

Erythropoietin improves glucose metabolism and pancreatic β -cell damage in experimental diabetic rats

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Abstract. Previous studies have implicated erythropoietin (EPO) signaling in the regulation of glucose metabolism. Whether EPO can be used treat diabetes and the underlying mechanism remain to be elucidated. The present study aimed to investigate whether EPO affects glucose metabolism, and the underlying mechanisms, in experimental diabetic rats. The effects of EPO (300 U/kg three times a week for 4 weeks) on glucose metabolism, hematopoietic function, blood selenium content and the ultrastructure of pancreatic β -cells were investigated in low dose (25 mg/kg body weight) streptozotocin-induced experimental diabetic rats provided with a high-fat diet. The results demonstrated that EPO significantly decreased the fasting blood glucose, the area under the curve of the oral glucose tolerance and insulin tolerance tests and L-alanine gluconeogenesis. Ultrastructural examination of the pancreatic islets revealed that EPO prevented the dysfunction of pancreatic β -cells in experimental diabetic rats, ameliorated

cytoplasmic vacuolation and fragmentation of mitochondria, and increased the number of secretory granules. EPO administration increased the activities of superoxide dismutase and glutathione peroxidase, and decreased the level of malondialdehyde. Additionally, EPO increased blood selenium in the diabetic rats and produced a hematopoietic effect. These results indicated that EPO modulated glucose metabolism and improved pancreatic β -cells damage by increasing anti-oxidation. The detailed mechanisms underlying these effects require further investigation.

Introduction

Erythropoietin (EPO), first identified for its key importance during erythropoiesis, is a glycoprotein hormone clinically used for the treatment of anemia, predominantly in chronic kidney disease (1). EPO is produced primarily in the peritubular fibroblasts in the kidney with a small contribution from the liver in adults (1-3). Its principal function is to regulate red blood cell production by binding to the EPO receptor (EPOR) on erythroid progenitor cells. Notably, the expression of EPO is not confined to the kidney and liver, and EPO mRNA has also been detected in the lungs, testis, uterus and brain in rodents, and skeletal muscles in humans (4-5). The expression of EPOR has been detected in non-erythroid cells, including endothelial cells, epicardium and brain neuroepithelium (2,3). Treatment with EPO can exert several extra-hematopoietic effects. Previous studies have revealed that EPO exerts multiple protective effects, including anti-oxidative (2,6), anti-inflammatory (7,8) and anti-apoptotic effects (9).

A total of ~366,000,000 individuals worldwide were affected by diabetes mellitus (DM) in 2011, and the numbers are expected to reach 522,000,000 by 2030, despite advances in treatment (10). It has been reported that 90% of DM cases are type 2 diabetes mellitus, the pathogenesis of which involves abnormalities in glucose metabolism, including inadequate insulin secretion from pancreatic β -cells and insulin resistance (11,12). In 2003, Fenjves *et al* demonstrated that pancreatic islets express EPOR protein (13). EPO exerts cytoprotective effects on non-erythroid tissues by modulating a variety of signaling pathways, which involve mitogen-activated protein kinase, nuclear factor (NF)- κ B and phosphatidylinositol 3-kinase (PI3K)/Akt (14). It is well known that activation

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Abbreviations: EPO, erythropoietin; EPOR, erythropoietin receptor; DM, diabetes mellitus; T2DM, type 2 diabetes mellitus; MAPK, mitogen-activation protein kinase; PI3K, phosphatidylinositol-3 kinase; rHuEPO, recombinant human erythropoietin; STZ, streptozotocin; FBG, fasting blood glucose; AUC, the area under the curve; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; ISG, insulin secretion granule; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration

Key words: erythropoietin, glucose metabolism, pancreatic β -cells, anti-oxidation, experimental diabetic rats

of the PI3K/Akt signaling pathway has protective effects on DM. Therefore, several previous studies have focused on investigating the effects of EPO on islet cells and, in particularly, the effect of EPO in the type 1 and 2 diabetes (1,2,13-15).

