Sex differences in diabetes-induced hepatic and renal damage

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Abstract. Diabetes mellitus (DM) is a disease that affects millions of individuals worldwide and is characterized by abnormal glucose metabolism that can induce severe damage to numerous organs throughout the body. Sex differences have been demonstrated in a number of factors associated with diabetes and its complications, such as diabetic kidney disease and diabetic liver disease. To investigate the sex differences in DM further, the changes in the weight, food and water intake, and blood sugar of mice were recorded for 8 weeks in the present study. Hematoxylin and eosin staining, Masson's trichrome staining and transmission electron microscopy were used to observe the pathological changes of liver and kidney tissues. There is no significant difference in the water intake and blood glucose concentration between db/db female and male mice was observed. However, sex differences in liver and kidney damage including glomerular injury and hepatic fibrosis were found. In conclusion, the present study characterized the features of liver and kidney damage in db/db mice and indicated that sex differences should be taken into account in experiments using female and male experimental animals. Furthermore, sex differences should be taken into account in the selection of drug interventions in experiments and in clinical drug therapy.

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Abbreviations: DM, diabetes mellitus; DN, diabetic nephropathy; NAFLD, non-alcoholic fatty liver disease; H&E, Harris's hematoxylin and eosin; OGTT, oral glucose tolerance test

Key words: DM, db/db mice, sex difference, liver, kidney

Introduction

Diabetes mellitus (DM) is a disorder of glucose metabolism characterized by hyperglycemia due to insufficient insulin or insulin resistance, and it is estimated to afflict 439 million individuals worldwide by 2030 due to lifestyle changes, such as extensive sitting and high fat diet (1). Chronic DM is associated with dysfunction and failure of most organs in the body such as the eyes, feet, nervous system, kidneys and liver (2).

Diabetic nephropathy (DN), a complication of diabetic microangiopathy, is a major cause of end-stage renal disease (3,4). Patients with DN have sustained proteinuria and exhibit a glomerular filtration rate in progressive decline when it is measured twice with an interval of 3-6 months, which is often associated with increased blood pressure and can eventually lead to end-stage renal disease (5,6). Liver fibrosis is another common complication that is associated with DM (7,8). It has been reported that the rate of advanced liver fibrosis is higher in individuals with diabetes compared with in those without diabetes (9). Furthermore, there is an intricate association between DM and non-alcoholic fatty liver disease (NAFLD) (10). NAFLD is characterized by a wide range of liver diseases, from benign steatosis, to inflammation, fibrosis, cirrhosis, liver failure and finally, hepatocellular carcinoma. However, this series of pathological processes is not associated with excessive alcohol, drugs or viral factors (11-14). Evidence has suggested that patients diagnosed with NAFLD have a two-fold increased risk of developing DM, particularly in those with tumors, cardiovascular disease and renal disease (15,16). Furthermore, studies have indicated that diabetes is an independent risk factor for NAFLD (17), and women with a history of gestational diabetes have an increased risk of NAFLD (18,19). Conversely, remission of hepatic steatosis may prevent the development of diabetes (20,21). From the aforementioned evidence, it was hypothesized that there may be sex differences in liver and kidney injury induced by diabetes. Therefore, db/db mice are spontaneous type 2 diabetic mice caused by Leptin receptor gene deficiency, and its pathogenesis is very similar to that of human type 2 diabetes so they were selected as a model of experimental diabetes in the present study to observe liver and kidney impairment by pathological sectioning to assess whether there are sex differences in tissue injury.

Materials and methods

Equipment and reagents. An advantage blood glucose meter and strips from Roche Diagnostics GmbH, Harris's hematoxylin and eosin (H&E) stain from Zhuhai Besso Biotechnology Co., Ltd. and a Masson staining kit (cat. no. G1340) from Beijing Solarbio Science & Technology Co., Ltd. were used in the present study. Furthermore, an optical microscope from Olympus Soft Imaging Solutions GmbH (BX53), the Hitachi H-7650 transmission electron microscope (Hitachi High-Technologies Corporation), the Leica EG1150H paraffin embedding machine (Leica Microsystems GmbH) and the Leica Leitz 1512 microtome (Leica Microsystems GmbH) were also used.

Animals. Male and female db/db (37-46 and 36-44 g, respectively) and db/m C57BLKS/J (BKS) (18-21 and 17-20 g, respectively) mice (8-9 weeks; total no., 32; n=8 for each group) were purchased from Changzhou Cavens Experimental Animal Co., Ltd. [experimental animal production license: : scxk (Su) 2016-0010] and housed in the Medical Experimental Animal Center of North China University of Science and Technology (Tangshan, China). db/db (C57BL/KSJ) mice with Leptin receptor point mutation are homozygous (-/-), db/m is heterozygous (+/-), WT is m/m mice (+/+). They were provided with Co60-irradiated chow and water, *ad libitum*. The animal experiments were performed in specific-pathogen-free barrier laboratory at the Experimental Animal Centre of North China University of Science and Technology (Tangshan, China). The room temperature was maintained at 23±1˚C (humidity, 55±10%) and there was a 12-h light/dark cycle. The diabetic model was defined as a blood glucose level >16.67 mmol/l. The random blood glucose concentration was periodically measured from the tail vein blood, using a scalpel to scrape through a small portion of skin at the end of the tail of each mouse. The volume of blood taken each time was 0.6 μl, and thus the damage to the experimental animal was minimal. Blood glucose testing was conducted at 0, 1, 3, 5, 6,7 weeks. The mice were starved for 8 h to carry out the oral glucose tolerance test (OGTT) at 4 weeks. Blood glucose levels were measured after glucose administration (i.g, 200 mg/kg) at time points of 0, 30, 60 and 120 min. Due to the short interval, that the tail was only scraped once before the blood samples were collected at the different time points. After feeding for 8 weeks, the mice were anaesthetized using ether, and then euthanized by cervical dislocation. Liver and kidney tissue specimens were dissected and fixed for 24 h at 4˚C in 4% neutral formaldehyde for paraffin embedding or in 2.5% glutaraldehyde for electron microscopy. The present study was approved by The Experimental Animal Ethics Committee of North China University of Science and Technology (Tangshan, China).

