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Original article

Female mouse model of diabetes mellitus induced by streptozotocin and high-carbohydrate high-fat diet

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Abstract

With the improvement of people's living standards and rapid economic development, the incidence of diabetes mellitus (DM) is increasing in most parts of the world. DM presents an important potential threat to human health. In the present study, a model of diabetes in female mice was established, and fasting blood glucose was detected at week 4, after which the biochemical profiles were evaluated by histopathological analysis. The success rate of modeling in the normal control (NC) group and the low/ middle/high-dose streptozotocin (STZ) group were 0, 0, 25% and 60%, respectively. In the middle-dose and high-dose STZ groups, the liver index was increased significantly compared with the NC group ($p<0.05$). The blood biochemical indicators of total cholesterol and low density lipoprotein cholesterol in three STZ injection groups were as follows: alanine aminotransferase and aspartate transaminase in middle- and high-dose STZ groups, high-density lipoprotein cholesterol and serum creatinine in the high-dose STZ group, and blood urea nitrogen in the middle-dose STZ group were significantly increased ($p<0.05$). The level of total triglycerides was lower, obviously, in the high-dose STZ group than in the NC group ($p<0.05$). The mice showed marked steatosis, green-dyed fiber tissue coloring in varying degrees, and the contour of the hepatic lobules basically disappeared in STZ injection groups. The results suggest that to establish a diabetes model for female ICR mice, the optimum dose of STZ is 100 mg/kg.

Key words: diabetes mellitus, female mouse, pathological analysis, streptozotocin, high--carbohydrate high-fat diet

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Introduction

Diabetes mellitus (DM) is a metabolic disorder of glucose and lipid metabolism caused by absolute or relative deficiency of insulin in the study of animal models of diabetes (Srinivasan et al. 2005, Gheibi et al. 2017, King et al. 2017). The incidence of DM is increasing all over the world, and it has been the third health killer after the heart disease, cerebrovascular disease, and tumor (Mu et al. 2008). It is crucial to establish an ideal animal model consistent with the pathogenesis and metabolic characteristics of human diabetes for exploring the pathogenesis of diabetes and evaluating the efficacy of hypoglycemic drugs.

Numerous studies have confirmed that high-carbohydrate high-fat diet combined with STZ injection are important conditions for replicating diabetes models (Seino et al. 2010, Talpur et al. 2010). High-fat high-carbohydrate diet with STZ injection may have the following mechanisms to participate in the formation of diabetes: diabetes is developed by a high-carbonhydrate high-fat diet to induce insulin resistance but no obvious fasting blood glucose increase. A low-dose STZ injection then casues mild dysfunction in β-cells and reduces insulin secretion. This produces abnormal glucose tolerance in rats,an example of hyperglycemia in animals (Gilbert et al. 2011, Shao et al. 2014, Guo et al. 2018). This model is very similar to the natural formation of Type 2 diabetes mellitus (T2DM), and has been used for investigating the mechanisms involved in T2DM (Sahin et al. 2007, Shatwan et al. 2013, David et al. 2018) .

Animal models are useful tools for studying the pathogenesis of diabetes and pharmacological therapies. Some model animals, including the rat, mouse, monkey, and rabbit, are susceptible to STZ, and show higher glucose levels (Wu et al. 2008, Kleinert et al. 2018). At the same time, there has been discussion that male mice are more prone to nutritional induced obesity than female mice, so female mice are not good candidates for diabetes models (Chen 2012). More than 460 published papers, establishing diabetes models by the diet-induced and STZ-treated method described above can be searched. The vast majority of subjects in these papers were male mice, female mice were rarely used in studies (Niu et al. 2008, Nath et al. 2017). This study is based on the theory of copying preclinical animal models close to the human body. Female mice were selected as subjects in this study, and three different doses of STZ were combined with high-carbonhydrate high-fat diet to induce the establishment of a diabetic model of ICR mice. The intention was to provide a scientific basis for further optimizing the diabetes model of female animals. Simultaneously, it is of great significance for studying diabetes mellitus in females.

