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Glycolipid metabolism and liver transcriptomic analysis of the therapeutic effects of pressed degreased walnut meal extracts on type 2 diabetes mellitus rats†

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Walnut meal (WM) is rich in polyphenols which exhibit multiple therapeutic effects. The purpose of this study was to investigate the therapeutic effects of walnut meal extracts (WMP) on glycolipid metabolism and liver transcriptomics in T2DM rats. A T2DM rat model was established by using a high-fat diet combined with streptozotocin. A 5-week WMP therapy showed the effects of decreasing water intake, excretion, fasting blood glucose, fasting insulin, and insulin resistance, increasing β-cell function and insulin sensitivity index; meanwhile regulating dysfunctional lipid metabolism and reducing inflammation; improving body weight, oral glucose tolerance test and insulin sensitivity; and increasing the activities of SOD and CAT while decreasing the MDA levels in the liver and serum of T2DM rats. Moreover, 10 key differentially expressed genes were identified by RNA-seq, including Gck, RT1-Ba, Fasn, Slc13a3, Cd74, Jun, Cyp4a1, Myh7b, Plin3, and Got1, and they were highly potentially related to glycolipid metabolism. Our results suggested that WMP exhibited the anti-diabetic effect and could regulate glycolipid metabolism in T2DM rats. This finding might assist in identifying potential therapeutic targets for T2DM prevention and intervention.

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1 Introduction

Diabetes, a worldwide prevalent chronic metabolic and endocrine disease, is characterized by persistent hyperglycemia.[1] According to the International Diabetes Federation, approximately 425 million people throughout the world are suffering from diabetes, and the number is predicted to rise to 438 million by 2030,[2] with 87%–91% being type 2 diabetes mellitus (T2DM) (http://www.diabetesatlas.org). T2DM is characterized by insulin resistance and/or insufficient insulin secretion from the pancreatic β cells, coupled with low-grade inflammation and impaired glucose tolerance.[3] The primary reasons for insulin resistance are obesity, modern dietary pattern, and bad life style.[4] T2DM being a major threat to public health is looming ahead and it consequently imposes a huge economic burden, and so the prevention and treatment of T2DM is essential.

Medicine therapy combined with exercise and diet is a routine strategy to fight diabetes.[5] Various drugs have been used to treat T2DM, such as biguanides, insulin secretagogues, and thiazolidinediones. Among these drugs, metformin, a biguanide derivant, is a widely-used oral hypoglycemic drug to control the blood glucose levels, and is reported to be associated with glycemic control and lipid metabolism (TG, TC and LDL-C).[6–8] Poor diabetes control can cause many complications, including nephropathy, retinopathy, myocardial infarction, stroke, organ damage, and even life-threatening conditions;[9] however, the anti-diabetic medicines used for T2DM have not only less choice but also various side effects. Considering the adverse effects of these medicines, alternative medicines from natural sources have gained attention due to their low toxicity, anti-diabetic effects and low cost. Chinese herbal medicines with a wide range of pharmacological activities have become prospective interventions for diabetes treatment.[10]

Walnut is one of the most popular tree nuts worldwide due to its nutritional and sensory attributes, and health benefits. Walnut kernel is not only a food, but also a traditional medicine recorded in the China Pharmacopoeia and is rich in com-

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mercial edible oil, essential fatty acids,[11] and phenolic compounds such as phenolic acids and condensed tannins, which are potentially able to scavenge free radicals.[12] Walnut meal (WM) is prepared by removing oil from the walnut kernel by cold pressing, and usually developed into products such as walnut food, beverage, and animal feed. However the usage of walnut is limited because of its bitter and astringent taste.[13] Pharmacological studies have shown that polyphenols in walnut kernels show the effects of antioxidants, reducing blood glucose and blood lipids, improving memory, *etc.*, and the extracts of WM (WMP) are rich in polyphenols.[14,15] The polyphenol substances of WMP, which are separated by HPLC, are tentatively identified as 9 phenolic components (vanillic acid glucoside, gallic acid, chlorogenic acid, cumaroylquinic acid, digalloylglucose, ellagic acid, glansreginin A, rutin, and glansreginin B).[16] Hepatic glycogenolysis and gluconeogenesis are the two major processes that enhance the fasting blood glucose (FBG) levels. Transcriptomics has been widely used to find therapeutic targets for T2DM.[17,18] Transcriptome sequence-based RNA-seq has become a comprehensive and accurate tool for gene expression pattern analyses with the superiorities of rapid development and low cost of next generation sequencing. This study employed RNA-seq to assess the effects of WMP on liver transcriptome in T2DM rats, and further analyzed the differentially expressed genes (DEGs) and their functional associations. The results obtained might aid in better understanding the role of livers played in the pathogenesis of T2DM, and assist in identifying potential therapeutic targets for T2DM prevention and treatment.

The aim of this study was to explore the therapeutic effects of WMP on the metabolism of glycolipids in rats with T2DM induced by a high-fat diet combined with STZ. RNA-seq was utilized to identify the differentially expressed genes, screen molecular targets, and understand the therapeutic mechanisms of WMP to reverse disease progression.

2 Materials and methods

2.1 Materials

Basic feed was purchased from Suzhou Shuangshi Experimental Animal Feed Technology Co. Ltd (China). High-fat feed was prepared by our research team. Pressed degreased walnut meals were purchased from Huizhiyuan Food Ltd, Yunnan, China. Streptozotocin (STZ) was purchased from MP Biomedicals, LLC, USA. Metformin (MET, positive drug) was purchased from Shandong Sibangde Pharmaceutical Co., Ltd, Kunming, Yunnan, China. A glucose meter was purchased from Sannuo Biosensor Co., Ltd, Hunan, China. Detection kits of total cholesterol (TC), triacylglycerol (TG), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glycated serum protein (GSP), alanine aminotransferase (ALT) and aspartate transaminase (AST) were purchased from Nanjing Jianchen Bioengineering Institute (Nanjing, Jiangsu, China). The concentrations of insulin (FINS), lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined by using ELISA kits purchased from Jianglai Biological Technology Co., Ltd, Shanghai. China.

2.2 Preparation of extracts from cold pressed degreased walnut meals (WMP)

The cold pressed degreased walnut meals (WM) were extracted twice using a condensation reflux extraction device with 50% ethanol (W/V) for 60 min at 73 °C followed by filtration, and the filtrate was then combined, concentrated, and freeze-dried to obtain the extracts (WMP).

2.3 Design for animal studies

Male Sprague-Dawley rats (weighing 180–200 g) were purchased from Hunan Sleek Jingda Experimental Animal Co. Ltd (the experimental animal production license: SCXK-(Xiang) 2016-0002). The animals were housed in the standard polypropylene animal cages at 22 ± 2 °C, with a relative humidity of 60 ± 5% and a 12 h day–night cycle throughout the study. Initially, all animals were fed basic feed and were provided with purified water libitum for 1 week to acclimatize to the environment. Rats that were set as the control group (CD, n = 10) had free access to water and basic feed. Rats of T2DM model were fed a high-fat diet (basal diet 68%, sugar 10%, lard 10%, egg yolk powder 10%, cholesterol 1%, pig-bile salt 0.5% and sodium salt 0.5%, with a total calorific value of 1777 kJ per 100 g) during the study, and were intraperitoneally injected with 35 mg kg^{-1} of STZ (dissolved in citrate buffer, pH 4.3) in the 4th week to establish a T2DM rat model.[19] A total of 50 T2DM rats (FBG levels ≥11.1 mmol L^{-1}) were randomly divided into 5 groups (n = 10): diabetic group (DM), diabetic with low-dose (125 mg kg^{-1}) WMP (WMP-L), diabetic with middle-dose (250 mg kg^{-1}) WMP (WMP-M), diabetic with high-dose (500 mg kg^{-1}) WMP (WMP-H), and diabetic positive control group which was administered with 150 mg kg^{-1} d^{-1} of metformin (MET). CD and DM rats were orally treated with the same volume of saline. Water intake, excretion, and body weights were detected every week. Blood samples of each group were collected from tail vein, and the blood glucose levels were measured using a glucometer according to the manufacturer's instructions. The levels of blood glucose were measured once every two weeks after WMP therapy.