Histological experiments. Tissue sections of 5-μm thickness were prepared, stained with Harris's hematoxylin for 20 min, washed three times in distilled water and differentiated with 1% hydrochloric acid-alcohol for 3 sec. The sections were then washed an additional three times in distilled water, stained with eosin for 10 min, dehydrated using an ethanol gradient, clarified using xylene, sealed with neutral gum and examined using light microscopy (all performed at room temperature) (22). All steps were performed at room temperature and Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) was used for analysis.

Other sections were dewaxed, fixed using Bouin's solution (Nanjing SenBeiJia Biological Technology Co., Ltd.) for 10 min and then, following the operation of a Masson staining kit, the sections were stained with Harris's hematoxylin for 5 min, washed with tap water for 2 min, differentiated with 0.5% hydrochloric acid-alcohol for 10 sec and washed with distilled water for 5 min. The sections were then stained with Masson's complex staining solution for 5 min, differentiated with 0.2% acetic acid solution for 5 min, differentiated with 5% phosphomolybdic acid solution for 5 min, rinsed 3 times with 0.2% acetic acid solution and rinsed with 2% aniline blue solution for 15 sec. Subsequently, the sections were rinsed with anhydrous ethanol for 15 sec, left for 2 h to dry, sealed with neutral gum and examined using light microscopy. All steps were performed at room temperature.

Ultrastructural observation of mouse specimens. Portions of liver and kidney tissues (1 mm^3) were fixed in 2.5% glutaraldehyde for 3 h (4˚C), treated with 1% osmic acid for 1 h (4˚C), dehydrated using a 50-90% acetone gradient (4˚C) and embedded in EPON 812 (37˚C). Ultrathin sections (80 nm) were prepared after orientation under a light microscope. The sections were examined by transmission electron microscopy after dual staining with uranium acetate and lead citrate (for 15 min, respectively) at room temperature (23).

Statistical analysis. SPSS 21.0 software (IBM Corp.) was used to analyze the data. Normally distributed data are expressed as the mean ± standard deviation (n=8 for body mass, food and water intake, blood glucose and OGTT experiment, respectively; n=3 for histology of the liver and kidney tissues) and comparisons between groups were made using two-way ANOVA and Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Body mass, food and water intake, and blood glucose. The weight and food intake of the mice over 7 weeks were recorded and there were no significant differences between db/m female and male mice ($P>0.05$), or db/db female and male mice ($P<0.05$) (Tables I and II). The body mass and food intake of the db/db mice were significantly higher compared with those of the db/m mice, irrespective of sex ($P<0.05$; Tables I and II). In addition, there were no significant differences in water intake or blood glucose concentrations between db/m female and male mice. However, differences in water intake and blood glucose were found between db/db female and male mice $_{\text{malefemale}}$ $P<0.05$; Tables III and IV).

OGTT. OGTT was performed at the end of the 7th week. Blood glucose levels in both female and male db/m mice were significantly lower compared with those in db/db mice at 30 and 60 min (Fig. 1A). Furthermore, the area under the curve calculated for db/m mice was significantly smaller compared with that for db/db mice $P<0.05$; Fig. 1B). From the results of the present study, abnormal glucose tolerance in db/db mice

Table I. Results of body weight of mice in each group (n=8).

	Body weight (g)					
Group	0 weeks	1 week	3 weeks	5 weeks	6 weeks	7 weeks
db/m male	21.3±1.12	22.8±1.04	23.9±1.13	25.3±1.21	25.0±0.85	26.3±0.76
db/m female	18.6±0.84	20.9±1.91	23.3±0.90	24.1±0.76	24.6±0.94	25.3±1.36
db/db male	42.0±2.48[a,b]	43.0±2.60[a,b]	46.9±1.93[a,b]	48.8±1.62[a,b]	51.4±1.44[a,b]	50.3±2.70[a,b]
db/db female	40.7±2.02[a,b]	43.2±2.46[a,b]	46.8±2.34[a,b]	50.2±4.58[a,b]	50.1±3.28[a,b]	49.9±3.53[a,b]

[a]P<0.05 vs. db/m male and [b]P<0.05 vs. db/m female. Mean ± SD.

Table II. Results of daily food intake of mice in each group (n=8).

	Daily food intake (g)					
Group	0 weeks	1 week	3 weeks	5 weeks	6 weeks	7 weeks
db/m male	3.8±0.28	3.2±0.12	3.7±0.23	4.0±0.21	3.8±0.25	3.9±0.18
db/m female	3.5±0.25	3.0±0.18	3.4±0.18	3.4±0.22	3.5±0.24	3.6±0.18
db/db male	8.0±0.21[a,b]	7.3±0.54[a,b]	7.7±0.23[a,b]	7.3±0.32[a,b]	7.5±0.35[a,b]	7.6±0.40[a,b]
db/db female	7.6±0.43[a,b]	6.7±0.07[a,b]	6.6±0.34[a,b]	6.5±0.48[a,b]	6.8±0.20[a,b]	6.3±0.50[a,b]

[a]P<0.05 vs. db/m male and [b]P<0.05 vs. db/m female. Mean ± SD.

Table III. Results of daily water intake of mice in each group (n=8).

	Daily water intake (ml)					
Group	0 weeks	1 week	3 weeks	5 weeks	6 weeks	7 weeks
db/m male	4.9±0.29	4.6±0.30	4.6±0.39	4.9±0.30	4.7±0.27	4.7±0.32
db/m female	4.0±0.27	3.4±0.26	3.8±0.32	3.7±0.41	3.6±0.31	3.6±0.30
db/db male	15.2±0.15[a,b]	14.4±1.01[a,b]	14.6±0.63[a,b]	14.9±0.21[a,b]	14.9±0.46[a,b]	14.6±0.83[a,b]
db/db female	10.1±0.16[a,b,c]	8.8±0.46[a,b,c]	9.3±0.18[a,b,c]	8.6±0.56[a,b,c]	9.0±0.29[a,b,c]	9.0±0.23[a,b,c]

[a]P<0.05 vs. db/m male, [b]P<0.05 vs. db/m female and [c]P<0.05 vs. db/db male. Mean ± SD.