Materials and Methods

Experimental animals and high-carbonhydrate high-fat diet

Clean-grade female ICR mice, 6 weeks old, were purchased from Shanghai Slack Laboratory Animal Co., Ltd. All animal use was approved by the Animal Care and Use Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (Reference Number: 2021-1404-1) and complied with the Institutional Guidelines for the Care and Use of Laboratory Animals. The mice were housed in IVC independent air supply cages with an ambient temperature of 25±1℃ and a humidity of 50% ± 10%. The mice ate and drank freely, maintaining a 12-hour day and night cycle. Before starting the experiment, the animals ate and drank water normally, and adapted to the environment for 1 week.

The high-carbonhydrate high-fat diet consisted of lard 10%, sucrose 5%, egg yolk powder 5%, cholesterol 1%, bile salt 0.1%, basic feed 78.9%.

Drugs and reagents

Streptozotocin (STZ), lot number S0130-1G, was purchased from Sigma. Citric acid and sodium citrate were both pure and purchased from Shanghai Bioengineering Co., Ltd.. We added 2.1 g citric acid to 100 mL double-distilled water to prepare the A solution, and added 2.94 g sodium citrate to prepare the B solution. We mixed A and B in a volume of 1:1, filtered it and adjusted the pH to 4.2 to 4.5. We used the Roche blood glucose meter, serial number 79521284686, purchased from Roche Diagnostics (Shanghai) Co., Ltd., and the Roche blood glucose test strip, batch number 474905, purchased from Roche Diagnostics (Shanghai) Co., Ltd..

Inducing diabetes in mice

The mice were randomly divided into two groups: the normal control group (NC group, n=20) and the high-carbonhydrate high-fat diet group (n=60). The mice of the NC group were fed on basic feed, the high-carbonhydrate high-fat group was fed a high-carbonhydrate high-fat diet before injection of STZ. At the end of week 2, the high-carbonhydrate high-fat mice were randomly divided into three groups (n=20): low-dose STZ group (L-STZ group), middle-dose STZ group (M-STZ group), high-dose STZ group (H-STZ group).

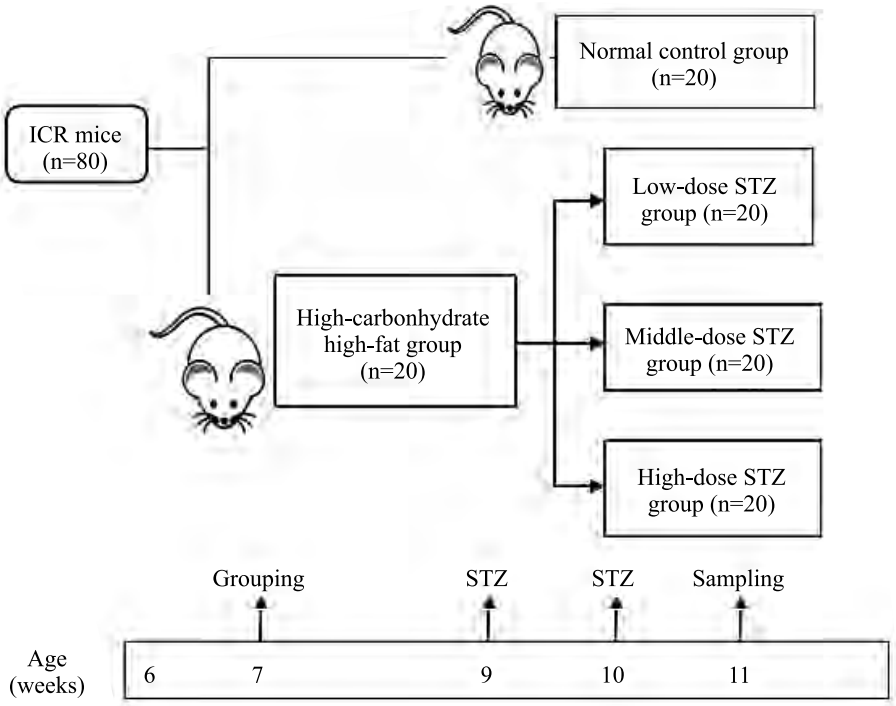

Fig. 1. Schematic diagram of experiment study design showing mice groups, chronogram.

