high cholesterol diet model(Schmitt et al. 2015). FXR activation in the liver reduces hepatic lipid content by decreasing lipogenesis and increasing fatty acid oxidation(Pineda Torra et al. 2003; Watanabe et al. 2004b). FXR-/- mice had increased liver fat accompanied by elevated triglycerides, cholesterol, non-esterified fatty acid and lipoproteins (VLDL and LDL) (Sinal et al. 2000). In contrast, in diabetic (db/db), obese (ob/ob) or wild-type mice, the use of bile acids or FXR agonists reduces plasma triglycerides, fatty acids and cholesterol, as well as reducing hepatic lipid/steatosis (Watanabe et al. 2004b; Zhang et al. 2006). In the present study, we found that reduced hepatic FXR mRNA and protein expression was observed in T2DM+CUMS animal models when compared to T2DM mice, suggesting a possible role for FXR in T2DM or CUMS. Reduced expression of sterol regulatory element binding protein 1c (SREBP1c) by inducing SHP and FXR activation, resulting in decreased expression of adipogenesis-related genes(Watanabe et al. 2004b). In this study, Hepatic FXR protein and mRNA levels were downregulated in T2DM+CUMS groups of mice, while the protein of SHP, the target gene of FXR, in the liver was not significantly different among the T2DM and T2DM+CUMS groups. As opposed to FXR，SHP showed an increased mRNA and protein levels in T2DM+CUMS group than T2DM. In previous study, liver-specific SHP deletion prevents hepatic steatosis and fatty liver development (Akinrotimi et al. 2017). Obviously, CUMS exacerbates hepatic steatosis in diabetic mice possibly through the FXR-SHP pathway. It is suggested that the lipid metabolism disturbance that occurs in diabetic mice after administration of chronic stress-induced depression-like phenotype may be mediated by FXR-SHP pathway.

FXR influences hepatic glucose metabolism in addition to regulating hepatic lipid levels. FXR activation reduces gluconeogenesis while increasing glycolysis(Ma et al. 2006). Insulin resistance was found in FXR-/- mice at an early stage, suggesting a role for FXR in glucose metabolism (Cariou et al. 2006; Zhang et al. 2006). Consistent with this, in wild-type or diabetic db/db or insulin-resistant ob/ob mice, the use of bile acids or FXR-specific agonists increased insulin sensitivity and reduced blood glucose concentrations (Cariou et al. 2006; Zhang et al. 2006). And our results showed the same performance, with downregulation of hepatic FXR protein and mRNA levels in both T2DM and T2DM+CUMS groups of mice. Enterocytes can activate the nuclear receptor FXR, which results in the production of FGF15 (Kliewer & Mangelsdorf 2015). In addition to the enterohepatic cycle, FGF15 can signal in an endocrine manner and is involved in lipid and glucose metabolism (Owen et al. 2015). Previous studies have shown that FGF15 secreted from the ileum after FXR activation has insulin-like effects and inhibits hepatic gluconeogenesis (Kir et al. 2011; Potthoff et al. 2011; Potthoff et al. 2012; Schaap 2012). In the present study, the mRNA and protein expression levels of FGF15 were downregulated in the ileum of both T2DM and T2DM+CUMS groups of mice.

In the brain, only about 20% of BAs are synthesized locally(Monteiro-Cardoso et al. 2021). BAs acts as a ligand for the nuclear receptor FXR. FXR activation in hippocampal tissue has been linked to neurological disorders. Previous research found that FXR expression is significantly downregulated in the medial prefrontal cortex of mice with a depression-like phenotype caused by chronic social defeat stress (CSDS)(Bao et al. 2021). Similarly, it is entirely consistent with the findings of this study. Simultaneously, there was a downward trend following the depression and T2DM comorbidity. Previous studies have found that FXR expression in the medial prefrontal cortex is significantly downregulated in mice with chronic social defeat stress (CSDS)-induced depression-like phenotype(Bao et al. 2021). In the present study, the results showed that mice in T2DM+CUMS group downregulated the expression of hepatic FXR protein and mRNA while upregulated the expression of hepatic SHP protein and mRNA. While in the ileum, mice in T2DM + CUMS group also downregulated FXR protein and mRNA expression, and the mRNA and protein expression of FGF15 also showed a decreasing trend in the T2DM+CUMS group.