Table IV. Results of random blood glucose of mice in each group (n=8).

	Random blood glucose (mmol/l)					
Group	0 weeks	1 week	3 weeks	5 weeks	6 weeks	7 weeks
db/m male	6.71±0.41	6.43±0.53	6.59±0.49	6.31±0.41	7.49±0.82	6.23±1.18
db/m female	6.49±0.45	6.38±0.58	6.30±0.68	6.41±0.59	6.08±0.46	5.86±0.77
db/db male	20.26±1.36[a,b]	21.79±1.55[a,b]	26.25±0.86[a,b]	27.14±0.57[a,b]	27.01±1.24[a,b]	26.86±2.31[a,b]
db/db female	18.30±0.79[a,b]	17.33±1.26[a,b,c]	17.55±1.25[a,b,c]	20.04±0.58[a,b,c]	20.68±1.30[a,b,c]	20.40±1.77[a,b,c]

[a]P<0.05 vs. db/m male, [b]P<0.05 vs. db/m female and [c]P<0.05 vs. db/db male. Mean ± SD.

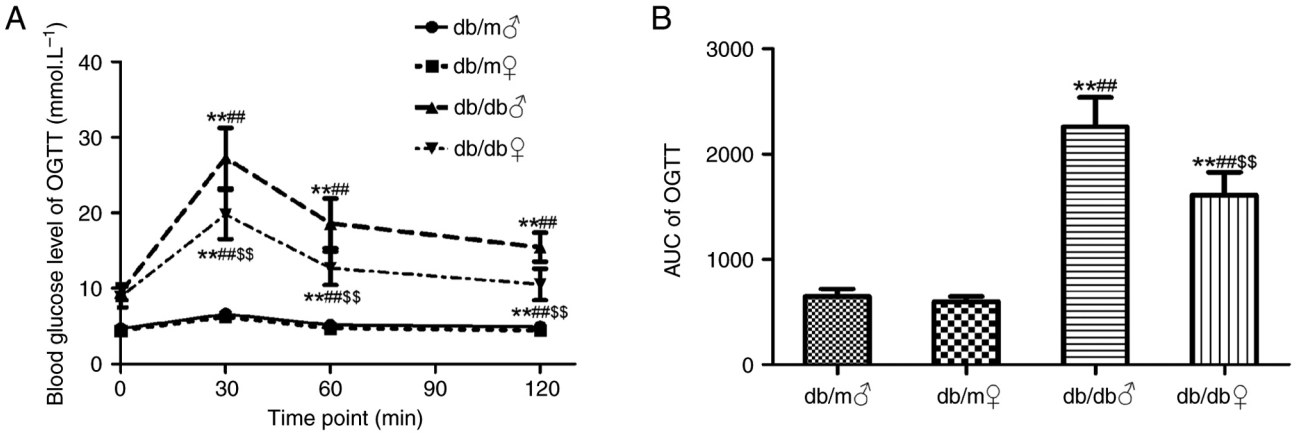

Figure 1. Results of the OGTT of mice in each group. (A) Levels of blood glucose (mmol/l) was determined in the different groups using the OGTT. (B) AUC of the OGTT was determined. All values are expressed as the mean ± SD (n=8). **P<0.01 vs. db/m male; $^{\#\#}$P<0.01 vs. db/m female; and $^{\$\$}$P<0.01 vs. db/db male. OGTT, oral glucose tolerance test; AUC, area under the curve. ♂, male; ♀, female)