All of the mice were fasted for 12 hours, then L-STZ group, M-STZ group, H-STZ group received introperitoneal injections of STZ in doses of 60 mg/kg, 80 mg/kg and 100mg/kg respectively, and the NC group were injected with citrate buffer at the same doses. A week later, all the mice were injected once more. One week after the second injection of STZ, blood was collected from tails and fasting blood glucose was measured twice with a blood glucose meter. When the blood glucose levels were both greater than 11.0 mmol/L (Ramer et al. 2012), the modeling was considered successful. The experimental design was shown in Fig. 1 (Gálvez et al. 2012).

Body weight and liver index

Body weight (BW) was measured prior to animal sacrifice (euthanasia). After the spinal cord was dislocated under isoflurane anesthesia, the abdominal cavity was opened, the liver was collected, rinsed in ice cold PBS and weighted. The precision balance scale gave the liver weight (LW). Using this we calculated the liver index (LW/BW).

Serum biochemical parameters

Blood samples were taken from the orbital vein in 12 h-fasted mice at week 4. In order to obtain serum, blood samples were kept at 4°C for 2 hours and centrifuged at 3500 g for 10 minutes. Application of automatic biochemical analyzer for testing: (1) the concentrations of serum total triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), serum urea nitrogen (BUN) and serum creatinine (SCr); (2) the activity of aspartate transaminase (AST), and alanine transaminase (ALT) were measured by commercial kits (Nanjing Jiancheng Bioengineering Inst.).

Histopathological analysis

Partial right lobe liver tissue was fixed in 10% paraformaldehyde for 24 h, embedded in paraffin, and sliced in 10 μm. Then the liver tissue was stained with (hematoxylin and eosin) HE and Masson. The pathological changes and fibrosis phenotype of the liver tissue were observed and analyzed under optical microscope.

Data statistics

All data was mean±standard deviation, and was analyzed by one-way ANOVA in SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). $p<0.05$ was considered significant, and $p<0.01$ was extremely significant.

Results

Success rate of mice model of diabetes

The definition of random blood glucose was greater than 11.0 mmol/L twice as a model for type 2 diabetes. There was no successful modeling in the NC group and

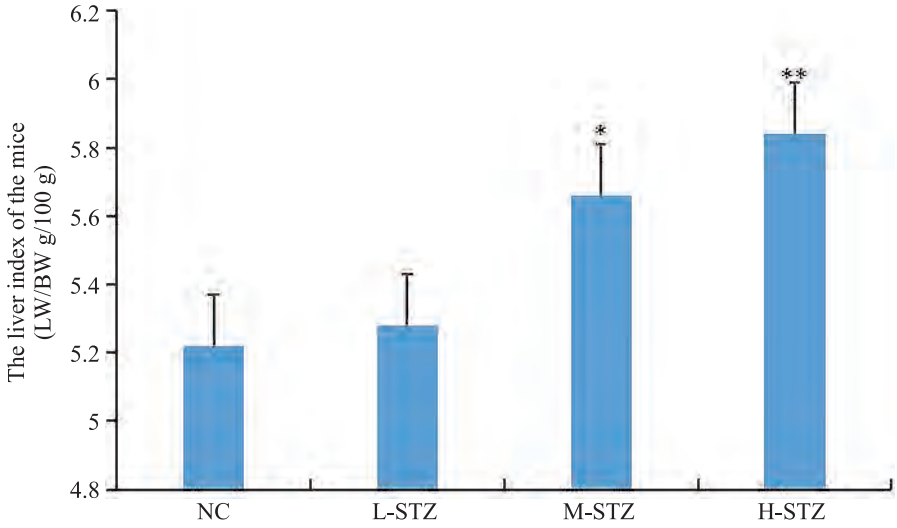

Fig. 2. Liver indices in mice at week 4.
Compared with the NC group, * and ** indicate p<0.05 and p<0.01, respectively.
NC: the normal control group. L-STZ: low-dose STZ group. M-STZ: middle-dose STZ group.H-STZ: high-dose STZ group.

