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RESEARCH ARTICLE

Intervening effects of progesterone combined with estradiol on streptozotocin-induced diabetes in ovariectomized mice and the underlying mechanisms

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ABSTRACT

Background: Estrogen and progesterone are closely related to the occurrence of diabetes, and the progestogenic and estrogenic effects in the development of diabetes are related to their blood levels and the organism state, and it is not clear what effect their combination will be. The present study was to investigate the interaction between estrogen and progesterone in the development of diabetes mellitus.

Methods: A model of type 1 diabetes mellitus（T1DM）was established in ovariectomized (OVX) mice by intraperitoneal injection of streptozotocin (STZ), and the levels of plasma glucose, insulin, C-peptide, progesterone, and estradiol, the changes of islet cells, the expressions of glucose transporter 4 (GLUT4), Glucokinase (Gck), Glucose-6-phosphatase (G-6-P) and phosphoenolpyruvate carboxylase (PCK) were detected after chronically injected different doses of progesterone and 17β-estradiol subcutaneously for 4 weeks.

Results: Progesterone and 17β-estradiol could delay the occurrence of T1DM induced by STZ in ovariectomized mice, but there was no dose-response relationship; progesterone (2mg/kg) and 17β-estradiol (0.1mg/kg) injected separately or jointly could make the plasma concentration of estradiol and progesterone reaches the physiological dose range, which markedly inhibited the increase of the blood glucose induced by STZ, improved the glucose tolerance, protected islet cells, promoted C-peptide and insulin secretion, up-regulated skeletal muscle GLUT4 and liver Gck expressions, but significantly down-regulated the expressions of liver PCK and G-6-P mRNA.

Conclusions: The physiological dose of progesterone combined with estradiol has certain synergistic effects in delaying the occurrence of diabetes, the mechanisms are probably related to the protection of islet cells, the promotion of insulin release, skeletal muscle glucose transport, and liver glucose metabolism.

Keywords: progesterone, estradiol, diabetes, pancreatic islet, glucose metabolism, GLUT4.

Introduction

Type 1 diabetes mellitus (T1DM) results from pancreatic β cell autoimmune destruction, leading to absolute insulin deficiency, which is defined as a complex nutritional-metabolic state characterized by reduced sensitivity of target tissues (liver, skeletal muscle, and adipose tissue) to the physiological effects of insulin[1,2]. Due to insufficient of insulin secretion, the blood glucose can't enter the peripheral target cells, some of the sugar in the blood will be filtered by the kidneys and discharged through the urine in T1DM. And because the cells can't obtain the energy from glucolysis, then they get energy by breaking down fat which results in the fatty acids to enter the blood, causing a metabolic imbalance and life-threatening diabetic ketoacidosis[3]. Therfore, T1DM is also known as insulin-dependent diabetes, and patients usually control their excessive blood sugar levels with insulin injections[4].

Sex hormomes are closely related to the occurrence of diabetes, including T1DM and Type 2 diabetes mellitus (T2DM)[5,6]. In studies of rodent models, estradiol treatment protects pancreatic β cells from various damage associated with T1DM and T2DM, including oxidative stress, amyloid polypeptide toxicity, lipid toxicity, and apoptosis[7]. It has been reported that obese women with T1DM show estradiol deficiency compared to healthy women[5]. Progesterone and estrogen can change the activity and function of islet β cells[8]. It has been reported that progesterone can promote the proliferation of islet β cells by binding specific receptors, improve the level of insulin secretion and promote the release of insulin after the body's glucose intake[9-11]. Three estrogen receptors have been identified in rodent and human islet β cells: estrogen receptor α （ERα）, estrogen receptor β (ERβ), G protein-coupled estrogen receptor (GPER)[12-16], and most of them belong to extranuclear receptors. Activation of ERα enhances insulin biosynthesis after glucose stimulation[12,17], and this effect may help the islets adapt to the increased metabolic demands of pregnancy by enhancing insulin biosynthesis. ERβ activation appears to enhance glucose-stimulated insulin secretion primarily by atrial natriuretic peptide receptors, ATP-sensitive potassium channels[18-20]. However, GPER activation protects β cells from lipid accumulation and promotes their survival[7,19,20]. The data have showed progesterone and estrogen can change the expression of glucose transporters (GLUT) in peripheral insulin-target cells, that is to say, GLUT1 expression is affected by progesterone, but not by 17β-estradiol. GLUT1 expression is increased in combination treatment with progesterone and 17β-estradiol. The two hormone treatments do not affect the expression of GLUT2, but both increase the expression of GLUT3 and GLUT4, indicating that progesterone and 17β-estradiol alone and in combination have different effects on glucose transport[21]. Other studies have shown that high doses of progesterone disrupt glucose homeostasis associated with diabetes[22], and the mice knockouted progesterone receptor are less likely to develop hyperglycemic disease[23]. When there appears insulin resistance and insufficient insulin secretion, progesterone can induce the up-regulation of phosphoenolpyruvate carboxykinase and

gluconeogenesis genes in mice, therefore, progesterone can induce gluconeogenesis in the liver and maintain relatively stable glucose levels[24,25].

According to the information described above, it is easily speculated that there is a close relationship between plasma concentrations of estrogen/progesterone and diabetes, and their effects and underlying mechanism are complex, however the detail effects of estradiol combined with progesterone are not very clear. Therefore, to further study the relationship between estrogen/ progesterone and diabetes, we first observed the blood glucose levels in ovariectomized and streptozotocin-induced diabetes mice treated subcutaneously with sex hormones and carried out the glucose tolerance test (GTT) and insulin tolerance test (ITT); secondly, in order to elucidate the molecular mechanisms, we focused on detecting the expression of skeletal muscle glucose transporter 4 (GLUT4), liver glucokinase (Gck), glucose-6-phosphatase(G-6-P) and phosphoenolpyruvate carboxylase (PCK) mRNA, and observing the morphology of islet cells as well. The aim was to provide a experimental basis for the prevention, diagnosis and treatment of diabetes and the role of sex hormones in glucose and lipid metabolism.

Methods

ANIMALS

Adult female Kunming mice that were not pregnant and weighed 22–26 g, were provided by the Laboratory Animal Center of Lanzhou University (Lanzhou University, Lanzhou, China). Animals were kept in a temperature- and light-controlled room with free access to food and water. All animal procedures performed on the mice in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of the Ethics Committee of Lanzhou University, China.