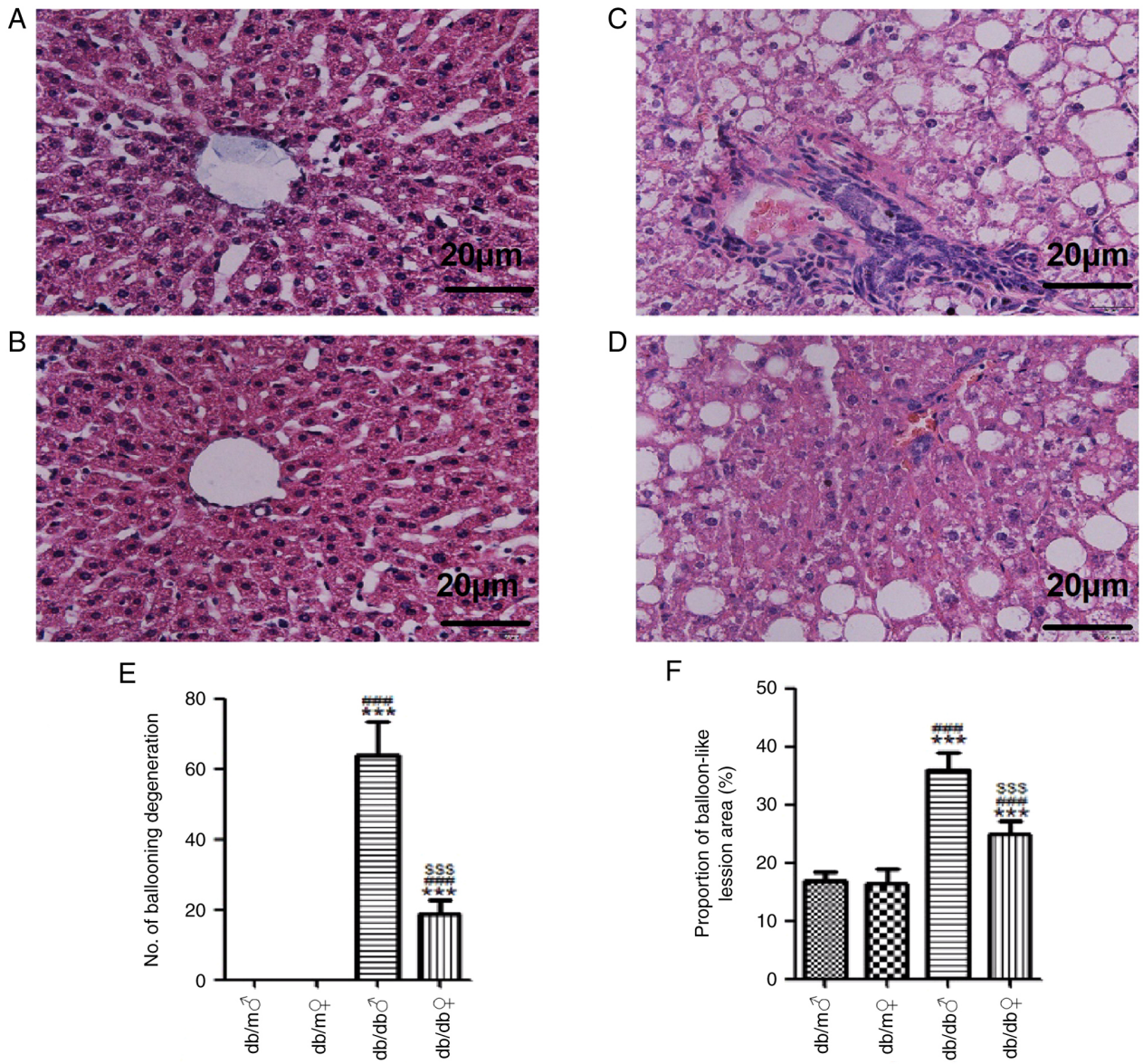

Figure 2. Representative images of liver tissue stained with H&E. H&E stained liver tissues of (A) db/m male, (B) db/m female, (C) db/db male and (D) db/db female mice (magnification, x400). (E) Mean number of ballooning degenerations and (F) proportion of balloon-like lesion area (%) in each group. Scale bar, 20 μm. All values are expressed as the mean ± SD (n=3). t=32.19, ***P<0.001 vs. db/m male; t=29.01, $^{\#\#\#}$P<0.001 vs. db/m female; and t=26.76, $^{\$\$\$}$P<0.001 vs. db/db male. H&E, hematoxylin and eosin. ♂, male; ♀, female.

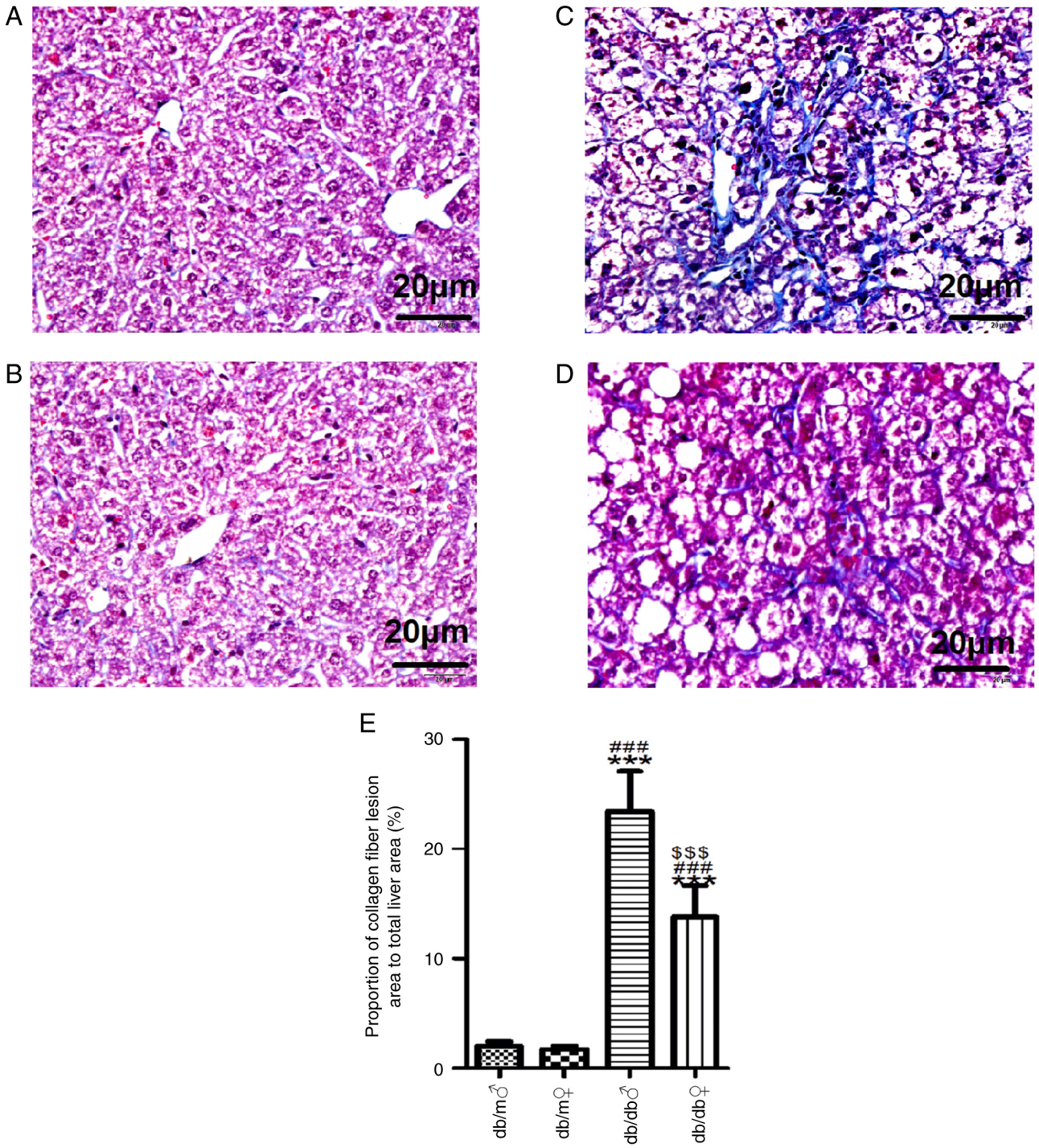

Figure 3. Representative images of liver tissue stained with Masson's staining. Masson's trichrome stained liver tissues of (A) db/m male, (B) db/m female, (C) db/db male and (D) db/db female mice (magnification, x400). (E) Proportion of collagen fiber lesion area to total liver area (%) in each group. Scale bar, 20 μm. All values are expressed as the mean ± SD (n=3). t=21.23. ***P<0.001 vs. db/m male; t=17.21, $^{###}$P<0.001 vs. db/m female; and t=24.27, $^{\$\$\$}$P<0.001 vs. db/db male. ♂, male; ♀, female.

was demonstrated; there was a notable sex difference ($_{malefemale}$; P<0.05).