Table 1. Body weight comparison before and after mouse modeling.

Group	Week 0	Week 1	Week 2	week 4
L - STZ	28.5±2.71	30.5±2.63	32.7±1.12*	31.2±2.72
M - STZ	27.6±1.16	29.7±1.19	31.8±3.52	30.1±1.11*
H - STZ	27.7±1.95	29.5±1.78	31.0±2.53	28.2±1.03**
NC	28.0±1.62	29.1±1.55	30.8±1.56	31.9±1.71

Compared with the NC group, * and ** indicate p<0.05 and p<0.01, respectively.

L-STZ group. The success rate of modeling in the M-STZ and H-STZ group (n=20) were 25% and 60%, respectively. After the second injection of STZ, there were two mice dead with a mortality rate of 10% in the H-STZ group.

Changes in body weight, physiological state before and after injection

Before the modeling, the mice were were given food and water normally, reacted sensitively, moved freely, and the body weight continued to grow steadily. After injection of STZ, the mice in the model group were observed to be wilting, the movement was slow, the back hair was chaotic and dull, and the mice gradually showed symptoms of "three more and one less," especially in the H-STZ group.

As shown in Table 1, there was no significant difference in the basal body weight of each group before the model establishment. The average body weight in the three STZ injection groups were higher than that in the NC group, and average body weight in the L-STZ group was significantly higher than the NC group (p<0.05). At the end of week 4, the M-STZ group was significantly lower in weight than that in the NC group (p<0.05), and the H-STZ group was also significantly lower than the NC group (p<0.01).

Hepatic gross morphology and liver index changes

As shown in Fig. 2, the mice in the NC group were observed to have sharp liver edges, and their livers had smooth surface, uniform dark red color, soft and elastic texture, no adhesion to surrounding tissues, and smooth cut surface. The liver color of the groups injected with STZ was uneven, the volume increased to varying degrees, and the edge was round and blunt. The cut surface was yellowish and greasy. Compared with the NC group, liver indexes of the mice in M-STZ group and H-STZ group were significantly increased (p<0.05).

Changes in serum lipid profiles

Cholesterol and triglycerides are indicators of lipid metabolism. As indicated in Table 2, compared with the NC group, the concentration of TC in the L-STZ group was significantly increased (p<0.05), and the TC level in the M-STZ and H-STZ groups was dramatically increased (P<0.01). The LDL-C index of three STZ injection groups and the HDL-C level in the H-STZ

Table 2. Blood lipids in mice at week 4.

Group	TC (mmol/L)	LDL - C (mmol/L)	HDL - C (mmol/L)	TG (mmol/L)
L - STZ	3.52±0.69*	0.47±0.096**	0.88±0.19	0.73±0.26
M - STZ	4.14±0.60**	0.54±0.097**	0.91±0.22	0.82±0.19
H - STZ	4.01±0.67**	0.52±0.12**	1.04±0.22**	0.65±0.11**
NC	2.78±0.36	0.2±0.06	0.67±0.17	0.90±0.14

Compared with the NC group, * and ** indicate $p<0.05$ and $p<0.01$, respectively.
TC: total cholesterol LDL – C: low-density lipoprotein cholesterol
TG: total triglyceride HDL – C: high-density lipoprotein cholesterol

Table 3. The hepatic and renal function levels in mice.