In vitro investigations have demonstrated that recombinant human erythropoietin (rHuEPO) protects against cytokine-induced islet dysfunction and cell death in rat islet cultures (13), and transfection of the EPO gene into human islets (15). Whether EPO modulates glucose metabolism and produces a protective effect on pancreatic β -cells *in vivo* remains to be elucidated. Therefore, the aim of the present study was to investigate the effects of EPO (300 U/kg, three times a week for 4 weeks) on glucose metabolism, insulin resistance, the ultrastructure of pancreatic β -cells, anti-oxidative indices, blood selenium content and hematopoietic function in low dose streptozotocin (STZ)-induced diabetic rats fed a high-fat diet.

Materials and methods

Animals, grouping and treatment. A total of 30 male Sprague Dawley rats, weighing 160-180 g (Experimental Animal Center of Xian Jiaotong University, Xian, China), were used in the present study, in accordance with the recommended guidelines on the care and use of laboratory animals issued by the Chinese Council on Animal Research. The present study was approved by the Ethics Committee of Xian Jiaotong University (Xian, China). The rats were housed in a temperature-controlled room (19°C) and were raised under a 12 h light/dark cycle with had free access to food and water.

Following acclimation for 1 week, the rats were randomly divided into two groups: Control (n=8) and diabetic model (n=22). The control rats were fed with a standard laboratory diet (carbohydrates, 30%; proteins, 22%; lipids, 12%; vitamins, 3%) *ad libitum*. The diabetic rats were fed a high-fat, high-glucose diet (10% refined lard, 20% sucrose, 2.5% cholesterol, 1% sodium cholate and 66.5% common food), which was provided by the Laboratory Animal Center of Xian Jiaotong University (Xian, China). After 6 weeks, the diabetic rats were administered with a peritoneal injection of freshly prepared STZ (25 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) to overnight fasted rats, while the control group was administered with an equivalent volume of citric acid buffer. At 4 weeks post-STZ injection, fasting blood glucose (FBG) was assessed and rats with FBG>16.7 mmol/l were considered to be diabetic (n=16). The diabetic rats were randomly divided into a diabetic model (model; n=8) and diabetic treated with EPO (EPO; n=8) group. rHuEPO (300 U/kg, three times a week; Shenyang Sunshine Pharmaceutical Co., Ltd., Shenyang, China) was administered to the diabetic treated rats by hypodermic injection for 4 weeks. The equivalent volume of normal sodium was administered to the control and untreated model rats.

At the end of the experiment, blood samples from the rats were obtained from the abdominal aorta following anesthesia with 10% chloral hydrate. The blood samples were separated into three samples, one collected with EDTA-normal saline solution for hematological analysis, the second collected with heparinized normal saline solution for selenium measurement, the third placed into Eppendorf tubes and centrifuged at

1,368 x g for 10 min at room temperature, followed by serum collection and storage at -80°C until biochemical analysis. The tail sections of the pancreatic tissue were thinly sliced to 1 mm³ and placed immediately into 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/l phosphate buffer.

Intraperitoneal glucose tolerance test (IPGTT). An IPGTT was performed at the fourth week. Following 12 h fasting, a glucose solution of 2 g/kg was administered by intraperitoneal injection and the blood glucose was measured at 0, 30, 60 and 120 min post-injection from tail veins using a glucose meter (Accu-Chek® Performa System; Roche Diagnostics GmbH, Mannheim, Germany) and the area under the curve (AUC) was calculated for FBG during the IPGTT.

Insulin tolerance test (ITT). Following 4 weeks of treatment, ITT was performed. Subcutaneous injection of 4 U/kg insulin (Jiangsu Wanbang Biochemistry Medicine Co., Ltd., Xuzhou, China) was administered following 12 h fasting, and the blood glucose was measured 0, 40 and 90 min following injection using a glucose meter. The AUC was calculated for FBG during the ITT.

Alanine gluconeogenesis test. Following 4 weeks of treatment, the animals were fasted for 12 h and intraperitoneally injected with L-alanine (1.5 g/kg; Shanghai Ding Guo Biological Technolog, Co., Ltd., Shanghai, China). The blood glucose was measured at 0 and 60 min following alanine load using the glucose meter. The AUC generated from the data collected were calculated.