Conclusions

To the best of our knowledge, the relationship between the comorbidity of T2DM and depression and activation of FXR or its target genes has not been reported. This study suggests that FXR and its downstream gene FGF15 are critical components in the development of depression-like phenotypes, and it identifies FXR-FGF15 as a potential novel therapeutic target for the treatment of T2DM and depression comorbidity.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Statement

The animal study was reviewed and approved by The Animal Care and Use Committee in Shanghai Sixth People's Hospital.

Author Contributions

Study conception and design: WC, CL and FX; acquisition of data: WC, CL, ZS, JC, ZC, YC and ZG; drafting of the manuscript: WC, CL, FX; critical revision: FX. All authors have read and agreed to the published version of the manuscript.

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730

Figure 1

Type 2 diabetic mice with depression-like phenotype comorbidity model validation

(A) Schedule of T2DM and chronic stress induced depression-like phenotype comorbidity model establishment. (B) Total distance, central distance and central durations in open field test, as well as the tracking map. (C) Immobility durations in tail suspension test. (D) Immobility durations in forced swimming test. (E) Sucrose preference rate in sucrose preference test. Data presented as mean ± SEM, n=10 per group. $^{\#\#}P<0.01$ compared with the Control group, $^{*}P<0.05$, $^{**}P<0.01$ compared with the T2DM+CUMS group.