Group	ALT (U/L)	AST (U/L)	BUN (mmol/L)	SCr (μmol/L)
L - STZ	66.3±28.2	58.3±8.7	5.08±0.33	38.7±14.2
M- STZ	112±53.8*	65.2±7.2*	5.51±0.93*	42.7±10.2
H - STZ	152.9±16.2**	83.0±13.7*	5.21±0.69	45.6±10.5*
NC	55.8±17.1	43.7±4.9	4.82±0.34	29.9±10.1

Compared with the NC group, * and ** indicate $p<0.05$ and $p<0.01$, respectively.
ALT: alanine transaminase AST: aspartate transaminase
BUN: serum urea nitrogen SCr: serum creatinine

group were dramatically increased ($p<0.01$). The TG content in the STZ injection groups was reduced to some extent, and the H-STZ group was significantly decreased ($p<0.05$). The increased content of CH suggests that the mice in the STZ injection groups had hypercholesterolemia, and the decrease in TG may be related to the body weight loss in diabetic mice. Compared with the NC group, * and ** indicate $p<0.05$ and $p<0.01$, respectively.

Changes in hepatic and renal function after injection

ALT and AST are indicators of liver damage. As indicated in Table 3, compared with the NC group, there was a dose-dependent increase in the level of ALT and AST in the M-STZ and H-STZ group ($p<0.05$). It is suggested that the larger the STZ dose, the greater the damage to the liver cells.

BUN and SCr are indicators of renal filtration function. As indicated in Table 3, the content of BUN and SCr in the STZ injection groups was increased. Simultaneously, the BUN content in the M-STZ group and the SCr content in the H-STZ group were significantly increased ($p<0.05$). It was revealed that there was glucose dysfunction in diabetic mice caused by a high-fat and high-sugar diet combined with STZ injection, but obvious structural disorder was not found in the pathological examination of the kidney.

Morphological observation of the mouse liver

1. *HE-stained results of the mouse liver*

As shown in Fig 3, the results of HE-stained liver revealed the characteristic architecture of the hepatic lobules of the mice in each group. In the NC group, the liver was structurally intact. Hepatocytes were round, and the nucleus was located in the center of the hepatocyte. Multiple hepatocytes were centered on the central vein, uniform in size, and the shape of the nucleus was round and regular. Hepatocyte cords were radially arranged tightly and neatly. The cytoplasm was stained with light powder. There was no fibrous tissue hyperplasia or inflammatory cell infiltration in the portal area, and the hepatic sinus was clearly visible (Fig. 3A). The structure of the hepatic lobule in the L-STZ group was still intact, and the morphology of the nucleus was not abnormal. However, the hepatocyte cords, although neatly arranged, were loose compared to the NC group. Occasionally a few hepatocytes had very slight cytoplasmic vacuolation (Fig. 3B). In the M-STZ group, obvious vacuolar degeneration and edema were observed in hepatocytes, and the cytoplasm was loose and lightly stained. Arrangement of hepatocyte cords was disordered (Fig. 3C). In the H-STZ group, the outline of the hepatic lobules almost disappeared, and the steatosis of the liver cells was the most severe. Part of the cytoplasm of hepatocytes was