2.4 Oral glucose tolerance test (OGTT)

The oral glucose tolerance test (OGTT) was performed in the last week of WMP therapy. Rats were fasted for 12 h overnight, and then given glucose at a dose of 2 g per kg body weight. Blood samples were collected from the tail vein, and the levels of blood glucose at 0, 30, 60, and 120 min were measured using a Sannuo glucometer. The area under the curve (AUC) in the time range from 0 to 120 min was determined to represent the glucose tolerance. At the end of this study, rats of each group were anesthetized by intraperitoneally injecting 3% pentobarbital sodium, and the abdominal aorta blood was collected and stored at −80 °C for measuring the biochemical indicators. Organs were quickly separated and weighed, of

which the liver and pancreas were fixed in 10% formalin for histopathological examination, and others were stored at −80 °C refrigerator for subsequent assays.

2.5 Homeostatic model assessment – insulin resistance, β-cell function and insulin sensitivity index

Based on FBG and FINS, the homeostatic model assessment of insulin resistance (HOMA-IR), β-cell function (HOMA-β), and insulin sensitivity index (ISI) were calculated by using the following formulas: HOMA-IR = (FBG × FINS)/22.5; HOMA-β = 20 × FINS/(FBG-3.5); and ISI = Ln [(FBG × FINS)$^{-1}$]; these were used for evaluating the degree of insulin resistance and islet β-cell function.[20,21]

2.6 Biochemical assay

FBG, GSP, FINS, HOMA-IR, and HOMA-β were tested during the intervention. The levels of TC, TG, HDL-C, LDL-C, ALT, AST, SOD, CAT, and MDA in the serum and liver were analyzed by using the commercially available kits respectively. The concentrations of LPS, TNF-α, and IL-6 were determined using ELISA kits. All measurements were carried out according to the manufacturer's instructions provided in the corresponding kits.

2.7 Histopathological analysis

Liver and pancreatic tissues were isolated from each group of rats, then fixed in 10% paraformaldehyde solution, followed by dehydration, embedding in paraffin, and sectioning to 5 μm slices. After dehydration, the sections were stained with haematoxylin and eosin (HE). To evaluate the histopathological damage, slides were observed under an upright microscope, and the counts of β-cell islets and hepatocyte steatosis were determined using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The values were expressed as mean ± SD (n = 3; three rats from each group were randomly selected for quantification). Differences were assessed by ANOVA.

2.8 Transcriptomic analysis of WMP in T2DM rats

In this study, liver samples of the CD, DM, and WMP-H groups were used for RNA-seq.

2.8.1 RNA extraction, library preparation, and sequencing. TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA from liver tissue samples according to the manufacturer's instructions, and an average RNA yield of approximately per 100 mg of liver tissue was 73 μg as reported in the previous study.[22] According to the manufacturer's instructions RNA quantification was performed using a Qubit® RNA assay kit in conjunction with a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). Preparation of RNA-seq libraries was carried out using the RNA Library Prep Kit for Illumina R (NEB, Ipswich, MA, USA). The library fragments were purified using QIAquick PCR Kits (Qiagen, Germany) following first-strand and second-strand cDNA synthesis. The cDNA was purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany). After the preparation of the library and adenylation of the 3′ ends, adapters were added, and the target products were selected and amplified to construct the sequencing library, and were qualified using the TaqMan fluorescent probe of the AB Step One Plus Real-Time PCR system. Finally, the Illumina HiSeq X platform (Illumina, San Diego, CA, USA) was used for sequencing and generating 150 bp paired-end reads. Methods of RNA extraction, library preparation, and sequencing were according to the previous studies.[23]

2.8.2 Filtering of reads. To obtain clean reads, raw data in FASTQ format were processed according to the previous study.[23] Using the I-sanger evaluated cooccurrence network, the differential gene expression analysis and the correlation analysis were performed on the free online platform of Majorbio Cloud Platform (Majorbio Bio-Pharm Technology Co. Ltd, Shanghai, China. http://www.i-sanger.com).

2.8.3 Differential gene expression analysis. Clean data of pair-end reads from each rat were aligned to the Rattus norvegicus reference genome (Genome build: rn6) using STAR2 (version 020201). Subsequently, StringTie (version 1.3.0) was used to assemble the transcripts. Ballgown version 2.10.0 was used to obtain the fragments per kilobase of exon per million fragments mapped (FPKM) of all annotated genes. Finally, in order to obtain the differentially expressed genes (DEGs), a Bayes-regularized t-test was performed with a false discovery rate (FDR) of 0.05 using Cyber-T bayesreg R.

2.8.4 Functional enrichment analysis. The Cytoscape plug-in ClueGO (version 3.5.1) was used for the enrichment analysis of the DEGs in terms of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. The GO terms and KEGG pathway were considered to be significantly enriched with an adjusted P-value <0.05.

2.9 Statistical analysis

GraphPad Prism 7.0 was used to perform all statistical analyses of the physiological indexes. All the results were expressed as mean ± standard deviation (SD). Differences between the groups were determined by repeated one-way analysis of variance (ANOVA) including Duncan's *post hoc* test at a 0.05 significance level.

3 Results

3.1 The effects of WMP therapy on the body weight, FBG level, and organ index in T2DM rats

During the experiment, a decrease in the body weight was a typical symptom in T2DM rats. The body weights of all rats in the CD, DM, MET and WMP treated groups were recorded weekly, as presented in Table 1. In comparison with CD rats (525.4 ± 33.78), DM rats (367.9 ± 15.79) showed a significant weight reduction (P < 0.001). Treatment with metformin and WMP (WMP-M and WMP-H) improved the body weight (P < 0.05 or P < 0.01) compared to DM rats, and the WMP-M group exhibited the same efficacy as the MET group. Treatment with WMP-L improved the body weight of T2DM rats, which was not significant when compared to DM rats.

Table 1 Effects of WMP on fasting body weight, blood glucose (FBG), organ index and insulin sensitivity index in T2DM rats

	CD	DM	MET	WMP-L	WMP-M	WMP-H
Body weight (g)						
0 Week	388.50 ± 23.92*	421.40 ± 26.01$^{\#}$	432.20 ± 34.05	435.40 ± 12.27	422.30 ± 45.52	432.80 ± 25.68
1 Week	469.80 ± 24.00***	391.30 ± 27.21$^{\#\#\#}$	399.90 ± 39.64	402.00 ± 36.10	415.00 ± 27.84	407.00 ± 27.08
2 Weeks	486.20 ± 28.48***	388.00 ± 28.61$^{\#\#\#}$	404.30 ± 42.73	398.40 ± 34.00	409.80 ± 31.76	418.10 ± 23.49*
3 Weeks	498.20 ± 34.59***	375.20 ± 30.96$^{\#\#\#}$	395.40 ± 46.92	383.30 ± 34.24	397.50 ± 35.53	411.80 ± 21.99**
4 Weeks	519.90 ± 37.84***	372.60 ± 37.93$^{\#\#\#}$	393.30 ± 56.81	384.30 ± 42.55	408.80 ± 28.28	416.70 ± 26.39*
5 Weeks	525.40 ± 33.78***	367.90 ± 15.79$^{\#\#\#}$	407.40 ± 57.19*	388.80 ± 43.94	410.90 ± 33.04*	414.30 ± 18.14**
FBG (mmol L^{-1})						
0 Week	4.37 ± 0.41***	17.25 ± 4.01$^{\#\#\#}$	18.36 ± 6.54	18.17 ± 3.81	17.60 ± 4.11	17.66 ± 4.74
2 Weeks	3.84 ± 0.27***	21.78 ± 2.37$^{\#\#\#}$	17.42 ± 3.93**	16.76 ± 2.52**	15.41 ± 5.16***	15.95 ± 3.27***
4 Weeks	4.83 ± 0.82***	24.80 ± 2.31$^{\#\#\#}$	15.20 ± 4.80***	20.68 ± 5.26*	19.5 ± 1.39**	19.92 ± 1.34**
5 Weeks	4.52 ± 0.47***	26.99 ± 1.61$^{\#\#\#}$	15.29 ± 6.55***	20.16 ± 4.92**	19.92 ± 4.97**	17.62 ± 4.44***
Organ index						
Heart	0.328 ± 0.031	0.38 ± 0.078	0.38 ± 0.04	0.36 ± 0.04	0.38 ± 0.07	0.37 ± 0.07
Liver	2.36 ± 0.28***	4.58 ± 0.39$^{\#\#\#}$	4.29 ± 0.31	4.32 ± 0.37	4.31 ± 0.32	4.00 ± 0.37**
Spleen	0.15 ± 0.02	0.16 ± 0.04	0.14 ± 0.01	0.16 ± 0.02	0.14 ± 0.025	0.14 ± 0.02
Kidney	0.61 ± 0.05***	1.09 ± 0.097$^{\#\#\#}$	1.05 ± 0.13	1.04 ± 0.13	1.04 ± 0.08	0.99 ± 0.16
Pancreas	0.31 ± 0.04	0.23 ± 0.04	0.26 ± 0.18	0.25 ± 0.04	0.25 ± 0.04	0.26 ± 0.04
Insulin sensitivity index						
FINS	29.33 ± 2.98***	42.02 ± 2.114$^{\#\#\#}$	30.87 ± 4.76***	36.63 ± 5.552*	34.99 ± 5.261**	31.46 ± 4.575***
HOMA-IR	4.76 ± 2.48***	50.3 ± 4.371$^{\#\#\#}$	21.69 ± 6.416***	33.2 ± 10.68***	28.68 ± 12.43***	24.92 ± 8.162***
HOMA-β	543.10 ± 248.10***	36.91 ± 3.58$^{\#\#\#}$	89.98 ± 63.28	49.48 ± 22.10	61.98 ± 59.35	63.54 ± 20.76
ISI	−4.87 ± 0.15***	−7.03 ± 0.09$^{\#\#\#}$	−6.15 ± 0.32***	−6.56 ± 0.38**	−6.51 ± 0.342**	−6.28 ± 0.36***