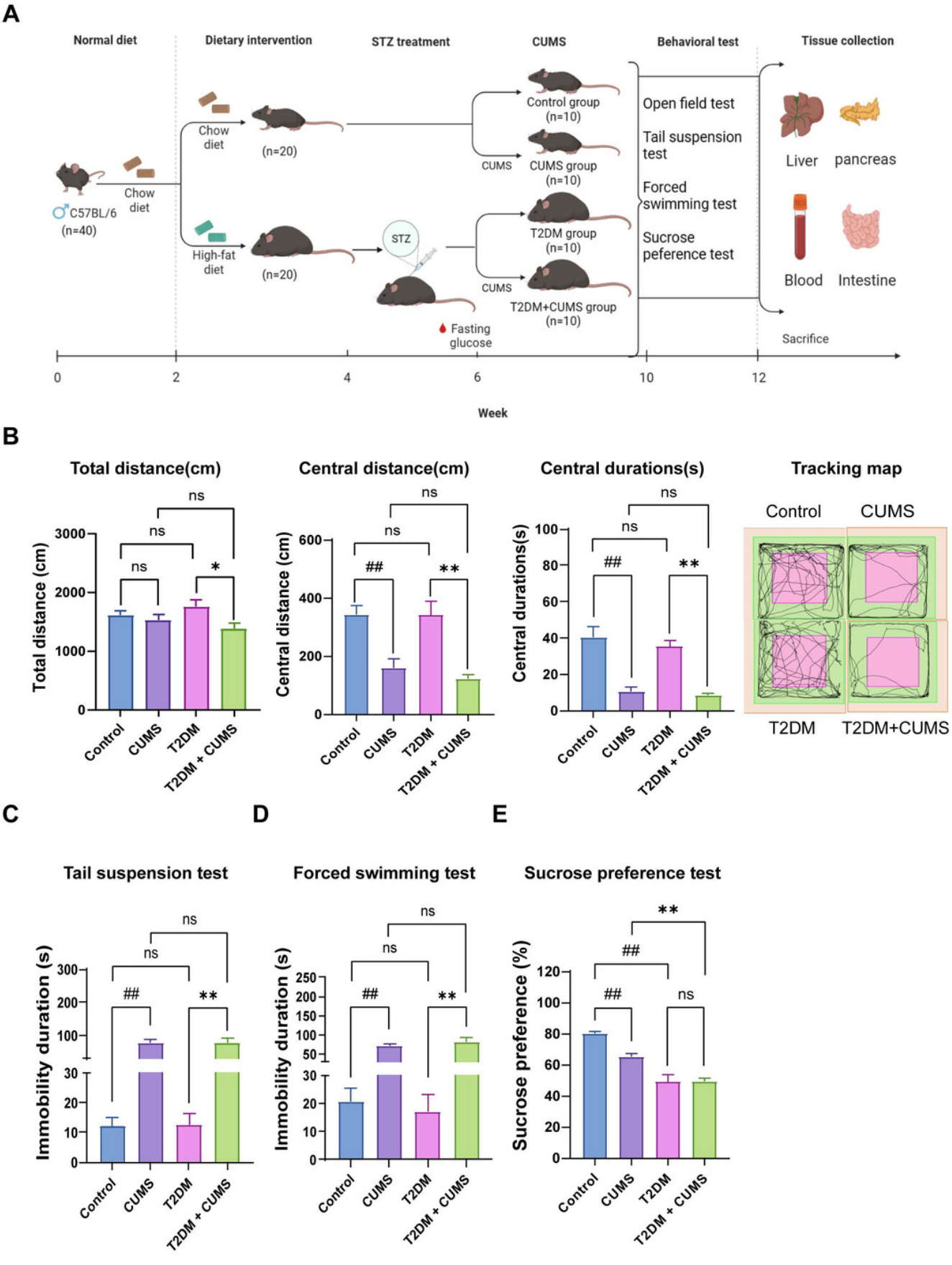

A
Normal diet
Dietary intervention
STZ treatment
CUMS
Behavioral test
Tissue collection
Chow diet
C57BL/6 (n=40)
Chow diet
(n=20)
High-fat diet
(n=20)
STZ
Fasting glucose
Control group (n=10)
CUMS
CUMS group (n=10)
T2DM group (n=10)
CUMS
T2DM+CUMS group (n=10)
Open field test
Tail suspension test
Forced swimming test
Sucrose peference test
Liver
pancreas
Blood
Intestine
Sacrifice
0
2
4
6
10
12
Week
B
Total distance(cm)
Total distance (cm)
Central distance(cm)
Central distance (cm)
Central durations(s)
Central durations(s)
Tracking map
Control
CUMS
T2DM
T2DM+CUMS
C
Tail suspension test
Immobility duration (s)
D
Forced swimming test
Immobility duration (s)
E
Sucrose preference test
Sucrose preference (%)
ns
##
**
*

Table 1(on next page)

The schedule of stressors.

1 **Appendix-table 1. The schedule of stressors.**

2

					Cage vibration Restraint stress	Damp sawdust Cage tilting
Noise No sawdust	Swimming at 4°C Cycle disturbances	Cage tilting Noise	Restraint stress Cage vibration	Damp sawdust Noise	No sawdust Noise	Swimming at 4°C Cycle disturbances
Restraint stress Cage vibration	Swimming at 4°C Cycle disturbances	Cage tilting Noise	Restraint stress Cage vibration	Cycle disturbance Noise	Swimming at 4°C Cycle disturbances	Restraint stress Cage vibration
Cage vibration Cycle disturbances	Damp sawdust Noise	No sawdust Cage vibration	Cage tilting Cycle disturbances	Swimming at 4°C Noise	Restraint stress Cage vibration	Noise Cage tilting

Swimming at 4°C Cycle disturbances	Restraint stress Cage vibration	Cage tilting Noise	Damp sawdust Restraint stress	No sawdust Noise

3

Table 2(on next page)

List of qPCR primer sequences.

Appendix-table 2. List of qPCR primer sequences.

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
FXR	ATGGCAACCAGTCATGTACAGA	ATTGAAAATCTCCGCCGAACGA
SHP	TAGATCTCTTCTTCCGCCCTA	AGACTCCATTCCACGGGTCA
FGF15	GACTGCGAGGAGGACCAAAA	CAGCCCGTATATCTTGCCGT
ACTB	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

FXR, farnesoid X receptor; *SHP*, small heterodimer partner; *FGF15*, fibroblast growth factor 15; *ACTB*, β-actin.