DIABETES MELLITUS INDUCTION AND DRUG PREPARATION

All mice were anesthetized with 0.3% pentobarbital sodium and then ovariectomized. An incision was made along the abdominal mid-line, the ovaries were removed from both sides after tubal ligation, and then the wound was stitched. After recovery for one week, all animals were injected intraperitoneally with streptozotocin (STZ) (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 50 mg/kg/day for 5 days in a row to induce pancreatic islet cell destruction and persistent hyperglycemia as described previously[21]. Some of the ovariectomized mice received 17β-estradiol or progesterone (both dissolved in sesame oil, Sigma Chemical Co.) subcutaneously once a day for 4 weeks, meanwhile, in the first 5 days, STZ was simultaneously injected at a dose of 50 mg/kg/day intraperitoneally (Fig 1). Fasting blood glucose levels were measured from the caudal vein using a portable glucose measuring device (Roche) once a week. Mice with hyperglycemia (blood glucose levels>300 mg/dl) were defined as a diabetic model, as before.

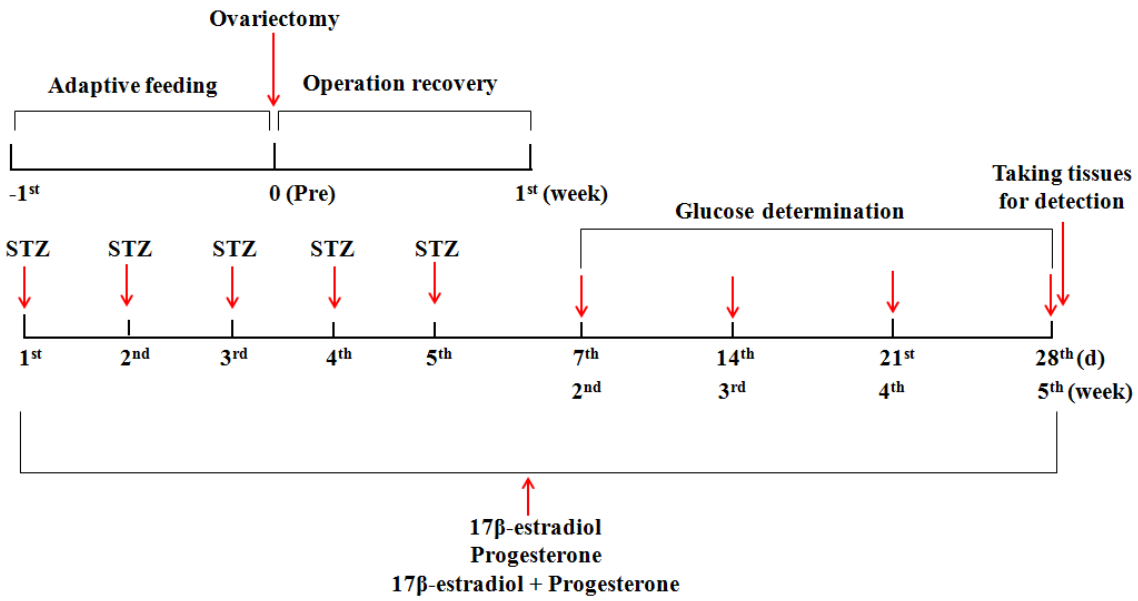


Figure 1. Temporal diagram of progesterone and 17β-estradiol treatment. Before operation (Pre); the recovery period after operation (1st week); streptozotocin (STZ) was injected intraperitoneally at a dose of 50 mg/kg/day in the 2nd week for 5 days, while 17β-estradiol and progesterone were administered subcutaneously from the 2nd week to the 5th week for 4 weeks once a day.

EXPERIMENTAL DESIGN

The mice were randomly divided into 9 groups: ovariectomy group (OVX), ovariectomy +intraperitoneal injection of STZ (OVX+STZ), ovariectomy+intraperitoneal injection of STZ +subcutaneous injection of 0.1 mg/kg 17β-estradiol (E0.1) which was determined based on our team previous experiments [2], ovariectomy+intraperitoneal injection of STZ +subcutaneous injection of 0.1 mg/kg progesterone (P0.1), ovariectomy+ intraperitoneal injection of STZ +subcutaneous injection of 2.0 mg/kg progesterone (P2), ovariectomy+intraperitoneal injection of STZ +subcutaneous injection of 8 mg/kg progesterone (P8), ovariectomy + intraperitoneal injection of STZ + subcutaneous injection of 0.1 mg/kg 17β-estradioland and 0.1 mg/kg progesterone (EP0.1), ovariectomy + intraperitoneal injection of STZ + subcutaneous injection of 0.1 mg/kg 17β-estradiol and 2 mg/kg progesterone (EP2), ovariectomy + intraperitoneal injection of STZ + subcutaneous injection of 0.1 mg/kg 17β-estradiol and 8 mg/kg progesterone (EP8), with 12 animals per group (n = 12). Five weeks after surgery, some animals were used to conduct the glucose tolerance test (GTT) and insulin tolerance test (ITT), and the rest of the mice were sacrificed by cervical dislocation and their plasma, livers, and skeletal muscles were collected. Fasting plasma insulin and C-peptide levels were determined using enzyme -linked immune sorbent assay (ELISA) kits.

GTT, ITT, AND MEASUREMENTS OF INSULIN, C-PEPTIDE, 17β-ESTRADIOL, AND PROGESTERONE LEVELS IN PLASM

Some mice fasted for 12 h before receiving an intraperitoneal injection of D-glucose (1.5 g/kg body weight) for the GTT or fasted for 4 h before receiving an intraperitoneal injection of human insulin (1.0 U/kg body weight) for the ITT (n=6). Blood samples from mouse tails were taken before and at 0, 30, 60, 90, and

120 min after the above injection, and plasma glucose was immediately determined with the Accu-Chek Aviva Blood Glucose Meter System (Roche Diagnostics). Fasting insulin and C-peptide levels were determined using enzyme-linked immune sorbent assay (ELISA) kits (Yuanye Institute of Biological Sciences, China); 17β-estradiol and progesterone levels were determined by chemiluminescent immunoassay (First Hospital of Lanzhou People's Liberation Army).

RT-PCR ANALYSIS

Total RNA was extracted from mouse livers and skeletal muscles using TRIZOL reagent (Life Technologies, Invitrogen Carlsbad, CA, USA.), and the reverse transcription reaction was performed with a reverse transcription kit (TaKaRa Co. Ltd, Kyoto, Japan) according to the manufacturer's protocol. The PCR reactions were heated at 95°C for 2min, and then immediately cycled 26-36 times through a 30s denaturation step at 95°C, a 30s annealing step at 60°C, and a 30s extension step at 72°C. After the cycling procedure, a final elongation step was performed for 10min at 72°C. The number of PCR cycles and annealing temperature required to produce PCR products in the linear logarithmic phase of the amplification curve was determined. The PCR products were electrophoresed in 2% agarose gels (Invitrogen) and visualized by staining with Gelred (Biotium). The reaction products were separated on 2% agarose gel, and then imaged using a Gel Imaging System. Normalization of the data was accomplished by quantifying the amount of amplified cDNA products, and by calculating the relative ratio of the amount of cDNA to the amount of β-actin cDNA. The following primers were used for PCR amplification (Table 1).