CD, control group; DM, diabetic model group; MET, metformin treatment group; WMP-H, high dose WMP (500 mg kg^{-1} d^{-1}) treatment group; WMP-M, middle dose WMP (250 mg kg^{-1} d^{-1}) treatment group; and WMP-L, low dose WMP (125 mg kg^{-1} d^{-1}) treatment group. The values were represented as mean ± SD. * indicates the significant difference compared with the DM group, respectively, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; # indicates the significant difference compared with the CD group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$.

FBG is an important indicator of insulin resistance in T2DM, as shown in Table 1. In order to test the effect of WMP on glucose metabolism, the levels of FBG of all rats were tested. During the WMP treatment, the FBG levels of all rats were measured once every two weeks and were also measured in the last week. In the 0th week of treatment, T2DM rats, with a significant FBG difference between the CD group and the DM group ($P < 0.001$), were randomly divided into 5 groups, among which there was no significant difference in FBG. In comparison with the CD group, the levels of FBG in DM rats were remarkably increased during the 5-week study ($P < 0.001$). In the 2nd week, FBG was significantly altered by WMP and MET compared with DM rats. In the 4th week, FBG was reduced by WMP-L ($P < 0.05$), WMP-M, WMP-H ($P < 0.01$), and MET ($P < 0.001$). In the last week, a significant decrease in FBG was observed in the MET and WMP groups compared with the DM group ($P < 0.01$ or $P < 0.001$), especially WMP which showed an obvious FBG reduction effect. There was no obvious difference in the FBG levels among the WMP-L, WMP-M, WMP-H and MET groups, suggesting that WMP exhibited the same efficacy as MET to control and decrease the FBG level. The organ index was calculated by using the formula: Organ index = organ weight/body weight × 100. In comparison with the CD group, the liver index of DM rats was significantly increased and the kidney index was significantly decreased ($P < 0.001$). High dose of WMP (WMP-H) decreased the liver index compared with DM rats ($P < 0.01$). In addition, there was no significant difference in the heart, spleen, and pancreatic indexes among all groups.

3.2 The effects of WMP therapy on water intake and excretion in T2DM rats

In comparison with CD rats, DM rats showed the typical symptom of diabetes: increased water intake and excretion (Fig. 1). These symptoms were significantly reversed by treatment with metformin and WMP. These results showed that WMP-L and WMP-M have the same efficacy as MET in the 0th week in decreasing the water intake of T2DM rats (Fig. 1A), while MET showed a more powerful decreasing effect of water excretion than the WMP-L and WMP-M groups, but the WMP-H group has the same efficacy as the MET group (Fig. 1B).

3.3 The effects of WMP therapy on GSP and OGTT in T2DM rats

GSP was measured using the commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The GSP level in the CD group was significantly lower than the DM group ($P < 0.01$). The WMP-H group presented the lowest GSP level in diabetic rats, and three doses of WMP have the same efficacy as MET ($P < 0.001$) (Fig. 2A). OGTT was performed to evaluate the ability of the tested animals to dispose of a glucose load. In comparison with CD rats, DM rats showed a statistically elevated blood glucose level before

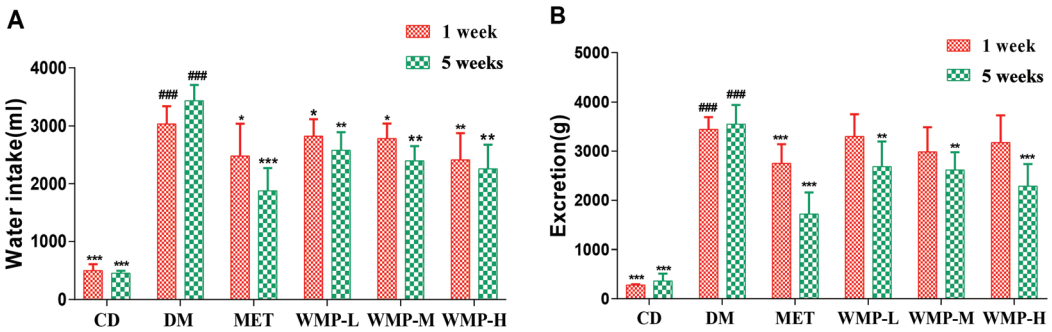

Fig. 1 The effects of WMP on water intake (A) and excretion (B) in T2DM rats. Data were presented as mean ± SD. * indicated the significant difference compared with the DM group in the 1st and 5th weeks, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; # indicated the significant difference compared with the CD group in the 1st and 5th weeks, ### $P < 0.001$.

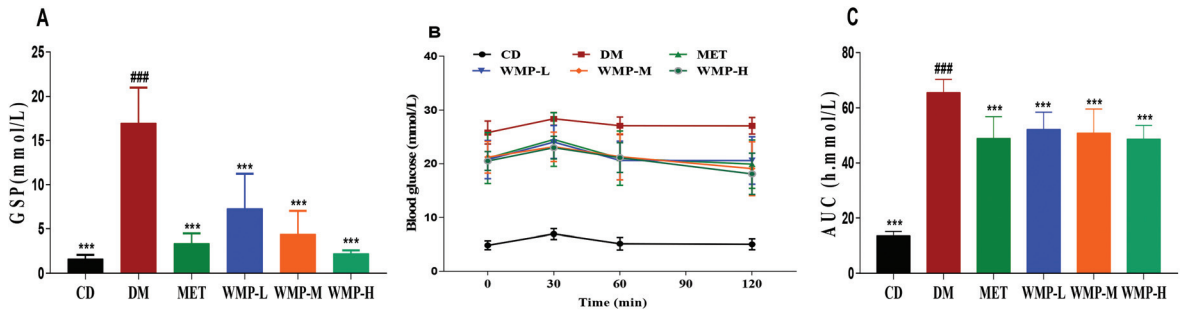

Fig. 2 The effects of WMP on glycated serum protein (GSP) and glucose tolerance tests (OGTT). (A) Glycated serum protein. (B) Glucose tolerance tests, following an overnight fasting, the rats were orally injected with glucose at a dose of 2 g per kg body weight, and the blood glucose level was measured in tail vein blood at 0, 30, 60, and 120 min. (C) Area under the curve (AUC), data were calculated by OGTT. Data were represented as mean ± SD. * indicated the significant difference compared with the DM group, *** $P < 0.001$; # indicated the significant difference compared with the CD group, ### $P < 0.001$.

and after glucose load (Fig. 2B). The AUC on the OGTT (Fig. 2C) was increased in DM rats, indicating their severe glucose intolerance ($P < 0.001$). DM rats treated with MET and three doses of WMP exhibited a reduction of AUC ($P < 0.001$). These results showed that three doses of WMP significantly improved the impaired glucose tolerance, and the high dose (WMP-H) group was found to have a better effect. In addition, the efficacy of WMP-H was better than MET. These results indicated that WMP could reduce postprandial blood glucose.