Figure 2

Type 2 diabetic mice with depression-like phenotype in glycolipid metabolism.

(A) Body weight weekly in CUMS procedures. (B) Body weight in mice after CUMS procedures. (C) Random blood glucose weekly in CUMS procedures. (D) Fasting blood glucose after CUMS procedures. (E) Serum CHO in mice. (F) Serum TG in mice. (G) Serum NEFA in mice. (H) Serum LDL in mice. (I) Serum HDL in mice. (J) GHbA1c in mice. Data presented as mean ± SEM, n=10 per group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the Control group, $^{*}P<0.05$, $^{**}P<0.01$ compared with the T2DM+CUMS group.

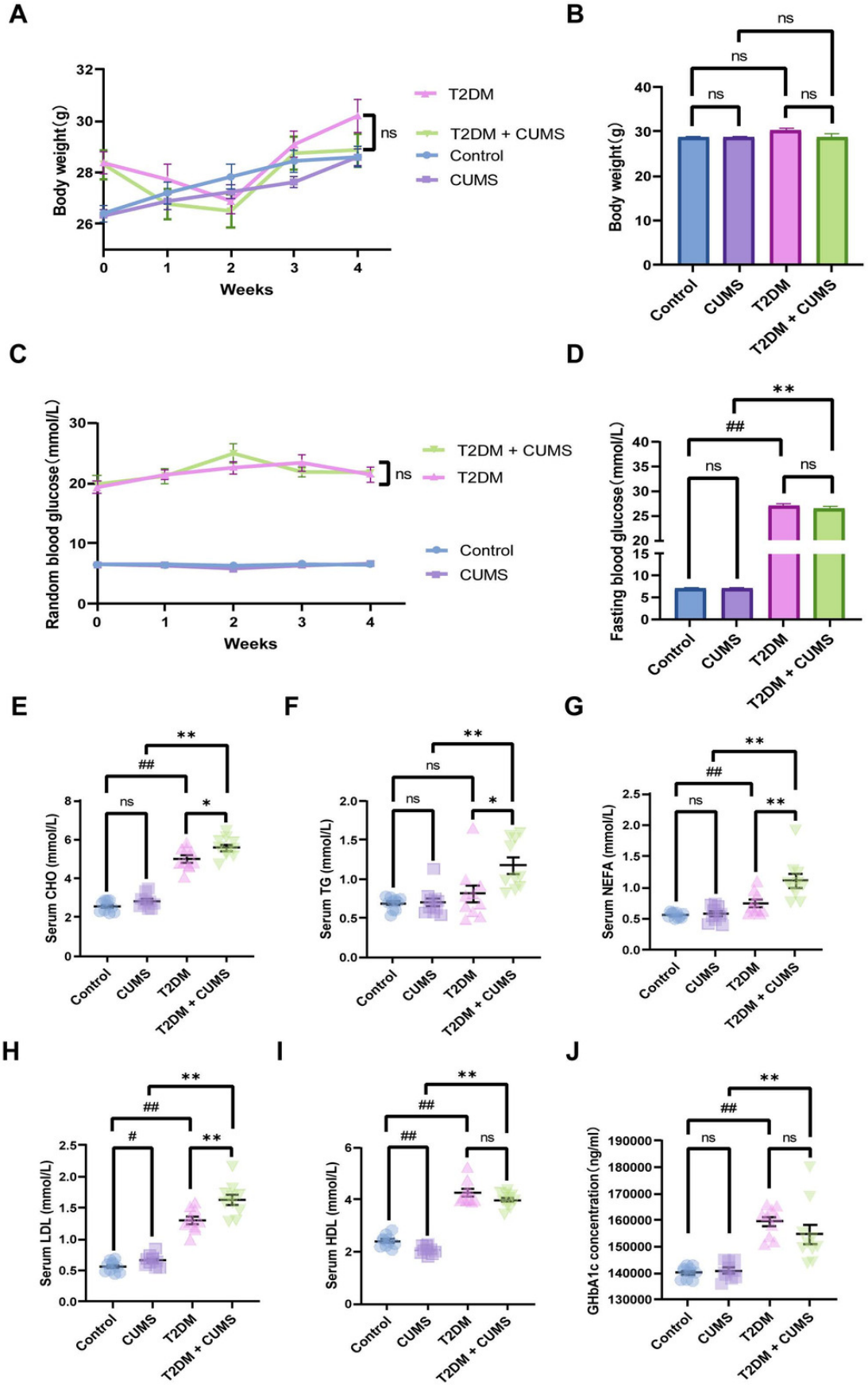

A
Body weight(g)
Weeks
T2DM
T2DM + CUMS
Control
CUMS
ns
B
Body weight(g)
Control
CUMS
T2DM
T2DM + CUMS
C
Random blood glucose(mmol/L)
Weeks
D
Fasting blood glucose(mmol/L)
E
Serum CHO (mmol/L)
F
Serum TG (mmol/L)
G
Serum NEFA (mmol/L)
H
Serum LDL (mmol/L)
I
Serum HDL (mmol/L)
J
GHbA1c concentration(ng/ml)

Figure 3

Insulin-related indicators and histopathological analysis of the pancreas in type 2 diabetic mice with depression-like phenotype.

(A) Serum glucose in mice. (B) FINS in mice; (C) HOMA-IR index in mice; (D) HOMA-β index in mice. (E) Representative images of pancreas sections from four groups of mice stained with H&E. Data presented as mean ± SEM, n=10 per group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the Control group, $^{**}P<0.01$ compared with the T2DM+CUMS group. Images were taken at 200× or 400× magnification.