Table 1. The primers for PCR amplification

Gene name	sequence(5′- 3)	Tm(℃)	Cycles
Actin –R	GCCACGATGGAGACATAGC	60	29
Actin– F	CATCCGTAAAGACCTCTATGCCAAC		
GLUT4 –R	ATGGAGCCACCGATCCACA	65	26
GLUT4 –F	GACGGACACTCCATCTGTTG		
Gck –R	TTGAGCAGCACAAGTCGTACCAG	65	28
Gck –F	AGTACGACCGGATGGTGGATG		
G-6-P –R	ATCCCAACCACAAGATGACGTTC	65	32
G-6-P –F	CAGCAACAGCTCCGTGCCTA		
PCK –R	GCCAGGTATTTGCCGAAGTTGTAG	65	30
PCK –F	TCTTTGGTGGCCGTAGACCTG		

OBSERVATION OF HEMATOXYLIN-EOSIN (HE) STAINING

The pancreas was fixed in neutral 4% paraformaldehyde (Solarbio) in 0.1mol/L sodium phosphate buffer (Solarbio), and slices were dehydrated and transparent with xylene and then embedded in paraffin blocks. Five-μm-thick slices were cut and placed on glass

slides and stained with HE by routine methods. Lastly, the slices were sealed using neutral gum. All Hematoxylin-eosin (HE) staining of the islet cells was evaluated using a microscope (Nikon Inc.) or using the BI2000 image analysis system (Taimeng Co. in Chengdu, China) equipped with a trinocular microscope (Olympus, America) and a digital camera (Nikon, Japan). The number of pancreatic islet cells was counted at a final magnification of 10×10 times in the visual field, and averaged from at least 3 representative parts in one section in a blinded fashion by 1 observer.

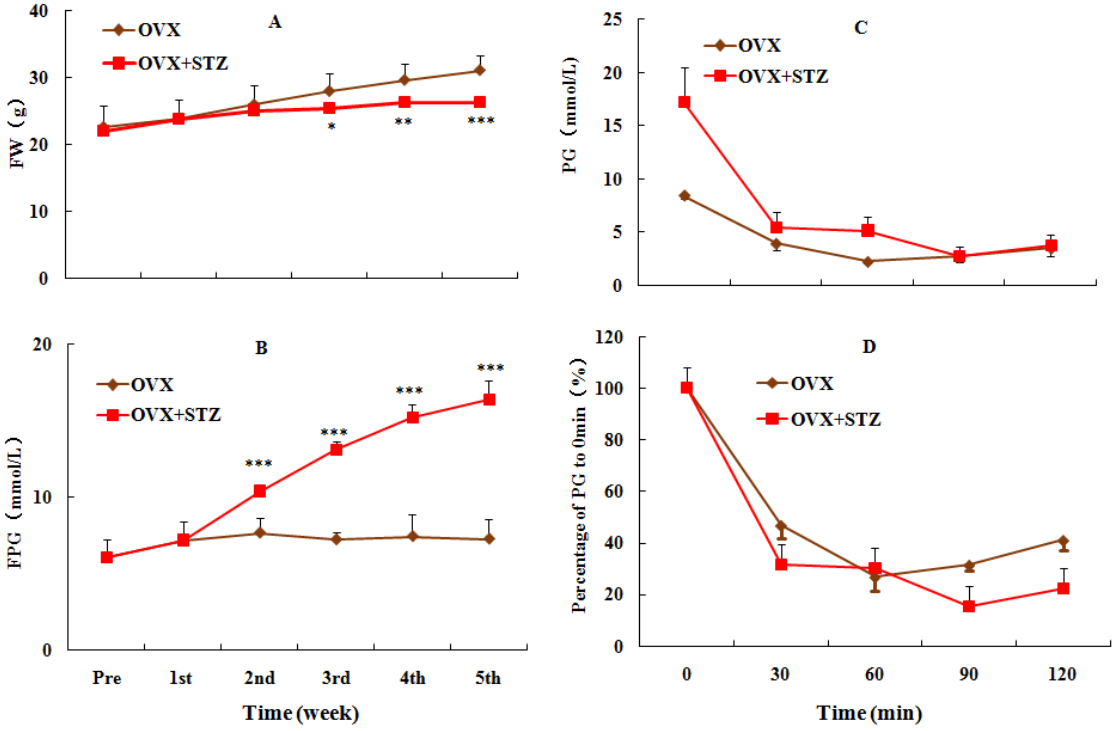
STATISTICAL ANALYSIS

All data were expressed as the average± standard deviation. The statistical differences were determined by Student t-test for comparison of two groups and one-way ANOVA for comparison of three or more groups with SPSS 21 software. $P<0.05$ was considered statistically significant.

Results

ESTABLISHMENT OF TYPE 1 DIABETES MICE

Compared with the OVX control group, the mice of a model group that received intraperitoneal injection of STZ exhibited slowly decrement on body weight, and it was significantly lower than the OVX group in the third, fourth, and fifth weeks (Fig. 2A); meanwhile the fasting plasma glucose (FPG) level of OVX+STZ group was significantly higher than the OVX group in the second, third, fourth and fifth weeks ($P<0.001$) (Fig. 2B); but there was no significant difference in the insulin intolerance test (Fig. 2C and 2D).



Figue 2. The fasting weight, fasting plasma glucose and the insulin tolerance test in STZ-induced mice. (A) Fasting weight (FW), (B) fasting plasma glucose (FPG), (C) the plasma glucose (PG) changes after insulin injection, (D) a percentage of plasma glucose at different time points after insulin injection relative to the plasma glucose level before administration insulin in ovariectomized mice (OVX) and streptozotocin-induced diabetic ovariectomized mice (OVX+STZ). Data are the mean±SD (n=6). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs OVX.

EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON FASTING BLOOD GLUCOSE AND BODY WEIGHT

Compared with the OVX+STZ group, FPG in the group of 2 mg/kg progesterone had a significant decrement after 4-week administration (P<0.001) (Fig. 3A) but there were not marked changes in the groups of 0.1 and 8 mg/kg progesterone. As shown in Fig. 3B, 0.1 mg/kg17β-estradiol alone (E0.1) could decrease FPG significantly versus OVX+STZ group in the third, fourth and fifth weeks (P<0.001) which was same as reported previously[2], and combination 0.1 mg/kg 17β-estradiol with 0.1mg/kg progesterone had a significant decline compared to E0.1 group in the fifth week (P<0.01).

As shown in Fig. 3, the body weight of diabetic mice in the OVX+STZ group decreased markedly, and the fasting body weight gain of the progesterone-administered groups (0.1, 2, or 8 mg/kg) was faster than that of the OVX+STZ group, but only 2 mg/kg progesterone had a significant difference in the fifth week (P<0.05) (Fig. 3C). When administered 0.1 mg/kg 17β-estradiol alone, there was a remarkable increase on body weight compared with the OVX+STZ group (P<0.05), and the weight gain of 0.1 mg/kg 17β-estradiol combined with 0.1, 2 or 8mg/kg progesterone (EP0.1, EP2, and EP8) were also faster than the OVX+STZ group, but compared with the 17β-estradiol alone group there had no significant difference (Fig. 3D).