3.4 The effect of WMP therapy on the insulin sensitivity index in T2DM rats

The HOMA-IR index was determined to evaluate the improvement of insulin sensitivity, which was calculated by using a formula based on FBG and FINS (Table 1). The HOMA-IR index was significantly reduced by MET and three doses of WMP ($P < 0.001$), and WMP has a comparable efficacy to MET. In the improvement of the HOMA-β index, there was no significant difference between the three WMP-treated groups and the MET group. ISI was used to assess the insulin sensitivity, which was decreased significantly in DM rats compared with CD rats ($P < 0.001$) and was able to be redressed by the supplements of MET and WMP ($P < 0.01$ or $P < 0.001$). In addition, the efficacy of WMP-H was comparable to MET. These results showed that WMP could decrease the FBG level and improve the insulin sensitivity of T2DM rats with the same efficacy as MET, indicating that WMP could improve the metabolism of glucose in T2DM rats.

3.5 Lipid profiles of serum and liver of T2DM rats

In this study, the parameters of serum and liver lipids were examined, including TG, TC, HDL-C, and LDL-C. Table 2 shows the levels of TC, TG, LDL-C, and HDL-C in the liver and serum of all the groups. A significant increase in the levels of serum and liver TC, TG, and LDL-C ($P < 0.01$ or $P < 0.001$) was observed in the DM group. The concentrations of serum TC, TG, and LDL-C in the MET group and WMP groups treated with doses of 125, 250 and 500 mg kg^{-1} were lower than those in the DM group ($P < 0.01$ or $P < 0.001$). Although there was no significant difference in serum HDL-C among the groups, the absolute values of HDL-C in the MET and three WMP groups were still higher than those in the DM group. The levels of liver TC, TG, and LDL-C in the MET and three WMP-treated groups were decreased when compared with those in the DM group ($P < 0.01$ or $P < 0.001$). There was no significant difference in liver HDL-C among the groups; however, MET elevated the HDL-C level further in DM rats. Thus, the efficacy of WMP was similar to MET in the inhibition of serum and liver lipids.

Table 2 Lipids profiles of serum and liver in each group

Index	CD	DM	MET	WMP-L	WMP-M	WMP-H
Serum (mmol L^{-1})						
TC	4.401 ± 0.5771***	70.340 ± 16.580$^{###}$	31.970 ± 17.420***	46.910 ± 12.750**	29.760 ± 21.800***	16.080 ± 9.669***
TG	0.486 ± 0.139***	17.240 ± 7.384$^{###}$	5.898 ± 6.377***	8.185 ± 5.518**	5.429 ± 4.737***	1.169 ± 0.573***
HDL-C	2.957 ± 1.536	2.286 ± 1.111	2.776 ± 0.962	3.527 ± 1.138	2.736 ± 1.255	2.241 ± 1.033
LDL-C	0.599 ± 0.317***	13.500 ± 4.824$^{###}$	4.569 ± 1.121***	5.041 ± 3.028***	3.782 ± 0.750***	3.83 ± 1.836***
Liver (μmol g^{-1})						
TC	6.144 ± 1.325***	9.245 ± 2.049$^{###}$	5.841 ± 1.239***	7.104 ± 1.062*	6.265 ± 1.287***	6.336 ± 1.013***
TG	3.957 ± 0.834**	7.416 ± 2.246$^{##}$	4.038 ± 1.833**	4.406 ± 2.008**	4.187 ± 1.872**	4.162 ± 2.110**
HDL-C	0.0203 ± 0.0046	0.0142 ± 0.0217	0.0186 ± 0.0083	0.0140 ± 0.0032	0.0146 ± 0.0070	0.0152 ± 0.0046
LDL-C	1.571 ± 0.4184***	4.237 ± 1.095$^{###}$	2.213 ± 0.7027***	1.772 ± 0.6169***	1.985 ± 0.511***	1.594 ± 0.7143***

Data were represented as mean ± SD. * indicates the significant difference compared with the DM group, *P < 0.05, **P < 0.01, ***P < 0.001; # indicates the significant difference compared with the CD group, $^{##}P$ < 0.01, $^{###}P$ < 0.001.

3.6 The effects of WMP therapy on MDA, SOD, CAT, ALT, and AST in T2DM rats

The activities of SOD and CAT in the serum and liver of DM rats were significantly decreased, while their MDA levels were higher (P < 0.001) than the CD group (Fig. 3). In comparison with the different intervention groups, the MDA levels in the serum and liver of the MET, WMP-L, WMP-M, and WMP-H groups were decreased (P < 0.01). These results showed that three doses of WMP could decrease the MDA level of diabetic rats with the same efficacy as MET (Fig. 3A and D). The activities of SOD and CAT in the serum and liver of the DM group were significantly decreased compared with those in the other groups (P < 0.01 or P < 0.001), and could be conspicuously reactivated by the supplements of MET and WMP (Fig. 3B, C E,

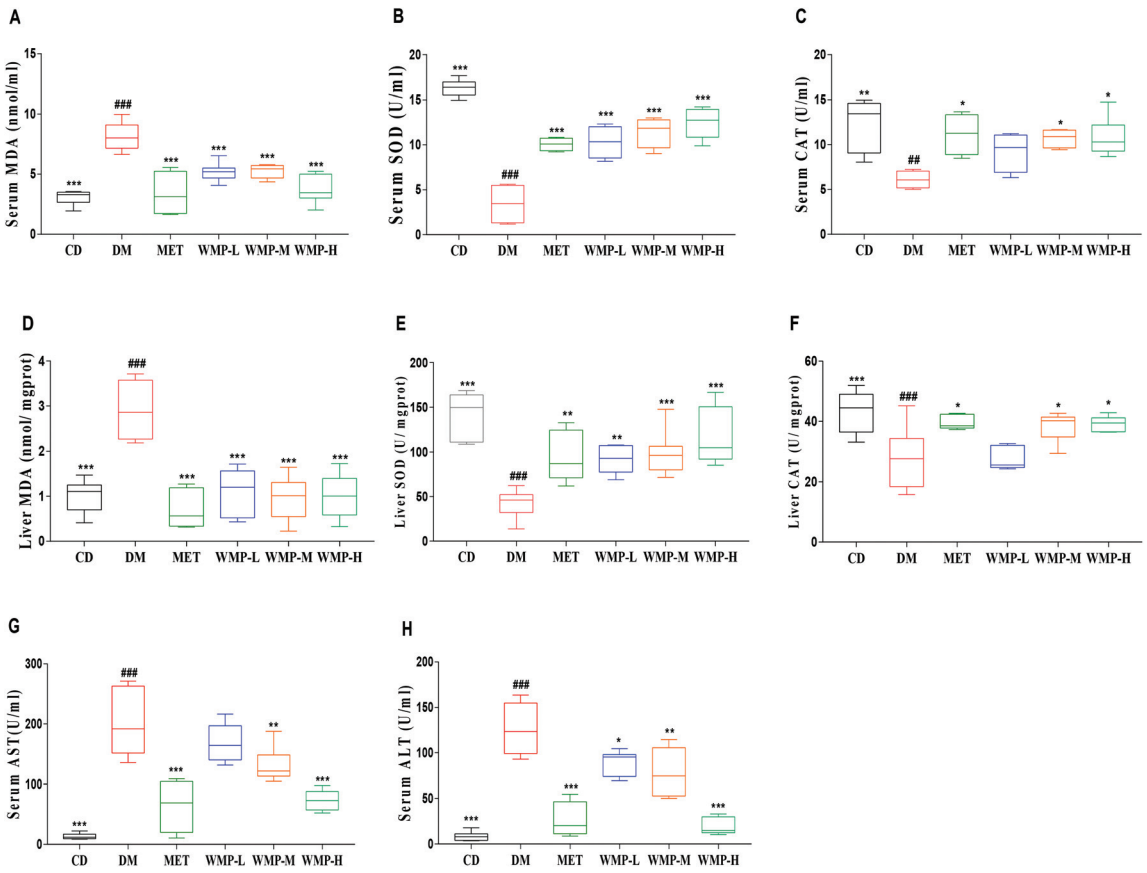

Fig. 3 Effects of WMP treatment on MDA, SOD, and CAT in the serum (A–C) and liver (D–E), and ALT (G), AST (H) in the serum levels of T2DM rats. Data were represented as mean ± SD. * indicated the significant difference compared with the DM group, * P < 0.05, ** P < 0.01, *** P < 0.001; # indicated the significant difference compared with the CD group, $^{##}$ P < 0.01, $^{###}$ P < 0.001.

and F). The serum ALT and AST activities, used as the biochemical markers of hepatic damage, are shown in Fig. 3G and H. As expected, the DM group showed significantly elevated ALT and AST levels compared with the CD group ($P < 0.001$). ALT was slightly reduced by the administration of metformin and WMP with the dose–effect relationship, and the level of ALT in the WMP-H group was similar to that in the CD group. Moreover, the ALT levels in the MET and three WMP-treated groups were significantly decreased compared to that in DM rats ($P < 0.05$). Additionally, the AST levels were elevated in the DM and WMP groups compared with that in the CD group. The AST levels were decreased in the MET, WMP-M and WMP-H treated groups ($P < 0.01$ or $P < 0.001$), although no statistical significance was found for the WMP-L group.