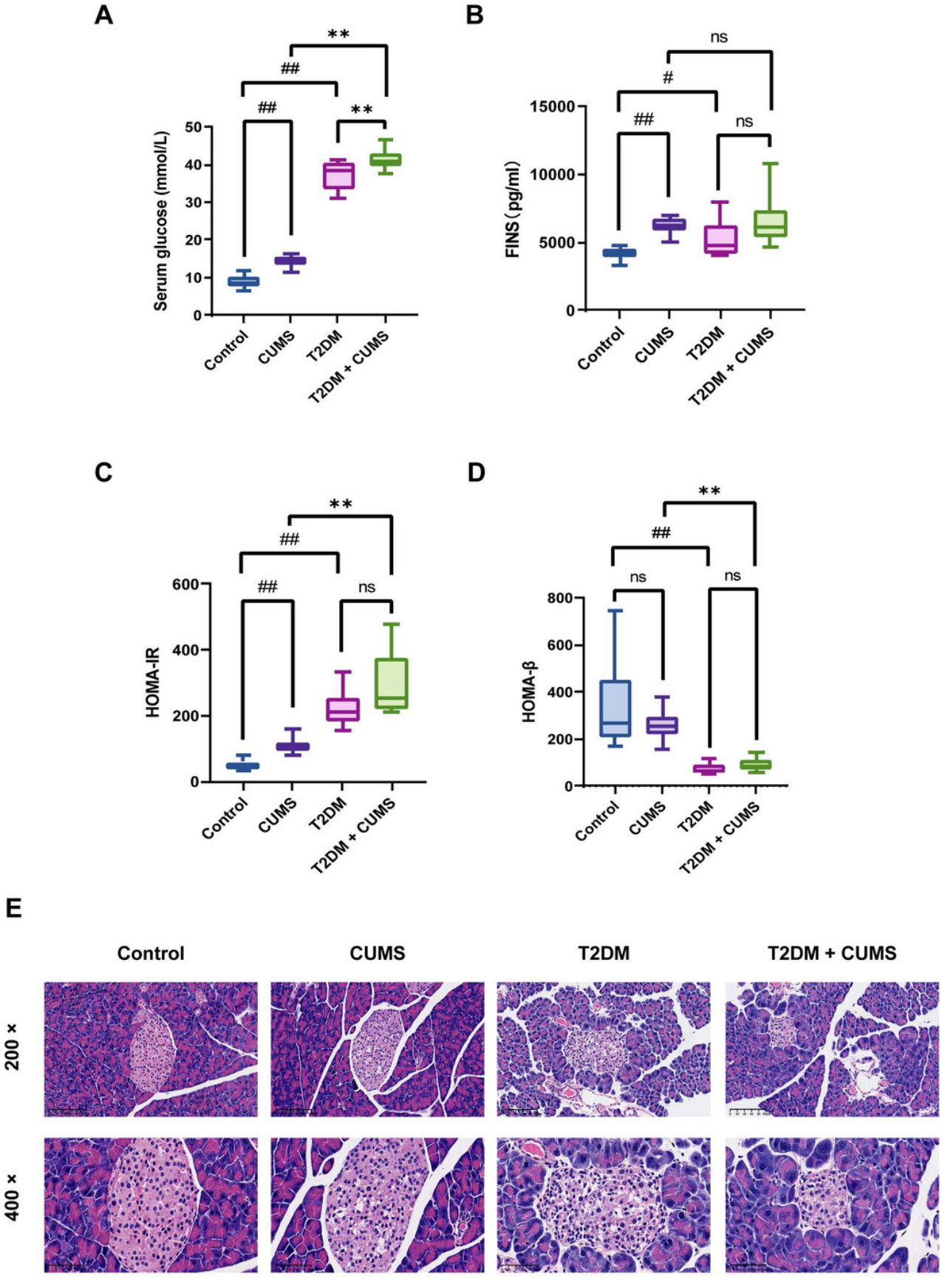

A
Serum glucose (mmol/L)
50
40
30
20
10
0
**
##
##
**
Control
CUMS
T2DM
T2DM + CUMS
B
FINS (pg/ml)
15000
10000
5000
0
ns
#
##
ns
Control
CUMS
T2DM
T2DM + CUMS
C
HOMA-IR
600
400
200
0
**
##
##
ns
Control
CUMS
T2DM
T2DM + CUMS
D
HOMA-β
800
600
400
200
0
**
##
ns
ns
Control
CUMS
T2DM
T2DM + CUMS
E
Control
CUMS
T2DM
T2DM + CUMS
200 ×
400 ×

Figure 4

Liver function, total bile acids and histopathological analysis of the liver in type 2 diabetic mice with depression-like phenotype.

(A) Representative images of liver sections from four groups of mice stained with H&E. (B) Lobular inflammation score in four groups. (C) Steatosis score in four groups. (D) Representative images of liver sections from four groups of mice stained with Oil Red O. (E) Hepatic lipids percentage in four group. (F) Liver weight/body weight ratio in mice. (G) ALT level in mice. (H) AST level in mice. (I) Total bile acid level in mice. Data presented as mean ± SEM, n=10. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the Control group, $*P<0.01$, $**P<0.01$ compared with the T2DM+CUMS group. Images were taken at 10× magnification.