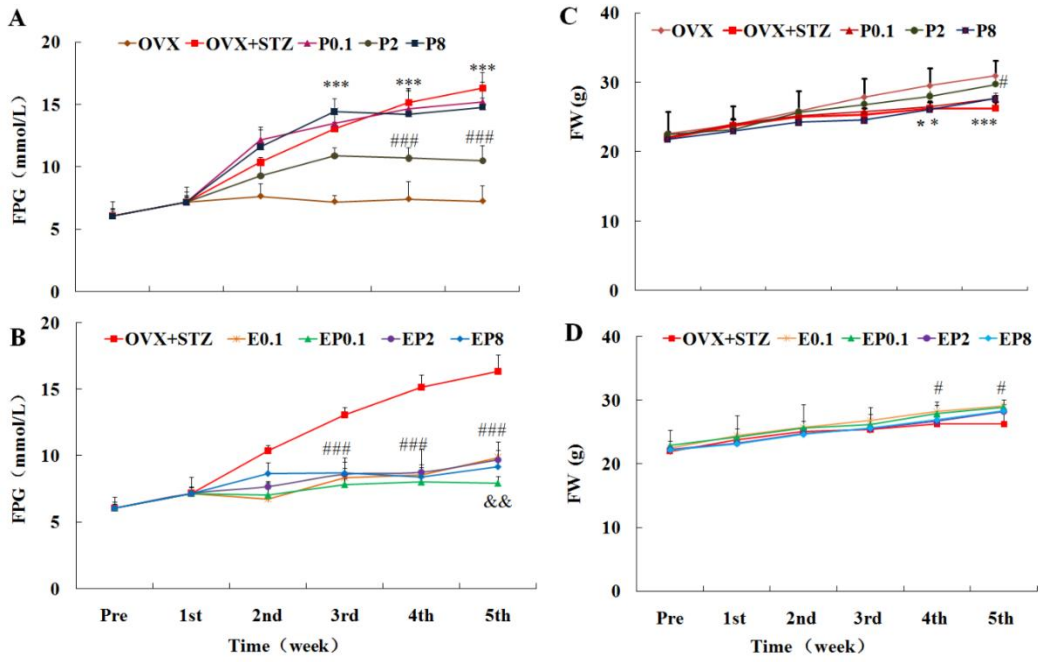


Figure 3. Effects of progesterone and 17β-estradiol on fasting blood glucose and body weight. Before operation (pre); the recovery period after operation (1st week); streptozotocin was injected intraperitoneally at a dose of 50 mg/kg/day in the 2nd week for 5 days, while progesterone and 17β-estradiol were administered subcutaneously from the 2nd week to the 5th week for 4 weeks once a day. (A) fasting plasma glucose (FPG) and (C) fasting weight (FW) in ovariectomized mice (OVX), in ovariectomized and streptozotocin-induced diabetes mice (OVX+STZ) and OVX+STZ diabetes mice treated with progesterone subcutaneously at the dose of 0.1 mg/kg (P0.1), 2 mg/kg (P2) and 8 mg/kg (P8). (B) FPG and (D) FW in OVX+STZ diabetes mice and OVX+STZ diabetes mice treated with 17β-estradiol subcutaneously at the dose of 0.1 mg/kg (E0.1) or the combination of 0.1 mg/kg 17β-estradiol with 0.1 mg/kg progesterone (EP0.1), 2mg/kg progesterone (EP2), or 8 mg/kg progesterone (EP8). **P<0.01, ***P<0.001 vs OVX; #P<0.05, ##P<0.01, ###P<0.001 vs OVX+STZ; &&P<0.01 vs E0.1. Data are the mean±SD (n=12).

EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON GLUCOSE TOLERANCE

As shown in Fig. 4, the mice fasted for 12 hours after administration of 17β-estradiol and progesterone for four weeks, a glucose tolerance test was performed by intraperitoneally injected glucose (1.5 g/kg), and the blood glucose value was acquired at 0 min, 30 min, 60 min, 90 min , and 120 min after glucose injected. We found that the OVX+STZ group exhibited significantly poorer glucose tolerance than the OVX group (P<0.001), although the three doses of progesterone did not significantly improve glucose tolerance in mice with type 1 diabetes (Fig. 4A), 0.1 mg/kg 17β-estradiol alone could improve glucose tolerance significantly versus OVX+STZ group (P<0.05), but there were no significant differences between E0.1 and combination 0.1mg/kg 17β-estradiol with 0.1, 2 or 8 mg/kg progesterone (Fig. 4B).

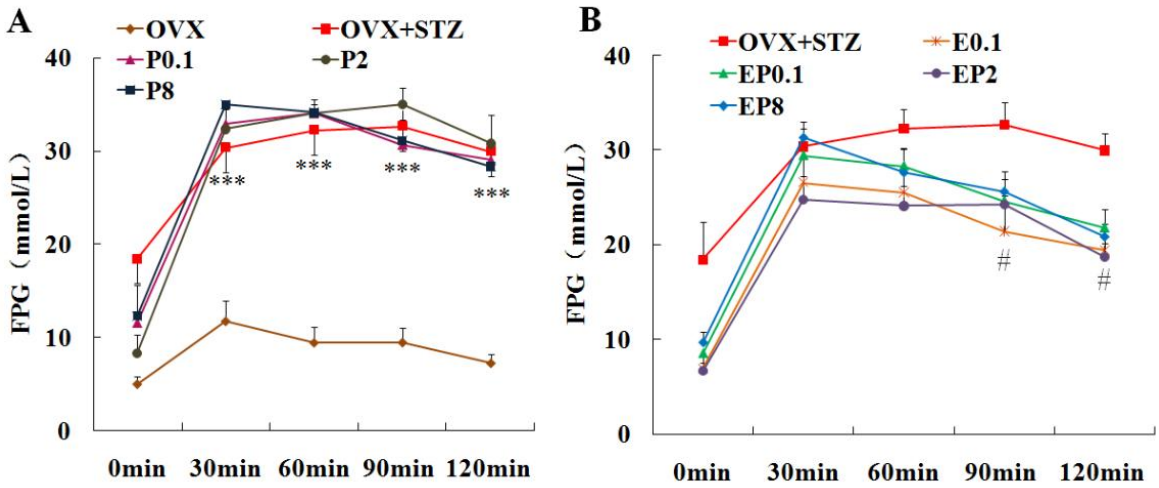


Figure 4. Effects of progesterone (A), 17β-estradiol or the combination of 17β-estradiol and progesterone (B) on glucose tolerance in diabetic mice. The blood glucose level was tested at 0, 30, 60, 90, and 120 minutes after glucose (1.5 g/kg body weight) was injected intraperitoneally. ***P<0.001 vs OVX; #P<0.05 vs OVX+STZ. Data are the mean±SD (n=6).

EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON PANCREATIC ISLET CELL MORPHOLOGY AND NUMBERS

Compared with the OVX group, the pancreatic islet were observed to be smaller, their shape was deformed, and the number of cells was significantly reduced in the OVX+STZ group. After treatment with 2 mg/kg progesterone, 0.1 mg/kg 17β-estradiol and the combination of 0.1 mg/kg 17β-estradiol with 2 or 8 mg/kg progesterone, the number of the islet cells increased significantly (Fig. 5).

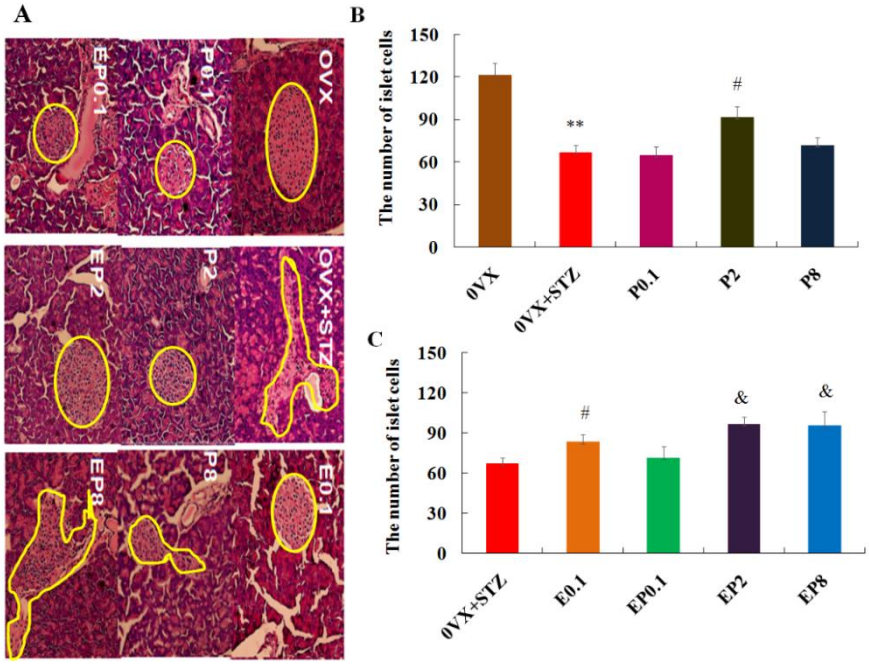


Figure 5. The picture of Islet morphology and the number of islet cells. Effects of 17β-estradiol and progesterone on the pancreas were visualized by hematoxylin-eosin staining (A) and the number of islets cells were counted (B, C) in OVX, OVX+STZ, P0.1, P2, P8, E0.1, EP0.1, EP2, or EP8. (n=3). ***P*<0.01 vs OVX; #*P*<0.05 vs OVX+STZ; &*P*<0.05 vs E0.1. Data are the mean±SD (n=3).

EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON SERUM C-PEPTIDE AND INSULIN

After administration of 17β-estradiol and progesterone for four weeks, the serum levels of 17β-estradiol and progesterone were measured by chemiluminescence, and the levels of C-peptide and insulin in the serum were detected by the Elisa method. The serum levels of 17β-estradiol and progesterone were shown in the table, and the serum levels of C-peptide and insulin were reduced significantly in the OVX+STZ group compared to the OVX group. After treatment with 2 mg/kg progesterone or 0.1mg/kg 17β-estradiol alone, C-peptide and insulin were increased obviously compared to the OVX+STZ group. A combination of 0.1mg/kg 17β-estradiol with 0.1 or 8 mg/kg progesterone also could increase the levels of C-peptide and insulin, particularly a combination of 0.1mg/kg 17β-estradiol with 0.1mg/kg progesterone showed a further markedly increment compared to E0.1 group (Table 2).

Table 2. Concentrations of plasma sex hormone, C-peptide, and insulin

Groups	Estradiol (pg/ml)	Progesterone (ng/ml)	C-peptide (ng/ml)	Insulin (MIU/ml)
OVX	86.77±13.3	0.37±0.3	5.75±1.1	29.56±2.3
OVX+STZ	84.49±12.2	0.37±0.3	3.63±0.8*	19.48±2.2***

P0.1	84.09±11.3	0.57±0.1	4.22±0.2	20.13±0.3
P2	88.5±22.2	1.78±1.3	5.31±0.3#	25.95±6.3#
P8	81.1±8.1	7.78±4.8	4.65±0.2	22.24±5.2
E0.1	186.53±66.1	0.34±0.3	5.99±1#	31.71±2.7###
EP0.1	128.85±6.1	0.65±0.0	19.28±1.5&&&	78.22±8.2&&&
EP2	210.28±99.6	1.77±1.3	5.12±0.4	27.81±9.8
EP8	221.62±77.5	7.87±4.8	4.78±0.1&	23.6±2.1&&&

Data are the mean±SD (n=6). *P< 0.05, ***P<0.001 vs OVX; #P<0.05, ###P < 0.001 vs OVX+STZ; &P<0.05, &&&P < 0.001 vs E0.1.

EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON SKELETAL MUSCLE GLUT4 mRNA EXPRESSION

As shown in Fig. 6A and 6B, the expression of skeletal muscle GLUT4 mRNA was dramatically down-regulated in the OVX+STZ group relative to the OVX group (P<0.001), 0.1mg/kg 17β-estradiol, 0.1 and 2 mg/kg progesterone made it obvious up-regulation. When combined with 2 mg/kg progesterone for treatment, the expression of skeletal muscle GLUT4 mRNA was markedly up-regulated in the EP2 group relative to the E0.1 group, there appeared a synergistic effect on the GLUT4 mRNA expression. However, in the combination of 0.1 mg/kg progesterone with 0.1 mg/kg 17β-estradiol, the expression of GLUT4 mRNA decreased (P<0.05), and there occurred an inhibitory effect on the GLUT4 mRNA expression.