3.7 The effects of WMP therapy on endotoxemia and inflammation in T2DM rats

To determine the effect of WMP on the inflammation in T2DM rats, the liver and serum LPS, IL-6, and TNF-α levels were measured using a rat-specific ELISA kit according to the manufacturer's instructions. LPS which is a cell-wall component of Gram-negative bacteria was delivered to the liver *via* the portal vein affected by endotoxemia. Such endotoxin production in T2DM rats could cause chronic low-grade inflammation. As shown in Fig. 4, the levels of LPS, IL-6 and TNF-α in the other 4 groups were lower than those in the DM group. Serum and liver LPS were significantly decreased in the high-dose, middle-dose, and low-dose WMP groups and the MET group ($P < 0.01$ or $P < 0.001$) (Fig. 4A and D). The IL-6 and TNF-α levels were significantly decreased in the three WMP-treated groups and the MET group ($P < 0.01$ or $P < 0.001$) (Fig. 4B, C and E, F).

3.8 Histopathological examination

As seen in Fig. 5, the islets of CD rats showed a typical histological structure, in which the boundary and number of β-cell islets in DM rats were unclear, irregularly arranged, and reduced significantly. After treatment with WMP and MET, the injury of the pancreatic tissue and the apoptosis of islet cells were both decreased, and the number of islet cells was significantly increased (Fig. 5A and C). The hepatic tissues of DM rats revealed extensive and severe hepatic lipid accumulation. After MET and WMP treatment, hepatic lesions were apparently reversed with improvements including a more intact hepatocyte structure, considerably lower accumulation of lipid droplets, and decreased number of hepatocyte steatosis (Fig. 5B and D). In particular, the WMP-M and WMP-H groups displayed a better repaired alteration and a notable trend of recovery of lipid droplets in CD rats. The liver morphology results indicated that WMP had reverse effects on the damaged liver of T2DM rats.

3.9 The transcriptomic analysis of WMP in T2DM rats

3.9.1 RNA-sequencing and mapping of the rat liver transcriptome. The cDNA libraries from CD, DM, and WMP ($n = 3$) liver samples were prepared and sequenced. The good quality of RNA with favorable integrity and purity for library construction was evaluated by agarose gel electrophoresis (Fig. 6A). As shown in Table 3, the total number of reads in each group were determined. To ensure the library reliability, quality control procedures were performed by using clean reads for each group. The obtained number of paired-end reads varied in the range of 49 558 812–57 377 606 per 100 bp per sample. These results confirmed that RNA-seq was reliable and this approach was reproducible. Furthermore, to

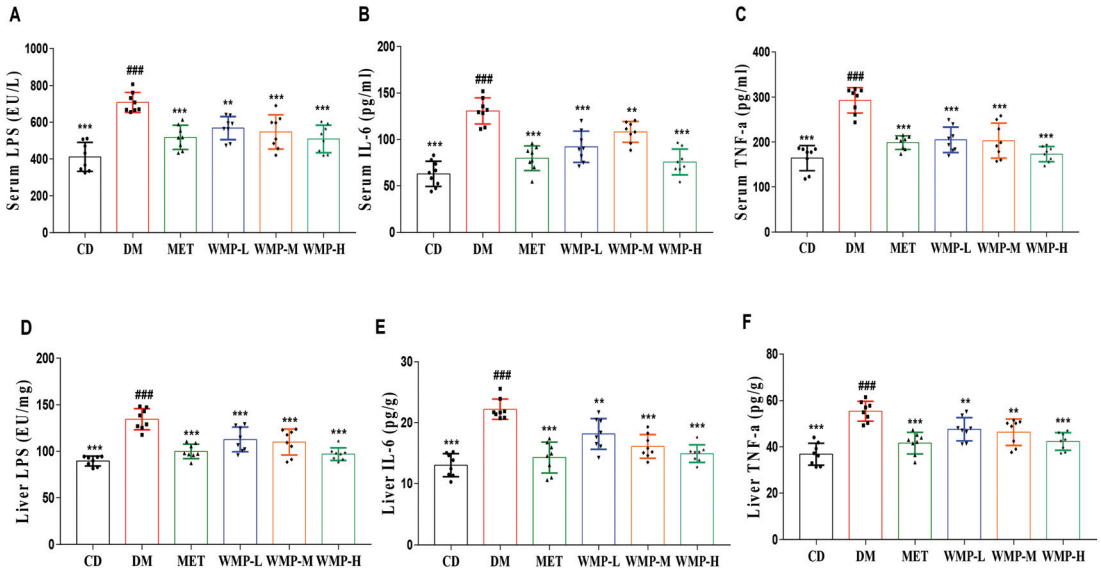

Fig. 4 Effects of WMP on LPS, IL-6 and TNF-α levels in T2DM rats. LPS, IL-6 and TNF-α in the serum (A–C) and liver (D–F) of rats. Data were represented as mean ± SD. * indicated the significant difference compared with the DM group, respectively, ** $P < 0.01$, *** $P < 0.001$; # indicated the significant difference compared with the CD group, ### $P < 0.001$.