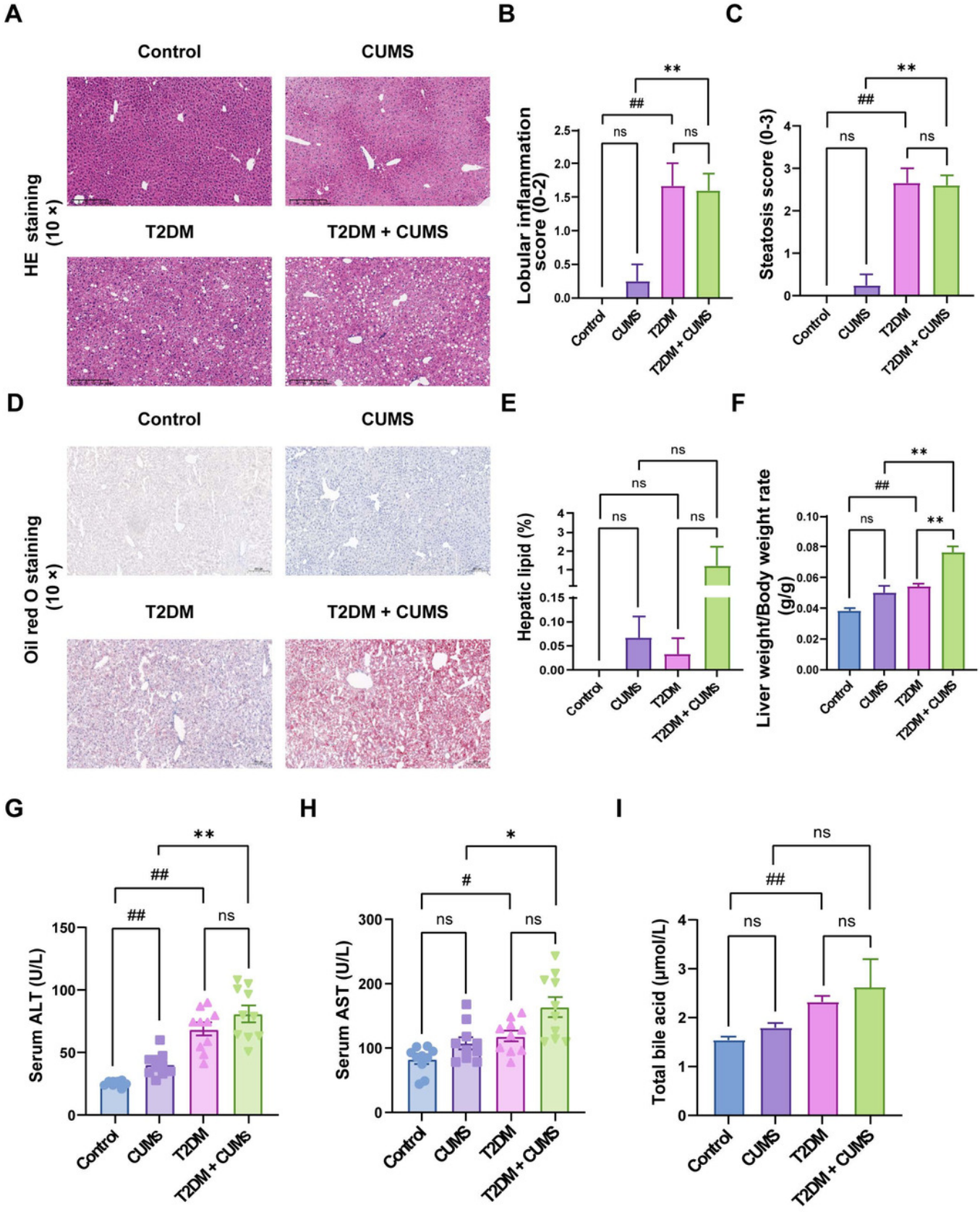