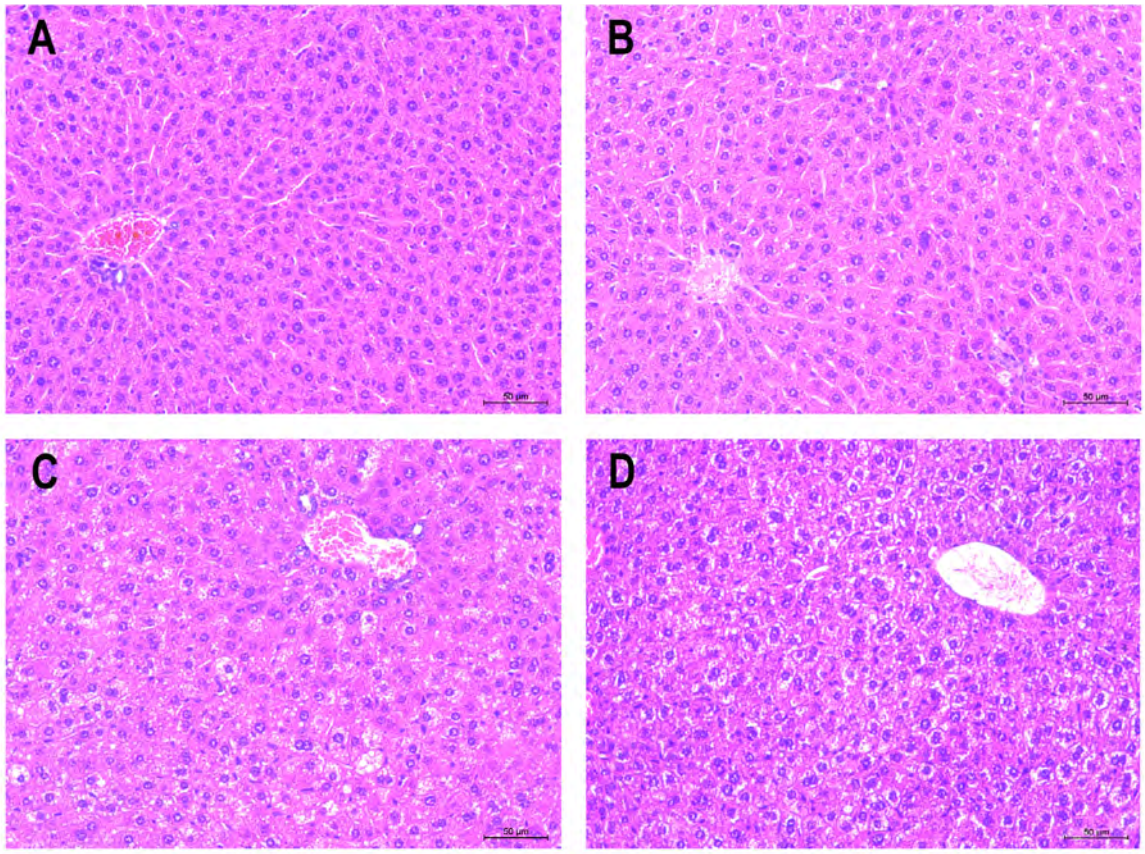

Fig. 3. Histological examination of HE-stained mouse liver sections.
Representative samples were stained with hematoxylin and eosin (HE). Original magnification: ×200. (A) normal control group. (B) low-dose streptozotocin group. (C) middle-dose streptozotocin group. (D) high-dose streptozotocin group.

squeezed by lipid droplets to disappear. The arrangement of hepatocytes was disordered and irregular, and there was no obvious arrangement of hepatocyte cords (Fig. 3D).

2. *Examination of the Masson-stained liver*

The Masson staining of the liver is shown in Fig. 4. A few reticular fibers were found in the liver tissue of mice in the NC group, which were found in the portal area and the wall of the central vein (Fig. 4A). The mice in the model groups with different doses of STZ had different degrees of blue-stained fibrous tissue staining. Compared with the NC group, there was a small amount of collagen hyperplasia around the portal area in the L-STZ group, and a very small amount of fibrous bridging was formed (Fig. 4B). In the M-STZ and H-STZ groups, the coloration of blue-stained fibers was darker, the reticular fibers and collagen fibers in the portal area were proliferated, the fiber cords were thickened, and the interval was widened (Fig. 4C-4D). And the phenomenon was more intense in the H-STZ group (Fig. 4D).