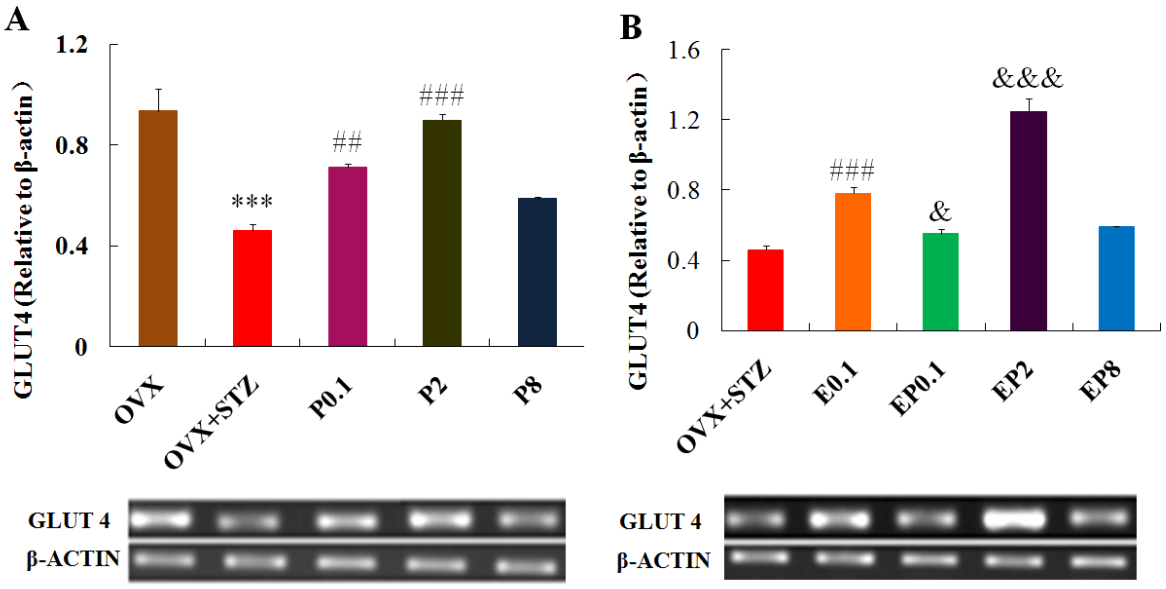


Figure 6. Effects of progesterone and 17β-estradiol on GLUT4 mRNA expression in skeletal muscle. ***P<0.001 vs OVX; ##P<0.01, ###P<0.001 vs OVX+STZ; &P<0.05, &&&P<0.001vs E0.1. Data are the mean±SD (n=3).

EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON HEPATIC GLUCOSE METABOLISM ENZYME EXPRESSION

Compared to the OVX group, liver Gck mRNA expression was significantly reduced in the OVX+STZ group (Fig. 7A, *P*<0.001); 0.1 or 2 mg/kg progesterone or 0.1 mg/kg 17β-estradiol treated respectively, there was a significant increment in Gck mRNA level (*P*<0.001). When treated by 17β-estradiol with progesterone also up-regulated liver Gck mRNA expression, but we found an antagonized effect in the low dose of 0.1 mg/kg progesterone and the large dose of 8 mg/kg progesterone combination groups compared to 0.1 mg/kg 17β-estradiol group (Fig. 7B).

As shown in Fig. 8, The expressions of G-6-P and PCK mRNA in the OVX+STZ group were significantly higher than OVX control group; after treatment with 0.1, 2, or 8 mg/kg progesterone or 0.1 mg/kg17β-estradiol respectively, there appeared significant decrement in G-6-P mRNA levels compared to OVX+STZ group (*P*<0.001), and the expression of PCK mRNA were also decreased in E0.1 group, but after combined 0.1, 2 or 8 mg/kg progesterone with 0.1 mg/kg 17β-estradiol, the expressions of G-6-P and PCK mRNA were same as E0.1group.

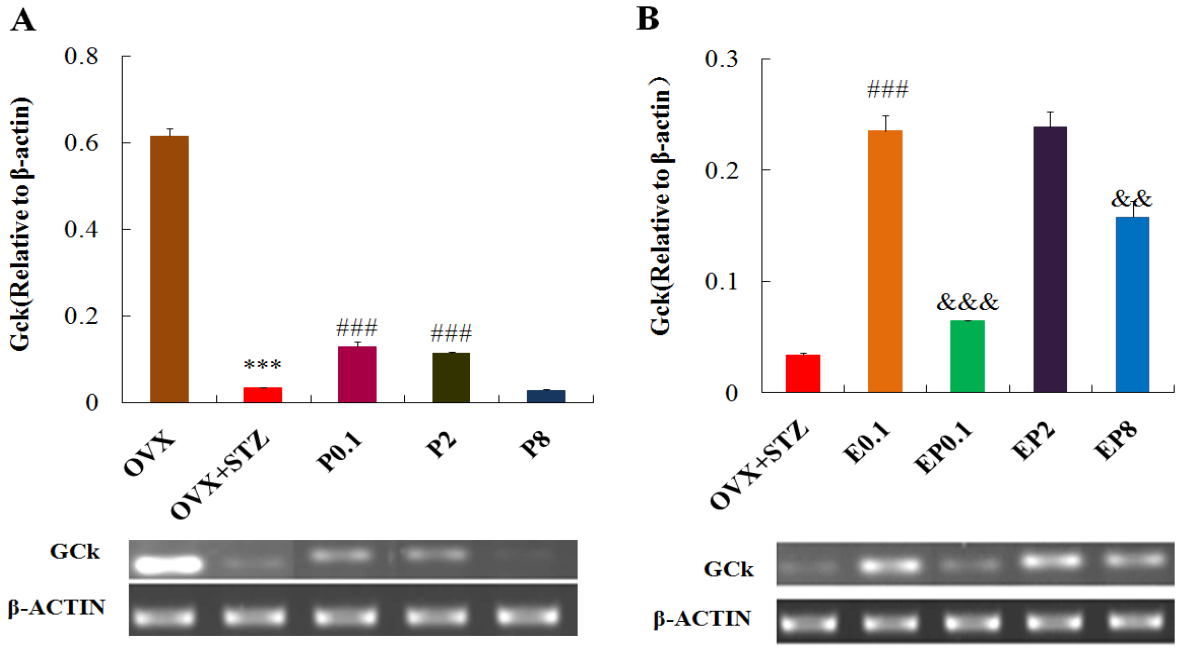


Figure 7. Eftects of progesterone and 17β-estradiol on liver Glucokinase (Gck) mRNA expression in mice. ***P<0.001 vs OVX; ###*P*<0.001 vs OVX+STZ; &&*P*<0.01, &&&*P*<0.001 vs E0.1. Data are the mean±SD (n=3).