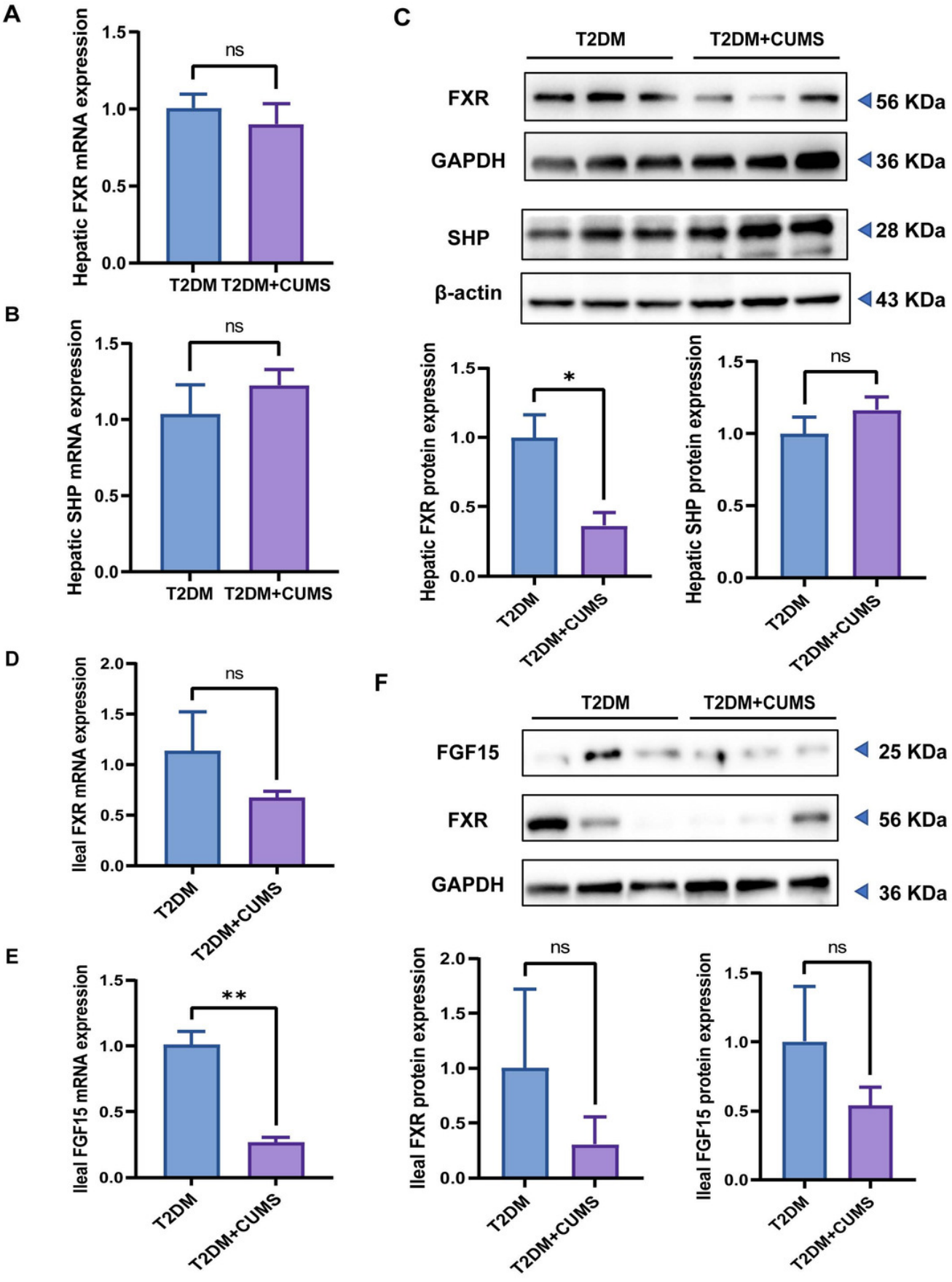