Discussion

The number of people with diabetes worldwide has increased in the past few years and it is expected to maintain a rapid growth. Although in vitro and computer studies have improved over the past few decades, they cannot completely replace the inferred information from animal models, given the complex etiology and multi-system interactions of diabetic patients. The present research studied the optimum dose of STZ to establish a female mouse model of diabetes. According to the results of this study, the success rate of the mouse model of diabetes showed a dose-dependent trend. The success rate of the mouse model increased with increases in dose, but the death rate of mice in the 100 mg/kg dose group was 10%. Considering the two factors of the survival rate and model success rate, the success rate of diabetic mice was too low after injection of STZ at 60 mg/kg and 80 mg/kg. There was a very small number of mouse deaths in the dose range of 100 mg/kg, while the success rate of diabetic mice was more ideal in that zone. In addition, the weight of mice in the H-STZ group was dramatically decreased ($p<0.01$), and the animals showed symptoms of “three more and one less”(significant symptoms of excessive drinking,

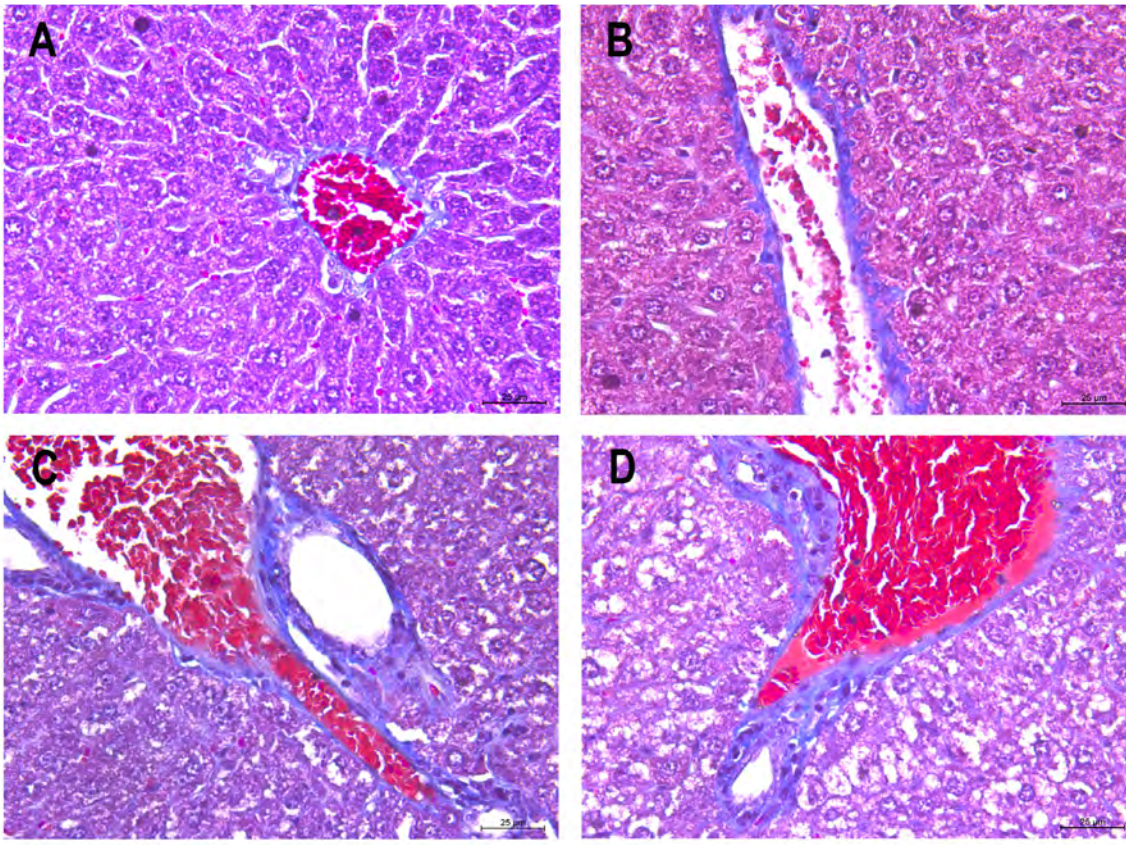

Fig. 4. Histological examination of Masson-stained mouse liver sections.
Original magnification: ×400. (A) normal control group. (B) low-dose streptozotocin group. (C) middle-dose streptozotocin group. (D) high-dose streptozotocin group.

excessive polydipsia, excessive polyuria, and weight loss) with depressed state of mind, which are typical signs of diabetes. The changing trend of pathological structure and biochemical detection in mice is similar to those in human T2DM. Therefore, it is recommended that when the diabetic mouse model is established using STZ, an injection dose of 100 mg/kg can be used as a reference dose.