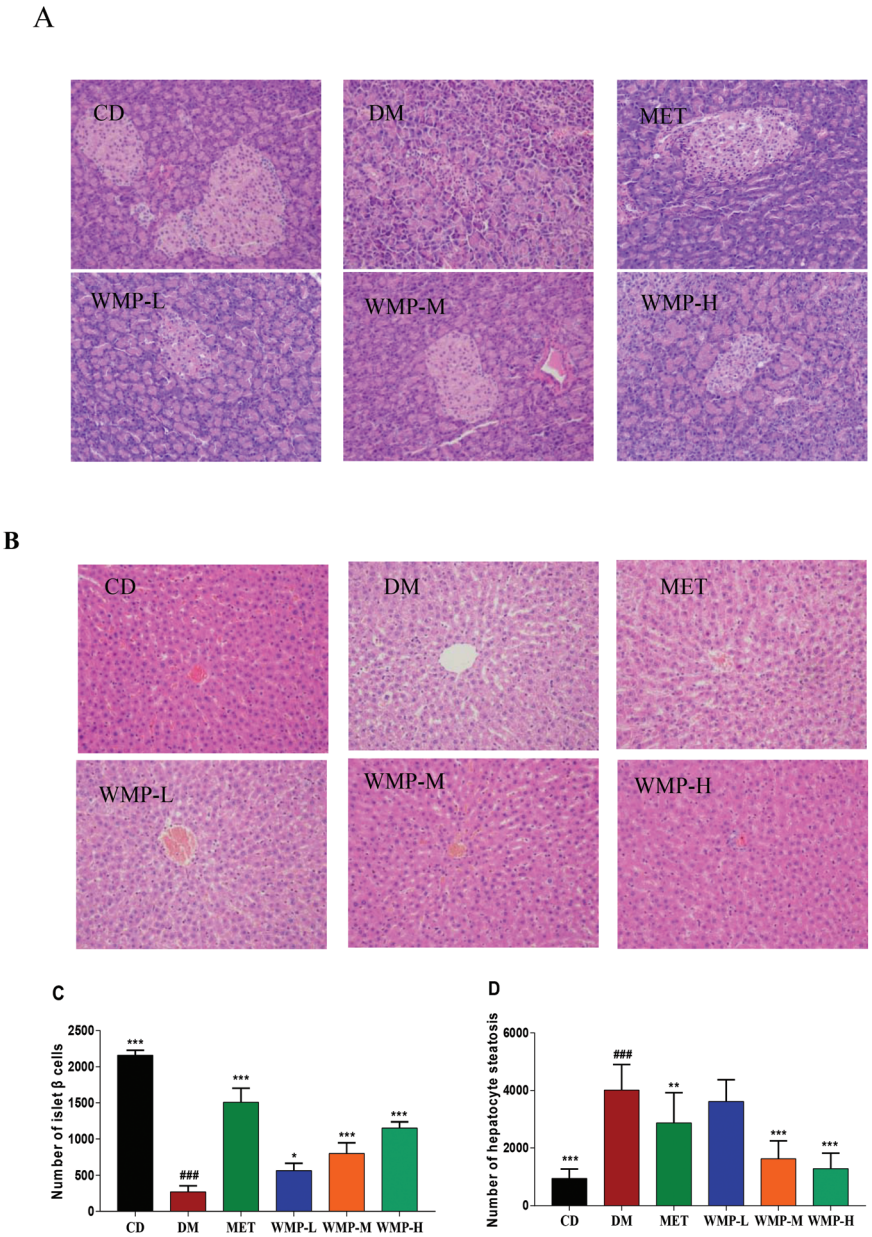

Fig. 5 Histopathological images and analyses of the pancreas and liver by HE staining. The image of the pancreas (A) and liver (B) were at 200× magnification. The number of β-cell islets (C) and hepatocyte steatosis (D) were calculated by using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The values were represented as mean ± SD (n = 3; three rats from each group were randomly selected for quantification). Differences were determined by ANOVA and denoted as follows: * $P < 0.05$, *** $P < 0.001$ compared with the DM group; ### $P < 0.001$ compared with the CD group.

eliminate the background noise associated with the individual-specific transcription, three rats from the same group were used.

3.9.2 Differential gene expression in the CD, DM, and WMP groups. Venn diagram analysis was used to show the commonly and specifically expressed genes among the samples (Fig. 6B). The number of genes commonly expressed among the CD, DM and WMP groups was 10 600, of which 88 were expressed in the CD and DM groups and 115 were expressed in the WMP and CD groups, indicating that the number of genes commonly expressed in the liver samples of T2DM rats was close to that in the CD group after WMP treatment. The principal component analysis (PCA) was performed to investigate the transcriptome (Fig. 6C) with the contribution ratios of the first principal component of 72.11% and the second principal component of 8.75%. The principal component points of the WMP and CD groups were mildly discrete and were partially mixed together, but were clearly separated from the DM group. It indicated that treatment with WMP could similarly improve the liver samples between DM and WMP groups, and return the liver transcripts of DM rats to a certain degree of normal.

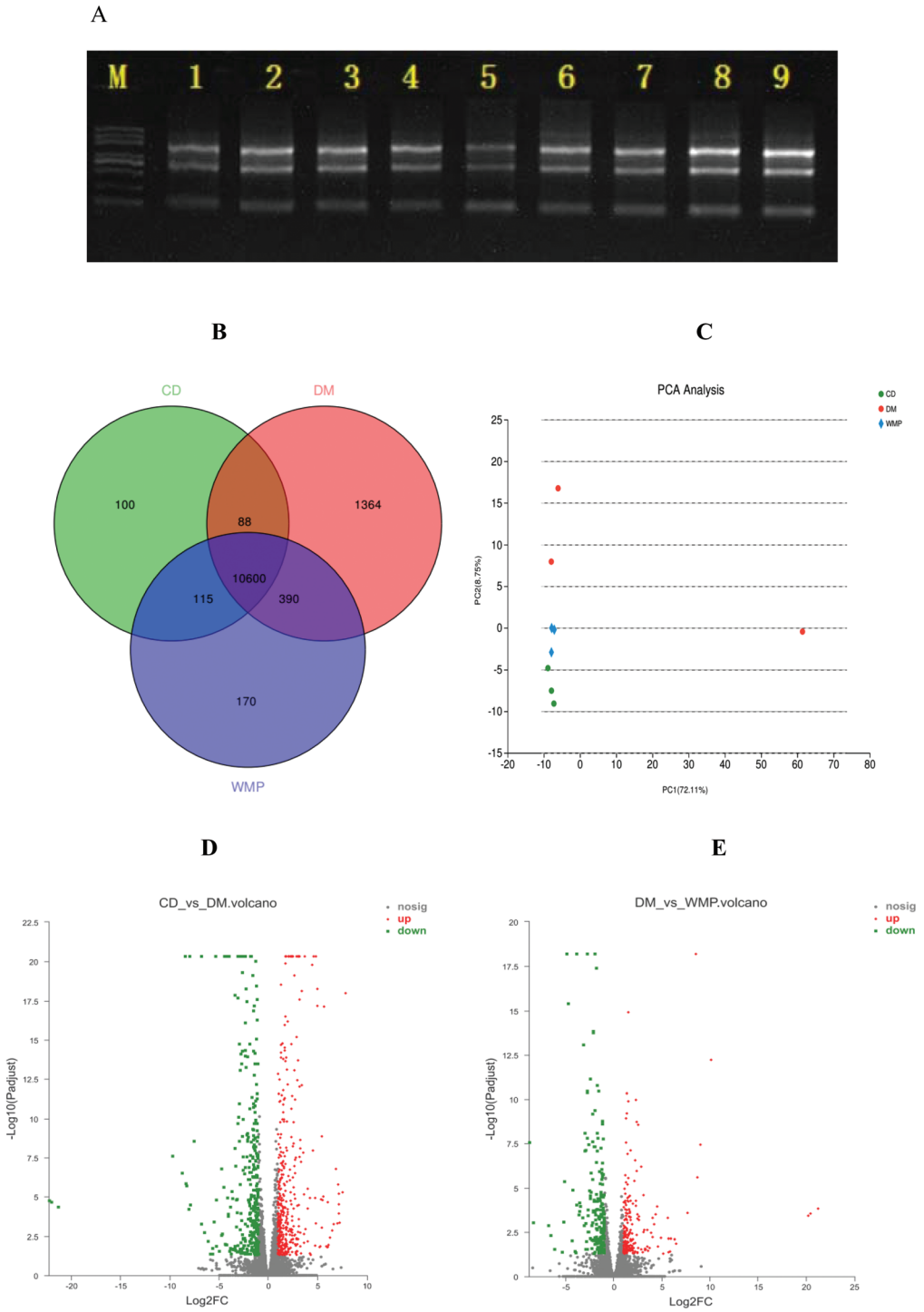

Fig. 6 1% agarose gel electrophoresis (voltage: 5 V cm^{-1} for 15 min), examination of RNA integrity in the CD, DM, and WMP groups. Lane M: RNA marker; lanes 1, 2 and 3: three CD samples; lanes 4, 5 and 6: three DM samples; and lanes 7, 8 and 9: three WMP samples (A). Genes differentially expressed in liver tissues of CD, DM, and WMP rats as shown by the Venn diagram (B) and the principal component analysis (PCA) of all quantifiable mRNAs (C). Volcano plot displaying differentially expressed genes between the CD and DM groups (D), and the WMP and DM groups (E) in liver tissues.

Differential expression was further examined by constructing the volcano plots (Fig. 6D and E), in which the *y*-axis corresponds to the mean expression value of log 10 (*P*-value) and the *x*-axis corresponds to the log 2 fold change value. The red dots correspond to significantly up-regulated transcripts ($P < 0.05$; false discovery rate, FDR, $q < 0.05$); the green dots correspond to significantly down-regulated transcripts ($P < 0.05$; FDR, $q < 0.05$); and the gray dots correspond to no stat-

Table 3 Assessment of assembly quality of CD, DM and WMP group cDNA libraries

Sample	Raw reads	Clean reads	Error rate(%)
CD_1	57 998 580	57 377 606	0.0232
CD_2	54 892 044	54 316 990	0.0231
CD_3	54 080 254	53 525 272	0.0233
DM_1	57 983 266	57 347 576	0.0233
DM_2	50 059 950	49 558 812	0.0232
DM_3	53 045 992	52 454 566	0.0232
WMP_1	52 452 808	51 837 732	0.0233
WMP_2	52 663 498	52 092 272	0.0231
WMP_3	55 144 936	54 536 450	0.0231

istically significant changes ($P < 0.05$; FDR, $q > 0.05$). The DEseq program was used to identify differentially expressed unigenes in the CD, DM, and WMP groups. For the DM group, 746 differentially expressed unigenes were identified relative to the CD group (402 up-regulated and 344 down-regulated). In comparison with the DM group, WMP therapy significantly altered 402 differentially expressed unigenes (195 up-regulated and 207 down-regulated). Evaluation of these genes demonstrated that treatment with WMP had a considerable impact on various biological processes, molecular functions, and cellular components in the liver.