A
Control
CUMS
T2DM
T2DM + CUMS
HE staining (10 ×)
B
Lobular inflammation score (0-2)
C
Steatosis score (0-3)
D
Oil red O staining (10 ×)
E
Hepatic lipid (%)
F
Liver weight/Body weight rate (g/g)
G
Serum ALT (U/L)
H
Serum AST (U/L)
I
Total bile acid (μmol/L)
ns
##
**
*
#
Control
CUMS
T2DM
T2DM + CUMS

Figure 5

T2DM and depression-like phenotype regulated by FXR/SHP/FGF15.

(A) Relative expression of FXR mRNAs in the livers of mice. (B) Relative expression of SHP mRNAs in the livers of mice. (C) Relative expression of FXR and its target gene SHP proteins in the livers of mice. (D) Relative expression of FXR mRNAs in the ileum of mice. (E) Relative expression of FGF15 mRNAs in the ileum of mice. (F) Relative expression of FXR and its target gene FGF15 proteins in the ileum of mice. Data presented as mean ± SEM**.** **P*<0.05, ***P*<0.01 compared with the T2DM group.


A
Hepatic FXR mRNA expression
ns
T2DM
T2DM+CUMS
B
Hepatic SHP mRNA expression
ns
T2DM
T2DM+CUMS
C
T2DM
T2DM+CUMS
FXR
56 KDa
GAPDH
36 KDa
SHP
28 KDa
β-actin
43 KDa
Hepatic FXR protein expression
*
Hepatic SHP protein expression
ns
D
Ileal FXR mRNA expression
ns
E
Ileal FGF15 mRNA expression
**
F
FGF15
25 KDa
Ileal FXR protein expression
Ileal FGF15 protein expression