Histology of the liver and kidney tissues. H&E staining demonstrated that both male and female db/m mice had well-arranged liver lobules and normal structures of liver lobules, hepatic cords and sinusoids, and the hepatocytes were regular in shape, uniform in size and distributed radially around the central vein (Fig. 2). However, both male and female db/db mice had irregular hepatic lobules, disordered hepatocytes and ballooning of a number of hepatocytes, and these changes were further marked in male db/db mice (Fig. 2). Masson's trichrome staining demonstrated that there was a small amount of collagen deposition in the hepatic interstitium of male and female db/m mice. Conversely, there were numerous collagen fibers in male db/db mice but significantly less in female db/db mice (Fig. 3).

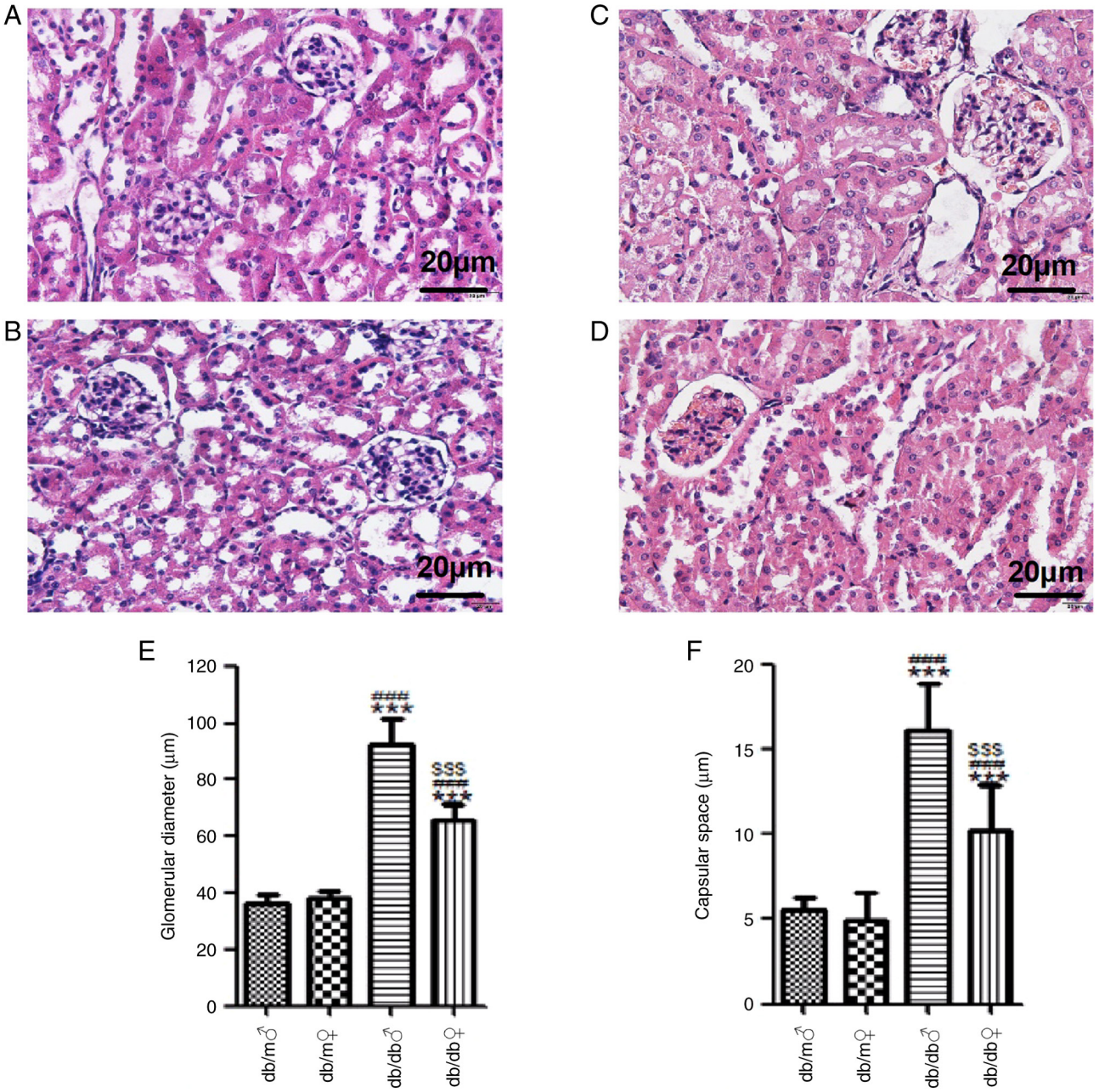

Figure 4. Representative images of renal tissue stained with H&E. H&E stained renal tissues of (A) db/m male, (B) db/m female, (C) db/db male and (D) db/db female mice (magnification, x400). (E) Mean glomerular diameter (μm) and (F) capsular space (μm) in each group. Scale bar, 20 μm. All values are expressed as the mean ± SD (n=3). t=18.25, ***P<0.001 vs. db/m male; t=15.29, $^{###}$P<0.001 vs. db/m female; and t=27.84, ^{SSS}P<0.001 vs. db/db male. H&E, hematoxylin and eosin; ♂, male; ♀, female.

H&E staining demonstrated that the renal structure of db/m mice of both sexes was normal, whereby the glomerular outlines were clear, the glomerular basement membrane was not thickened, the mesangial matrix indicated no sign of proliferation, the tubular epithelial cells were ordered and regularly shaped, and the renal interstitium had no abnormal features. Conversely, male db/db mice had larger glomeruli (92.32 μm) and wider renal capsular space (16.11 μm) compared with db/m mice (36.33 and 5.57 μm, respectively). In female db/db mice, the glomerular volume (65.36 μm) was larger compared with db/m mice (38.46 μm) values, but the difference from the db/m mice was less marked compared with that in the male db/db mice (Fig. 4). Masson's trichrome staining demonstrated that there was a small amount of collagen deposition in the renal interstitium of db/m mice, whereas there were large numbers of collagen fibers in the renal interstitium of db/db mice. However, the renal interstitial fibrosis in the male db/db mice was significantly increased compared with that in the female db/db mice (Fig. 5). The results of the present study showed that male db/db mice had more severe liver and kidney damage compared with female db/db mice.