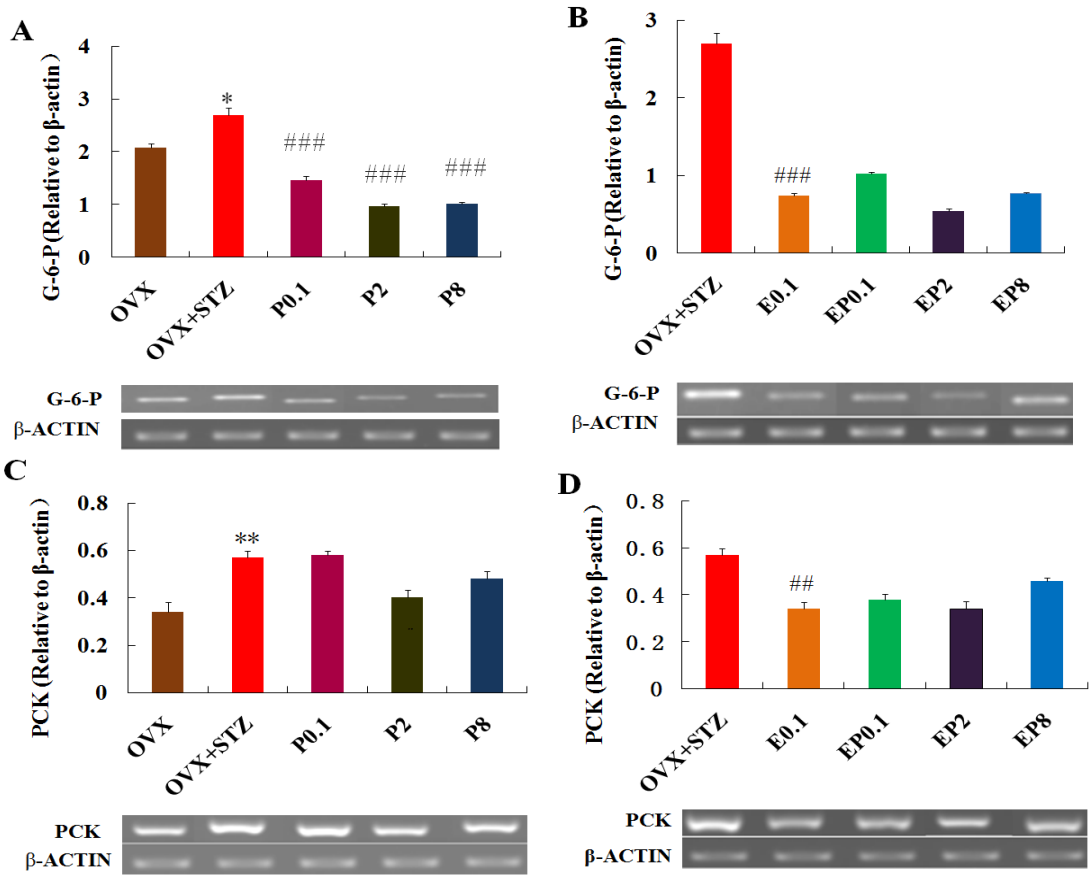


Figure 8. Effects of progesterone and 17β-estradiol on liver Glucose-6-phosphatase (G-6-P) and phosphoenolpyruvate carboxylase (PCK) mRNA expressions in mice. **P*<0.05, ***P*<0.01 vs OVX; ##*P*<0.01, ###*P*<0.001 vs OVX+STZ. Data are the mean±SD (n=3).

Discussion

T1DM is a chronic disease whose etiology involves genetic predisposition and a variety of pathogenic environmental factors. The characteristics of T1DM are T cell-mediated autoimmune destruction of islet β cells, and insulin hyposecretion[26]. Clinical treatment is mainly dependent on exogenous insulin, although T1DM patients account for less than 10% of the number of people with diabetes, its pathogenesis is complex and associated with a variety of complications, which seriously affect the patient's physical condition and quality of life. Therefore, relevant research on T1DM is of great significance for its effective prevention and treatment. Modeling with STZ is a commonly used conventional method, which selectively destroys islet β cells, resulting in a type 1 diabetes model when injected in high doses[27,28]. In the present experiment, the model of type 1 diabetes was established by intraperitoneal injection of STZ, and according to the difference between type 1 and Type 2 diabetes, the type of diabetes model was distinguished and identified by the insulin resistance experiment. The results showed that the modeling method adopted in this paper could lead to increased blood glucose, weight loss, and significantly increased urine in mice, and there was no insulin resistance compared with the OVX group. The preliminary experimental results were consistent with the basic manifestation

of type 1 diabetes "three more and one less", and the serum C-peptide and insulin levels in the model group decreased significantly. These results indicated that the model was consistent with the essential characteristics of insulin deficiency in T1DM. The method had the advantages of a short mold-making period, a high mold-forming rate, high operability and repeatability, and low mortality (5.6% mortality rate in the present study).

The role of estrogen in the regulation of glucose homeostasis has been shown previously by relatively more research data including our lab preliminary results[2,29]. However, there are few controversial studies on the effect of progesterone alone or combined with estrogen on diabetes. For example, Nunes et al.[30] showed that progesterone at high micromolar concentrations was able to decrease the proliferation rate of insulin-producing cells, but Candolfi et al.[31] showed that progesterone is able to antagonize the apoptosis induced by TNFα in somatotrophs and lactotrophs. Meanwhile, Cheng et al.[32] results using hepatoma HuH-7 cells, indicate that the beneficial or deleterious effects of estradiol and progesterone may be tissue-specific. Recent clinical and experimental evidence suggests that sex steroids protect from insulin resistance associated with diabetes[32,33]. Ordonez et al.[34] found the combined therapy of progesterone with estrogen had a synergic effect on insulin sensitivity when their plasma levels were low or high. In our experiment, progesterone, similar to 17β-estradiol, could moderately, yet persistently inhibit the increase of blood glucose in ovariectomized mice induced by STZ over the experimental period of 4 weeks. Particularly, after subcutaneous administrated progesterone alone at the dose of 2 mg/kg or combined with 0.1 mg/kg 17β-estradiol, obvious protection from hyperglycemia and delaying onset of diabetes appeared in STZ-induced diabetic mice, but the doses of 0.1 mg/kg and 8mg/kg progesterone had no obvious anti-hyperglycemic effect, this may be consistent with the fact that postmenopausal and pregnant women are prone to diabetes, and only the level of progesterone, which is close to the physiological range, can reduce the blood glucose caused by diabetes. In the present study, we found that the dose of 0.1 mg/kg 17β-estradiol could induce the level of blood estrogen to reach the physiological concentration range (about 200pg/mL), and the dose of 2 mg/kg progesterone made the level of blood progesterone get the physiological range (1~5ng/mL).

Estradiol protects islet β cells, inhibits apoptosis, and promotes insulin secretion, improving glucose tolerance and insulin resistance[35]. Le May et al.[36] reported that estrogen replacement therapy can control hyperglycemia and reduce the incidence of type 2 diabetes in postmenopausal women. We observed the HE staining of islet β cells and found that 17β-estradiol and progesterone can protect islet β cells from injury caused by intraperitoneal injection of STZ, promote the repair of islet β cells and increase the number of islet β cells. At the same time, progesterone and 17β-estradiol alone or in combination can promote the secretion of C-peptide and insulin, and the effects of 2mg/kg progesterone, 0.1mg/kg 7β-estradiol alone or 0.1mg/kg 17β-estradiol combined with 0.1 or 2mg/kg progesterone on c-peptide and insulin secretion were significantly enhanced. The insulin resistance test showed that there

were no significant differences in insulin resistance between the diabetic model group and the OVX control group. In the glucose tolerance test, 17β-estradiol and 17β-estradiol combined with progesterone improved glucose tolerance in diabetic mice significantly. Therefore, estradiol and progesterone improve glucose tolerance and reduce blood glucose in diabetic mice by protecting pancreatic beta cells and promoting endogenous insulin secretion.