We calculated the liver index, measured the serum biochemical parameters and observed morphological changes in the mouse liver 2 weeks after the injection to explore the feasibility for establishing a diabetes model with female mice. The liver morphology and the liver indexes suggested that the higher the STZ dose, the greater the likelihood that the mouse will develop fatty liver, or that the liver may have functional compensatory hypertrophy. The results of biochemical tests showed that the high-fat diet combined with STZ would cause damage to hepatocytes in mice and creat hypercholesterolemia in mice. The content of TCH, ALT, BUN and SCr in the type 2 diabetes model mice reported by Zeng Weisen (Zeng et al. 2014) showed an increasing trend, and liver injury indicators such as vacuolar-like structure and abnormal lipid metabolism were observed in the liver tissue, which were basically consistent with the results of this experiment. It was indicated that diabetic mice have fatty liver disease. After liver cells were damaged, intracellular ALT and AST entered the blood, resulting in an increase in the serum level of these two enzymes. The extent to which the enzyme from hepatocytes rises in the blood reflects the extent of damage to the hepatocytes. This study figured out the situation of female mice with diabetes in a specific environment and provided basic data for establishing a better model of female diabetes.

Obesity is an important and risk factor contributing to the development of diabetes, so obesity is usually induced by feeding a high-fat diet as a part of the formation of metabolic disorders for diabetes study. Although three STZ injection groups were fed a high-carbonhydrate high-fat diet before injection of STZ, there was no significant difference in the body weight between two STZ injection groups (the M-STZ and H-STZ group) and the NC group. A study indicated that the success rates of the obesity model of female and male mice were 16.67% and 66.67% respectively, when an ICR mouse obesity model was developed under the same conditions (Li et al. 2012). This indicates that the success rate of obesity modeling is affected by the sex of mice, and that male ICR mice are more likely

to fatten. The result is consistent with the report by Niu et al. (2008) that female mice are not easy to fatten. It may be that food resistance exists in some mice, or that different ages of mice have different sensitivities to high-fat diets.

There is no doubt that the success rate of obesity modeling is closely related to the success rate of diabetic mice. The highest success rate of diabetic mice in the H-STZ group was only 60%. As it has been reported by researchers (Li et al. 2012, Navarro et al. 2018) that male rats are more sensitive to STZ than females, the male modelling rate is nearly double that of female rats when using the same dose of STZ modeling, and androgen excess predisposes women to type 2 diabetes. At the same time, the diabetic model of rats fasting at 10 to 12 o'clock in the evening was twice as that of fasting rats at 8 o'clock in the evening, and the blood glucose in the model rats was further increased. Therefore, screening the suitable modeling method for ICR mice by optimizing the dose, fasting time, and mouse sex are important conditions for establishing a mouse model of diabetes with high yield of diabetic mice and low mortality. In addition, the high-fat and high-sugar diet of the model group before the injection of STZ in the experiment lasted only two weeks, and the fasting time was not appropriate. The next study will prolong the time of high-fat and high-sugar feeding, and fix the fasting time between 10 o'clock in the evening to 10 a.m.. This will be combined with the STZ optimal dose of 100 mg/kg. This will further develop the modeling method of mouse diabetes.

Conclusions

Our results demonstrate that female mouse model of DM induced by streptozotocin and high-carbohydrate high-fat diet was established, with the optimum dose of STZ 100 mg/kg. The results may provide some theoretical basis for studying diabetes mellitus in females.

Acknowledgements

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