Electron microscopy findings in the liver and kidney tissues. Electron microscopy demonstrated that the podocyte nuclei of db/m mice were regular in shape and that the chromatin was evenly distributed. In addition, normal nucleoli,

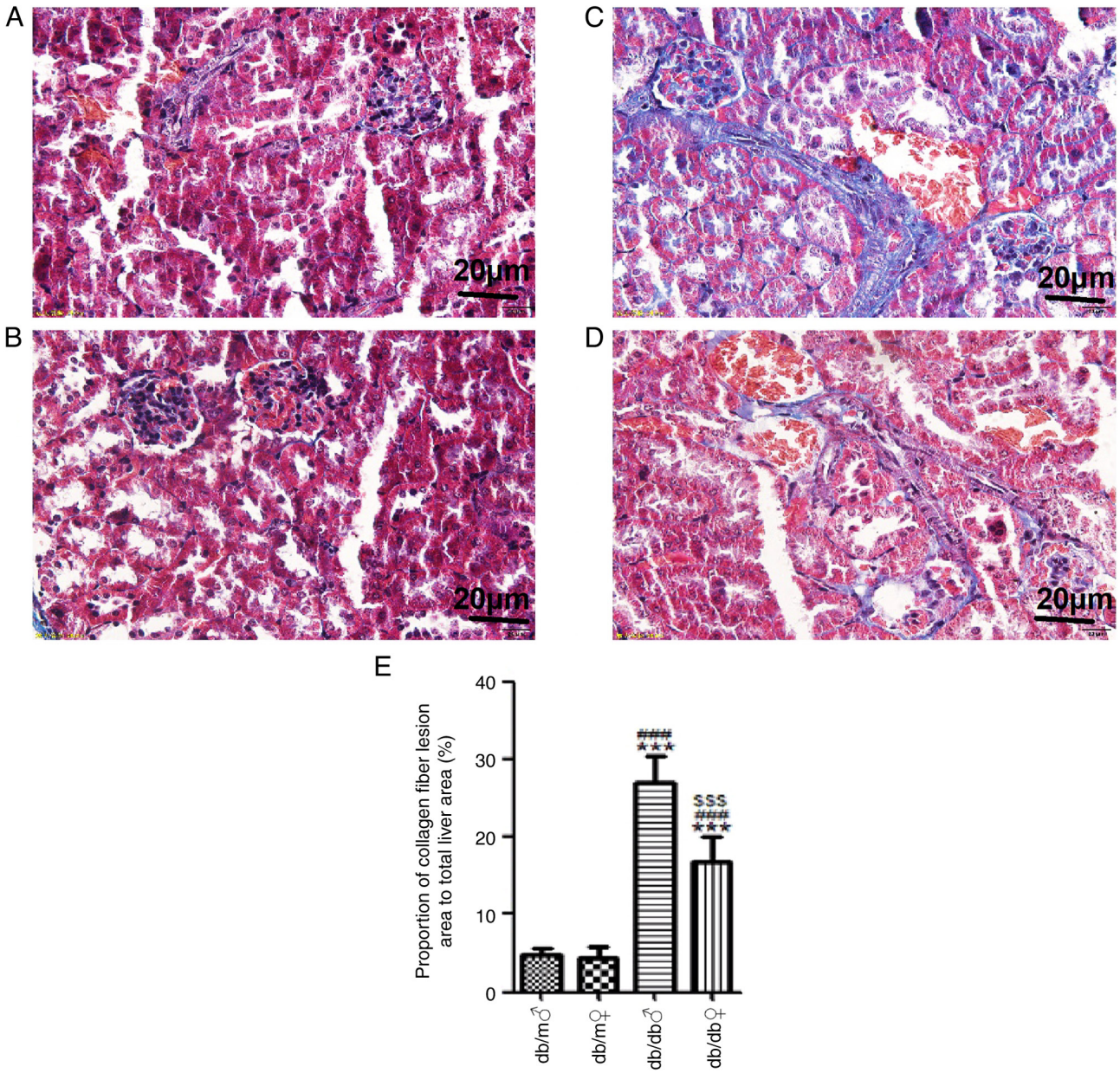

Figure 5. Representative images of renal tissue stained with Masson's staining. Masson's trichrome stained renal tissues of (A) db/m male, (B) db/m female, (C) db/db male and (D) db/db female mice (magnification, x400). (E) Proportion of collagen fiber lesion area to total renal area (%) in each group. Scale bar, 20 μm. All values are expressed as the mean ± SD (n=3). t=24.19, ***P<0.001 vs. db/m male; t=32.11, $^{###}$P<0.001 vs. db/m female; and t=33.18, $^{\$\$\$}$P<0.001 vs. db/db male. ♂, male; ♀, female.

mitochondria, endoplasmic reticulum and lysosomes were observed. However, in female db/db mice, a number of the mitochondria were enlarged and collagen deposition was observed compared with db/m mice. Furthermore, in male db/db mice, the mitochondria indicated oedema and vacuolar degeneration and a large number of collagen fibers were present (Fig. 6). Transmission electron microscopy demonstrated that the glomerular basement membranes of db/m mice of both sexes were intact and indicated no thickening, and the foot processes were evenly distributed. Conversely, there was collagen deposition in the stroma of the kidneys of female db/db mice, with a larger amount of collagen present in the stroma of male db/db mice (Fig. 7). These results indicated that there are sex differences in liver and kidney ultrastructure damage in db/db mice, and male db/db mice have more severe liver and kidney ultrastructure damage compared with female db/db mice.