Glycemic homeostasis results from an orchestrated regulation of territorial glucose fluxes, which includes flows into and out of the extracellular/blood compartments. Glucose fluxes are highly variable, even being tightly regulated, and they can alter blood glucose quite rapidly. These include glucose fluxes to blood from the intestine (postprandial absorption), liver (glucose production), and kidney (glucose reabsorption); and also glucose fluxes from blood to liver, skeletal muscle, and adipose tissue, highlighting these fluxes as the most variable and regulatable[37,38]. All these fluxes involve several distinct and complex mechanisms, and, in each territory, one or more glucose transporter isoforms play a key role[39,40]. In epithelial cells of the proximal intestine and renal proximal tubule, sodium-glucose cotransporter 1 and 2 (SGLT1 and SGLT2), respectively, uptake glucose at the luminal membrane; whereas the facilitative glucose transporter 2 (GLUT2) effluxes glucose into the interstitium/blood side[38,39]. In hepatocytes, GLUT2 performs a bidirectional flux of glucose, accordingly to the substrate concentration gradient, which is critical for cellular glucose production[38]. Finally, the glucose uptake by muscle and adipose tissue occurs through the GLUT4, which can be acutely translocated to the plasma membrane in response to insulin[40]. GLUT2 is a transporter with low affinity and high capacity which is closely related to the insulin receptor[41]. Collectively, data from Seyer et al.[42] shows that hepatic glucose uptake depends on GLUT2 expression, but the presence of this transporter is dispensable for normal glycemic control during the absorptive phase and a normal rate of hepatic glucose production in the fasted state. However, during fasting, GLUT2 is required for the equilibration of cytosolic glucose with the external space to allow normal glycogen mobilization and suppression of glycolytic and lipogenic gene expression, processes activated by elevated glucose levels. Skeletal muscle is the major tissue accounting for 70-85% of whole-body glucose disposal following a glucose challenge. GLUT4 is expressed mainly in skeletal muscle and white adipose tissue, and its inappropriate expression, translocation, or anchorage to the membrane can lead to insulin resistance, and consequently, impaired glucose homeostasis[40]. Insulin is the most important hormone for the maintenance of euglycemia. It regulates carbohydrate metabolism in the liver and glucose uptake in insulin-sensitive tissues, i.e., skeletal muscle and white adipose tissue. By binding to its receptors on the cell membrane of skeletal muscle and white adipose tissue cells, insulin initiates a signaling cascade of phosphorylation leading to the translocation of vesicles containing glucose transporter GLUT4 to the plasma membrane. Once GLUT4 is anchored to the membrane, glucose can enter the cell by facilitated diffusion. In the present study, the expression of GLUT4 mRNA in skeletal muscle was down-regulated in the diabetic

mouse model group. Progesterone, 17β-estradiol, or both combined could promote the expression of GLUT4 in skeletal muscle. When administered alone, 2mg/kg progesterone and 0.1mg/kg 17β-estradiol significantly up-regulated the expression of GLUT4 mRNA in skeletal muscle, and after combining 2mg/kg progesterone with 0.1mg/kg 17β-estradiol, the expression of GLUT4 mRNA was also increased. But the dose of 0.1 or 8mg/kg progesterone antagonized the up-regulation of 17β-estradiol on GLUT4 mRNA expression. Therefore, the mechanism of 17β-estradiol, progesterone and their combination to reduce blood glucose and delay the occurrence of diabetes probably contributes to the promotion of skeletal muscle sugar transportation.

Glucokinase (Gck) is a hexokinase isoenzyme that catalyzes the phosphorylation of glucose to glucose-6-phosphate, which is the first rate-limiting step in glucose metabolism. Gck is predominantly expressed in pancreatic β cells, where it regulates insulin secretion by altering the rate of glucose phosphorylation according to the glucose concentration. Gck is, therefore, a glucose sensor of the pancreas, it has unique kinetics, such as its lower affinity for glucose (Km ~10 mmol/L), moderate cooperative binding with glucose, and lack of inhibitory feedback by glucose-6-phosphate (G-6-P)[43], which distinguish it from other hexokinases and make it function as an effective glucose sensor. Hepatic glycogen plays a major role in the maintenance of blood glucose homeostasis. The breakdown of glycogen in hepatocytes can release glucose into the bloodstream[41]. Phosphoenolpyruvate carboxylase (PCK) is a key rate-controlling enzyme that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the rate-limiting step of gluconeogenesis, while G-6-P catalyzes the conversion of glucose-6-phosphate to glucose in the last step of gluconeogenesis. Westermeier et al.[44] suggested that the lack of PCK gene expression may cause defective insulin signaling and induce hyperglycemia in diabetic animal models. In the present study, the expression of Gck has decreased in STZ-induced type 1 diabetic mouse, but the expressions of liver G-6-P and PCK increased significantly. Progesterone alone or combined with 17β-estradiol could reverse the expressions of Gck, PCK, and G-6-P, which possibly contribute to regulating carbohydrate metabolism in the liver, increasing the local insulin sensitivity, and reducing hepatic glucose production and efflux[45]. Estrogen replacement treatment (ERT) is always associated with a higher risk for breast, endometrial, and ovarian cancer. Due to these side effects of long-term ERT use, the number of ERT users has fallen dramatically[46]. However, Getoff et al.[47] found that progesterone in combination with 17β-estradiol can strongly reduce the number of carcinogenic 17β-estradiol-metabolites. This fact might offer a new pathway for applying hormones in the medical treatment of patients.

In conclusion, 17β-estradiol and progesterone alone can delay the occurrence of type 1 diabetes in mice induced by STZ, and the effects are different with their doses, but there is no dose-effect relationship. A medium dose of 17β-estradiol (0.1mg/kg body weight) and progesterone (2mg/kg body weight) alone or jointly can make the blood concentration reach the physiological dose range, which shows an obvious preventative effect on

streptozotocin-induced diabetes in ovariectomized mice and has certain synergistic effects. The mechanisms of 17β-estradiol and progesterone delaying the occurrence of diabetes are multifaceted, which is probably related to protecting islet cells, promoting insulin release, skeletal muscle glucose transportation, and liver glucose metabolism.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Authors' Contributions:

YY and LJJ performed the research; LHF designed the research study; YY, LJJ, WYD, and HXJ analyzed the data; MY and WYD performed the formal analysis; YY and LHF wrote the manuscript.

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