3.9.3 Gene ontology (GO). GO analysis was performed (http://www.geneontology.org/GO.database.Shtml) to investigate the functional associations of the differentially expressed unigenes. The primary biological processes affected by WMP included the cellular process, the single-organism process, the biological process regulation, and the response to stimulus and metabolic process (Fig. 7A). The most affected cellular

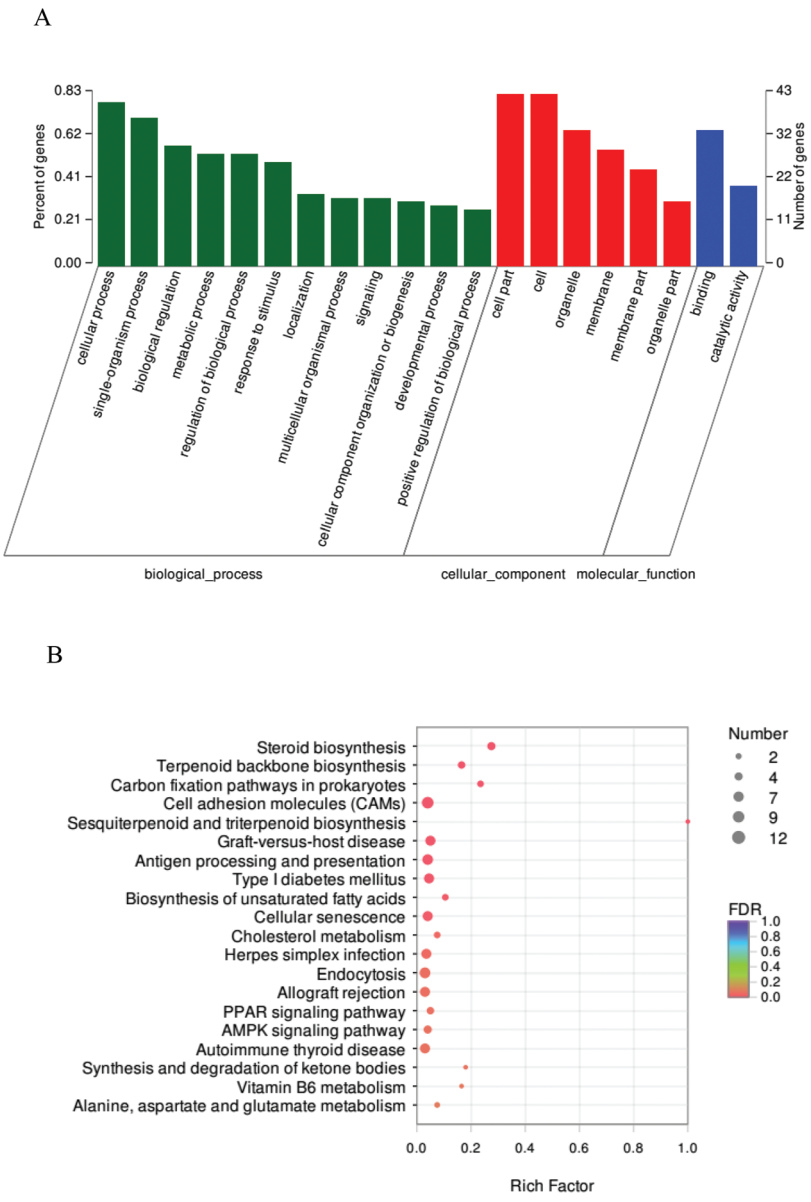

Fig. 7 GO analyses (A) and KEGG enrichment (B) of the differentially expressed genes reversed by WMP in liver tissues.

component ontologies included the cell, cell part, organelle, membrane, membrane part, and organelle part. The molecular function ontologies associated with WMP therapy were the binding and catalytic activity. KEGG analysis provided a basic platform for the systematic analysis of gene function in terms of the networks of gene products. To further identify the metabolic pathways related to T2DM, unigenes were analyzed using the KAAS (KEGG Automatic Annotation Server). A total of 32 pathways were revealed to be significantly altered by WMP ($P < 0.05$) (Table 4). In the KEGG pathway enrichment analysis, the main significant pathways were related to diabetes, including cell adhesion molecules (CAMs), antigen processing and presentation, endocytosis, human T-cell leukemia virus 1 infection, the AMPK signaling pathway, PPAR signaling pathway, and insulin signaling pathway, insulin secretion, endocytosis, steroid biosynthesis, cholesterol metabolism, and so on (Fig. 7B).

3.9.4 The details of key DEGs. In this study, WMP therapy associated with glucose metabolism was cataloged *via* whole transcriptome profiling of T2DM rat livers. Transcripts were sequenced deeply and analysis was performed using two commonly utilized packages: DESeq and Cuffdiff. Only those DEGs

Table 4 Key pathways associated with the gene expression profiles of WMP-treated rat livers, as determined by KEGG analysis

Pathway ID	KEGG pathway	Num	*P*-Value
map04514	Cell adhesion molecules (CAMs)	12	0.0001
map04612	Antigen processing and presentation	10	0.0002
map04144	Endocytosis	10	0.0039
map05166	Human T-cell leukemia virus 1 infection	10	0.0087
map05332	Graft-*versus*-host disease	9	0.0001
map04940	Type I diabetes mellitus	9	0.0002
map04218	Cellular senescence	9	0.0009
map05168	Herpes simplex infection	9	0.0029
map05330	Allograft rejection	9	0.0042
map05320	Autoimmune thyroid disease	9	0.0054
map05167	Kaposi sarcoma-associated herpesvirus infection	7	0.0230
map05163	Human cytomegalovirus infection	7	0.0450
map00100	Steroid biosynthesis	6	0.0000
map04152	AMPK signaling pathway	6	0.0060
map00900	Terpenoid backbone biosynthesis	5	0.0000
map03320	PPAR signaling pathway	5	0.0046
map05144	Malaria	5	0.0185
map04910	Insulin signaling pathway	5	0.0340
map00720	Carbon fixation pathways in prokaryotes	4	0.0000
map01040	Biosynthesis of unsaturated fatty acids	4	0.0008
map04979	Cholesterol metabolism	4	0.0025
map04911	Insulin secretion	4	0.0191
map04512	ECM-receptor interaction	4	0.0191
map00250	Alanine, aspartate and glutamate metabolism	3	0.0089
map04960	Aldosterone-regulated sodium reabsorption	3	0.0139
map00280	Valine, leucine and isoleucine degradation	3	0.0265
map00909	Sesquiterpenoid and triterpenoid biosynthesis	2	0.0001
map00072	Synthesis and degradation of ketone bodies	2	0.0059
map00750	Vitamin B6 metabolism	2	0.0070
map00910	Nitrogen metabolism	2	0.0173
map00220	Arginine biosynthesis	2	0.0249
map02020	Two-component system	2	0.0270

Table 5 Identified differentially expressed transcripts in liver tissues

	DM *vs.* CD			WMP *vs.* DM		
Gene	Log 2 fold change	FDR	Regulate	Log 2 fold change	FDR	Regulate
Gck	−8.368	1.49×10^{-6}	Down	10.153	6.27572×10^{-13}	Up
RT1-Ba	−2.684	2.92×10^{-33}	Down	2.864	6.54074×10^{-7}	Up
Fasn	−2.678	3.42×10^{-14}	Down	3.602	0.034478555	Up
Cd74	−1.868	2.81×10^{-7}	Down	2.157	4.09931×10^{-6}	Up
Jun	−1.169	0.007167	Down	1.725	5.4171×10^{-5}	Up
Plin3	1.114	0.000346	Up	−1.501	0.000640849	Down
Slc13a3	1.341	3.67×10^{-7}	Up	−1.393	0.000183054	Down
Got1	1.694	1.09×10^{-16}	Up	−2.712	5.11787×10^{-41}	Down
Cyp4a1	1.745	3.09×10^{-17}	Up	−2.133	0.000197497	Down
Myh7b	2.275	1.86×10^{-5}	Up	−1.992	0.000208995	Down

FDR, false discovery rate.

in the top half of the expressed genes were considered, while those in the bottom half were rooted out as previously suggested.[24] A total of 10 most significant DEGs were identified (FDR, $q < 0.05$ with log 2 fold change >1 or <−1), of which 5 were up-regulated in the WMP group relative to the DM group, while the other 5 were down-regulated (Table 5).