Discussion

To investigate whether there are sex differences in liver and kidney damage in diabetic mice, db/db mice were selected as diabetic model animals in the present study. The db/db mouse is an autosomal recessive leptin receptor gene-deficient mouse that was selected in the C57BL/KSJ strain (24). The homozygous db/db mouse spontaneously develops diabetes and shows hyperphagia and obesity from 4 weeks of age (25). Thus, the pathogenesis of its phenotype resembles that of human type 2 DM (26,27). Homozygous db/db mice are infertile, but heterozygous db/m mice can be bred by introducing the 'misty'

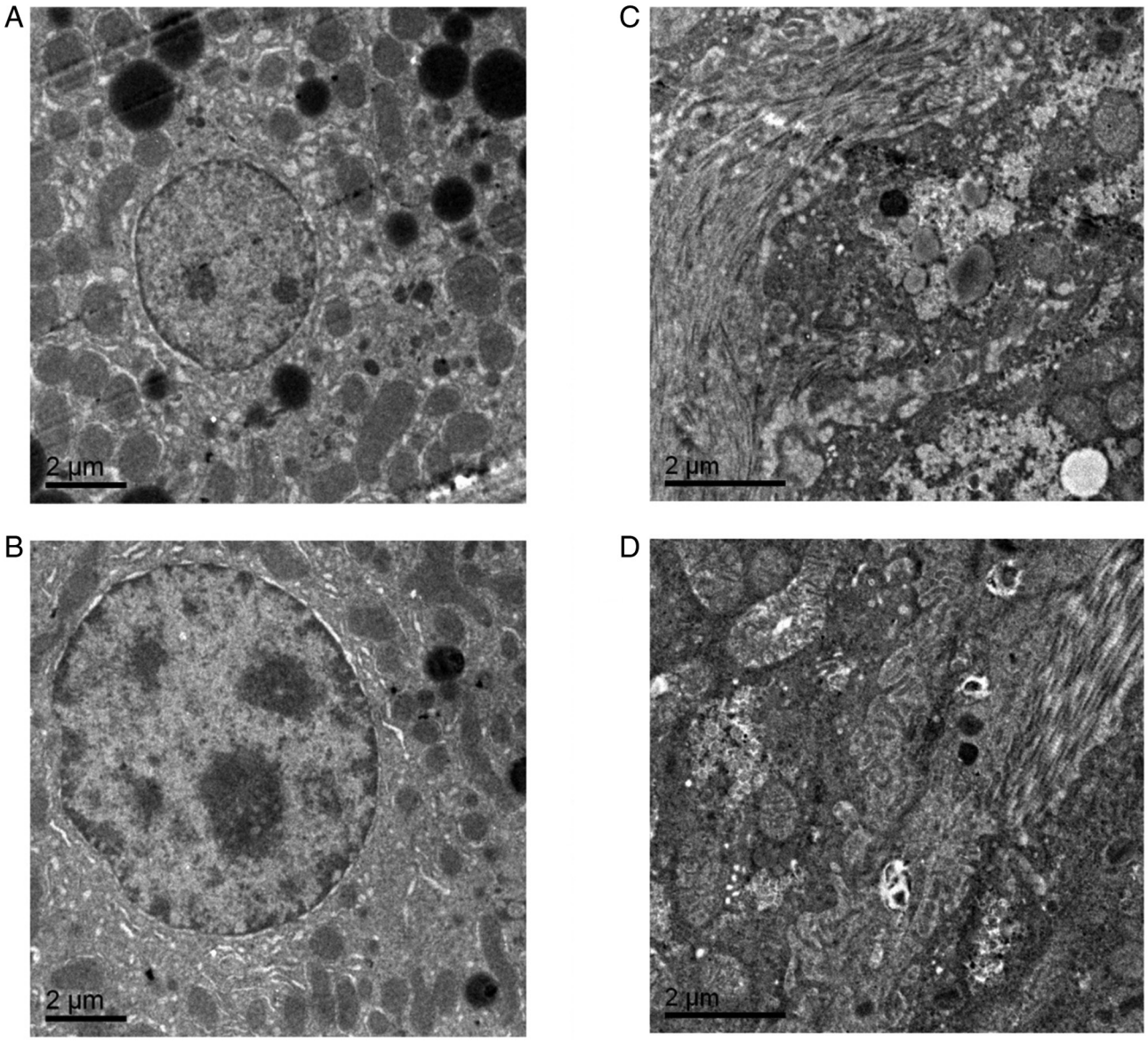

Figure 6. Ultrastructure of mouse liver. Electronic microscopy structure images of liver tissues in mice including (A) db/m male, (B) db/m female, (C) db/db male and (D) db/db female.

gene (28). The latter has normal body mass, blood glucose and insulin concentrations, such that it can be used as a control strain in studies of db/db mice (29).

In the present study, the effects of diabetes on the liver and kidneys of db/db mice of both sexes were characterized with reference to db/m controls. As expected, the body masses and blood glucose concentrations of the db/db mice were significantly higher compared with those of the db/m mice of both sexes. However, the results of the present study demonstrated that there were sex differences in body weight and food intake of mice, but the differences were not statistically significant. This result may have been caused by the small sample size of the present study. Future studies should use an increased sample size to track sex differences in body weight. Compared with the control group, db/db mice showed hepatocyte swelling, vacuolization and collagen deposition in the liver and kidney tissues, but the degree of pathological changes in the kidney tissue was milder. In previous pathological studies conducted in humans, patients with diabetic kidney disease also showed glomerular basement membrane thickening and interstitial fibrosis (30), and those with diabetes complicated by NAFLD showed steatosis and fibrosis (31). Thus, the present findings are consistent with those in humans, which implies that the db/db mouse is a suitable model for the study of diabetes and its complications.