4 Discussion

T2DM is a complex chronic metabolic disease, and our previous study has reported that WMP shows a preventive effect on T2DM rats.[25] Our research team has also found that WMP shows a hypolipidemic effect on high-fat diet-induced obese mice and also the effect of antioxidation, and improves glucose and lipid metabolism, and the levels of TC and TG in postnatally monosodium glutamate (MSG)-induced obese mice, thus suppressing their fat accumulation and increasing the activities of glutathione peroxidase and superoxide dismutase in their livers.[26] A T2DM rat model used in this study simulated the insulin resistance and hyperglycemia in T2DM patients, according to the methods described by Reed *et al.*[27] and was employed to investigate the anti-diabetic effect of WMP. A T2DM rat model was established by using a high-fat diet combined with low dose STZ, which could cause insulin resistance, leading to impaired glycolipid metabolism, causing oxidative stress and inflammation, and damaging the pancreas and liver. MET was used as the positive control drug in the model for insulin sensitization.[27] The oral administration of MET (150 mg kg^{-1} d^{-1}) and treatment with three different doses of WMP (125, 250 and 500 mg kg^{-1} d^{-1}) have revealed a comparable ability to reduce the blood glucose levels, to improve lipid metabolism, insulin resistance, oxidative stress, and inflammation, and to protect the pancreas and liver of T2DM rats.

During the 5 week therapy, WMP treatment resulted in the reduction of blood glucose in diabetic rats, which was observed using OGTT, HOMA-IR, HOMA-β, and ISI. Being used as the positive control, MET exhibited the similar activities, which suggested that WMP was able to improve glucose metabolism through the enhancement of insulin sensitivity to T2DM and its efficacy was similar to MET. HOMA-IR, HOMA-β, and ISI were used in the evaluation of the degree of peripheral insulin resistance and islet β-cell function. In addition, as an indicator of insulin resistance, OGTT was improved by WMP in T2DM rats, which was consistent with the changes in HOMA-IR and HOMA-β. Pancreatic slice analyses of diabetic rats showed a decreased number of islet β cells, and this symptom could be improved by WMP. In our study, diabetic rats with a continuous decrease in body weight with time as reported previously[28] may be related to the inadequate capability of glucose utilization to produce energy and to reinforce the breakdown of fats and proteins. At the endpoint of therapy, the body weight of WMP rats showed a smaller decline compared with the DM rats, demonstrating a significant amelioration on reversed emaciation, although it did not reach the levels of CD rats.

A long-term high-fat diet could cause hyperlipidemia, and it has been reported to be accompanied by hyperglycaemia.[29] Moreover, it is well known that the consumption of a high-fat diet will lead to the increased TC, TG, and LDL-C levels.[15] Our results suggested that the lipid metabolism disorder was caused in the T2DM rat model. MET and WMP displayed a powerful ability to ameliorate the serum and liver lipids. Numerous previous studies have reported that an increase in the lipid level is an important risk factor for the development of cardiovascular disease, and a decrease in the LDL-C levels would significantly reduce the risk of cardiovascular disease and mortality in T2DM patients.[30,31] The disorder of lipid metabolism and pancreatic injury could lead to an increase in lipid peroxidation and free radical production.[32] In T2DM rats, hyperglycemia could lead to oxidative stress through glucose autooxidation.[33–35] The formation of free radicals in T2DM rats was increased and the antioxidant potential was decreased. Our results suggested that WMP was able to reduce oxidative stress in T2DM rats, which might contribute to the improvement of insulin sensitivity. The imbalance of oxidant/antioxidant defense systems was observed in alterations of antioxidant enzymes including SOD and CAT, which are common markers in the study of oxidative stress.[36] MDA is the oxidative stress marker that could be detected in the salivary glands of diabetic rats, and was significantly higher in DM rats than in the other groups.[37] Oxidative stress was also implicated in promoting low-grade systemic inflammation in T2DM rats. It is now broadly accepted that low-grade chronic inflammation plays an important role in the development of diabetes.[38] T2DM also causes low-grade chronic inflammation resulting in the increased level of LPS in individuals. LPS could further lead to the secretion of a variety of pro-inflammatory mediators, including TNF-α and IL-6.[36] Low-grade inflammation in T2DM rats through LPS-associated endotoxaemia could encourage insulin resistance (IR) and cytokine secretion.[39] Our study revealed that WMP could decrease the LPS levels in the blood and liver of T2DM rats. It is well known that IL-6 and TNF-α were elevated in IR and were found to be significantly reduced by WMP. Therefore, our results suggested that WMP relieved IR by reducing inflammatory markers. In addition, oxidative stress could cause liver damage in T2DM rats with the elevated biomarker enzymes, such as ALT and AST,[40] indicating liver damage induced by the high-fat diet combined with STZ. In contrast, WMP significantly decreased the serum AST and ALT levels. Besides, the pathological observation of liver showed that WMP obviously reduced hepatic lipid accumulation in T2DM rats and attenuated the swelling of adipocytes, significantly reduced the steatosis grades, and the hepatic TC, TG, and LDL-C levels. Thus, WMP seemed to exhibit restorative and protective effects on the liver of T2DM rats.

The liver serves a crucial role in the maintenance of material metabolism, as the "metabolic center" of the human body, and is an important organ associated with glucose modulation. RNA-seq, as a comprehensive and accurate tool for gene expression pattern analyses,[41] was utilized to obtain whole transcriptomes from the livers of CD rats, DM rats and

DM rats treated with WMP. Ten differentially expressed genes (DEGs) were identified in the WMP treated group relative to the DM group *vs.* CD group (FDR, $q < 0.05$ with log 2 fold change >1 or <−1), in which the downregulated order was Gck ≥ Fasn ≥ RT1-Ba ≥ Cd74 ≥ Jun in the DM group while the upregulated order with almost the same fold-change as in the WMP group followed the order Myh7b ≥ Cyp4a1 ≥ Got1 ≥ Slc13a3 ≥ Plin3 in the DM group (Table 5). Among them, GCK (Glucokinase) is the most promising target, which is the rate-controlling step in insulin-stimulated hepatic glycogen synthesis *in vivo*.[42] It takes part in the insulin signaling pathway, insulin secretion pathway, valine, leucine and isoleucine degradation pathway, and alanine, aspartate and glutamate metabolic pathway (in Table 4 and Fig. 1S†). The second potential target is RT1-Ba which is involved in 8 key pathways related to inflammation or immunity regulated by WMP in T2DM rats (in Table 4 and Fig. 1S†), and herein it is reported for the first time that RT1-Ba is so important for the treatment of diabetes.

5 Conclusion

Generally, this study showed that the therapy of WMP improved glycolipid metabolism in STZ-induced and high-fat diet maintained T2DM rats. Moreover, WMP had an important role in regulating the body weight, blood glucose, lipids, the insulin sensitivity, oxidative stress, and inflammation, and in protecting the pancreas and liver of T2DM rats. The research has provided a global view of the complexities of rat liver transcriptome and has identified 10 differentially expressed genes (DEGs) in WMP-treated rats relative to diabetic rats, which might serve as promising targets to affect glycolipid metabolism. This study indicates that WMP is a new type of resource with great potential for developing treatment for T2DM.

Ethics statement

The investigation was conducted according to the ethical standards and national and international guidelines. The animal protocol was approved by the Animal Experimental Ethics Committee of Yunnan University of Traditional Chinese Medicine, and animals were cared for and utilized in accordance with the guidelines approved by the Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine.

Conflicts of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

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