In the present study, there were significant differences in the body mass or blood glucose concentration of the male and female db/db mice except for week 0, and the severity of hepatic and renal fibrosis in male db/db mice was significantly higher compared with that in female db/db mice. There is controversy regarding sex differences in diabetic complications (32). However, the experimental results of the present study observed a number of sex differences in diabetic mice. The present study was a preliminary investigation, and in future studies sex differences and organ damage will be investigated more thoroughly and over a longer

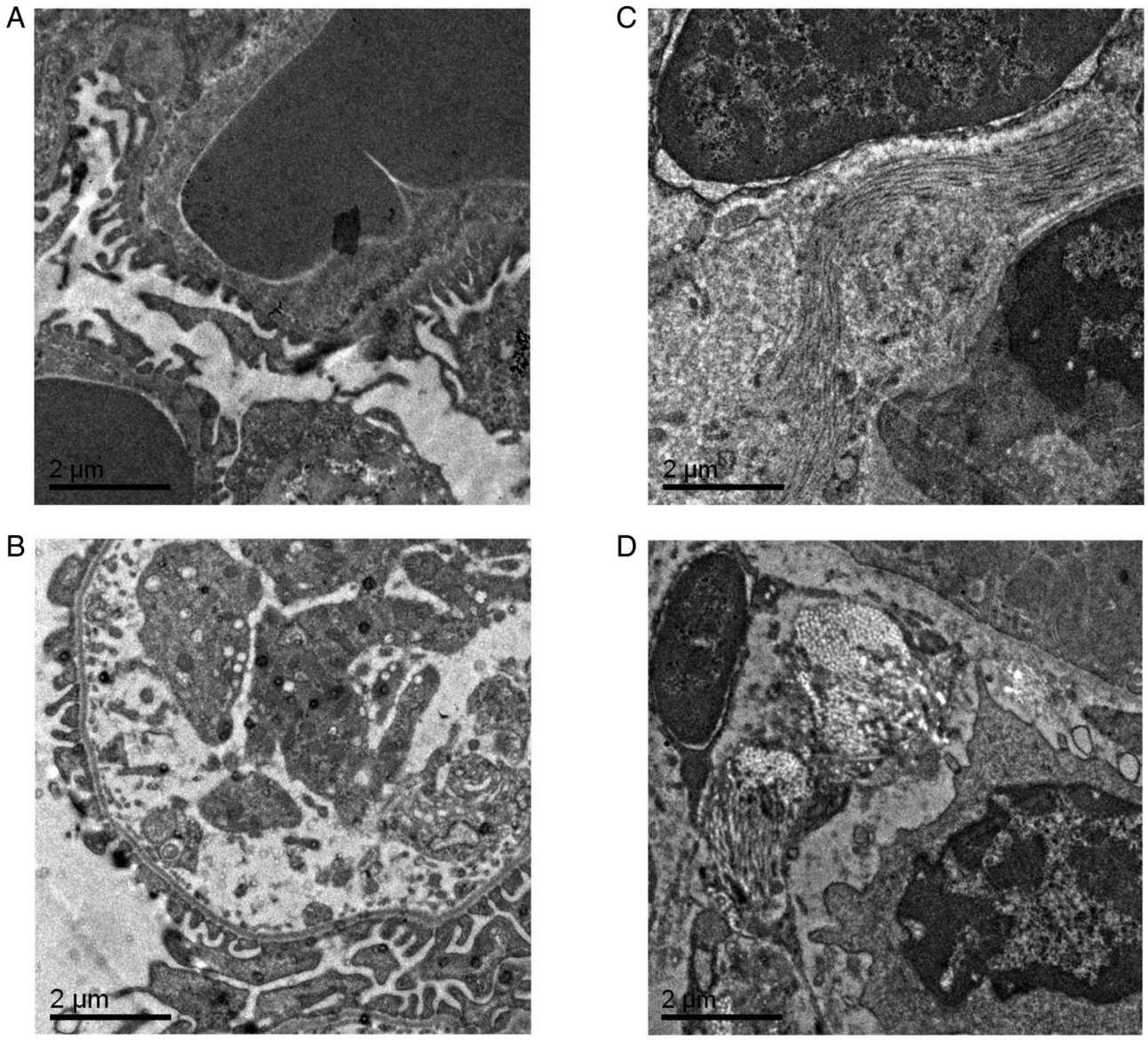

Figure 7. Ultrastructure of mouse kidney. Electronic microscopy structure images of kidney tissues in mice including (A) db/m male, (B) db/m female, (C) db/db male and (D) db/db female.

period of time. In addition, lipids accumulate in the liver, and the accompanying vacuolation was more pronounced in male compared with in female db/db mice. Thus, the liver and kidney fibrosis in male db/db mice also appeared to be more pronounced compared with that in female db/db mice. Consistent with this, previous studies have demonstrated that the level of proteinuria in male db/db mice is usually twice that of female mice (33), female mice demonstrate less proteinuria and vascular remodeling in their kidneys compared with male mice (34) and mice lacking estrogen receptor-α demonstrate obvious proteinuria and glomerulonephritis (35). This suggests that estrogen may limit the renal injury that is caused by diabetes. A previous clinical study has also shown that male patients with diabetes are at a higher risk of microalbuminuria and are more likely to have multiple microvascular diseases (36), which suggests that among patients with diabetes, men are more likely to develop more serious kidney disease compared with women. Engin (37) found that menopausal hormone therapy with estrogen can delay the onset of type 2 diabetes in women and can improve β-cell insulin secretion, blood glucose utilization and insulin sensitivity. Thus, the protective effect of estrogen may explain why the complications of female patients with diabetes are less severe compared with those of male patients.

A small sample size was used in the present study, and follow-up experiments should aim to determine whether estrogen is the cause of the difference in the severity of the secondary pathological changes between male and female db/db mice. However, the results of the present study suggested that the db/db mouse represents a useful model of the pathogenesis of human type 2 diabetes and secondary liver and kidney pathology. Furthermore, the lesions in the male db/db mice were significantly increased compared with those in the female mice. In future studies, physical and chemical indicators such as proteinuria should be obtained, to more specifically analyze

liver and kidney injury in mice. Furthermore, the potential impact of sex should be considered when animal models are selected for the study of diabetes and other diseases, so that research can be appropriately targeted.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, LGa and SH designed the study. GW made substantial contributions to acquisition of data. XW and LGu performed the experiments. WZ conducted the analysis of data. YZ and SH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by The Animal Experimental Ethical Inspection Form of North China University of Science and Technology (approval no. LX201935; Tangshan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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