Ultrastructure of the pancreatic β -cells. The ultrastructure of the pancreatic β -cells was investigated using transmission electron microscopy. The tail sections of pancreatic tissue in all groups were thinly sliced to 1 mm³ and placed immediately into 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/l phosphate buffer and were post-fixed in 2% osmium tetroxide (Johnson, Matthey Chemicals, Ltd., Royston, UK). Following dehydration in graded ethanol, the samples were embedded in spur resin (SPI Supplies Division of Structure Probe, Inc., West Chester, PA, USA). Semitic sections were stained with 1% toluidine blue. The ultrathin sections were collected onto 200-mesh copper grids (Beijing Xinxing Brain Technology Co., Ltd., Beijing, China) and double stained with uranyl acetate and lead citrate (Ted Pella, Inc., Redding, CA, USA). Each section was observed using a H7650 TEM (Hitachi, Tokyo, Japan).

Biochemical analysis. The levels of serum superoxide dismutase (SOD), malonicdialdehyde (MDA) and renal glutathione peroxidase (GSH-Px) were measured using a Total Superoxide Dismutase Assay kit (hydroxylamine method), Malondialdehyde Assay kit (TBA method) and Glutathione Peroxidase Assay kit (colorimetric method), according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Selenium measurement. At the end of the investigation, whole blood samples were collected with heparinized normal saline solution. The blood samples (0.5 ml) were solubilized by

mixing with 0.5 ml $\text{HNO}_3/\text{H}_2\text{O}_2$ mixture (2:1), followed by agitation and heating to 350-400°C in an electric oven until 0.1-0.2 ml solid residue remained. The final clear residue was adjusted to a final volume of 1 ml with 1 mol/l HNO_3 . The selenium contents were determined by hydride generation-atomic fluorescence spectrometry (AFS-202; Beijing Kechuang Haiguang Instrument Co., Ltd., Beijing, China) (16).

Measurement of erythropoiesis. A blood cell count was performed using a Coulter JT automated hematology analyzer (Beckman Coulter, Fullerton, CA, USA). EDTA-blood (200 μl) was collected at the end of the experiment ($n=8$ per group). The quantification of red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured (17).

Statistical analysis. The results are expressed as the mean \pm standard error of the mean. An analysis of variance single-factor test was performed using SPSS v13.0 software (SPSS, Inc., Shanghai, China) for the statistical analyses of intergroup comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EPO improves the general conditions of diabetic rats. The rats in the control group were in good health, with lively movements, robust bodies, luster fur and soft, thick, clean hair. However, in the diabetic model group, the rats were weak, sweaty and unclean, with lackluster fur and thin hair, which easily fell out. The rats were slow to respond to external stimuli. These rats also drank and urinated more frequently, compared with the control group, and skin appeared to be easily damaged. However, experimental treatment with EPO markedly improved the general conditions of the diabetic rats.

EPO alleviates glucose and insulin tolerance in diabetic rats. Glucose tolerance is an indirect indicator for insulin sensitivity and healthy pancreatic β -cell response. In the present study, IPGTT, ITT and gluconeogenesis assessments were performed for all groups following the experimental procedures. The results demonstrated that glucose tolerance and insulin tolerance were severely impaired in the diabetic model rats, whereas gluconeogenesis was increased, compared with the control rats ($P<0.01$; Fig. 1). However, the diabetic rats treated with EPO exhibited a significantly higher glucose tolerance ($P<0.01$; Fig. 1A and B) and insulin tolerance ($P<0.001$; Fig. 1C and D), compared with the untreated model. Treatment with EPO decreased L-alanine gluconeogenesis, compared with the diabetic model ($P<0.001$; Fig. 1E and F).

EPO decreases FBG in diabetic rats. To obtain further insight into the observed effects of EPO on glucose metabolism, the FBG levels were compared between all groups (Fig. 2). It was revealed that FBG significantly decreased from the first week of treatment with EPO (EPO group, 16.2 ± 2.1 mmol/l; model, 23.5 ± 1.4 mmol/l; $P<0.01$) and was maintained at a relatively stable range until the end of the study (EPO group,

14.3 ± 2.7 mmol/l; model, 24.4 ± 1.2 mmol/l; $P<0.001$). By contrast, the FBG in the control rats were in the normal range (4.4-6.6 mmol/l).

EPO relieves ultrastructural injury of pancreatic β -cells in diabetic rats. Transmission electron microscopy investigation of the pancreatic tissue of the control group revealed a normal pancreatic architecture (Fig. 3). By contrast, the β -cells of the diabetic model rats exhibited several pathological findings (Fig. 3). The cytoplasm appeared vacuolated and degranulated, with an increased number of empty granules. The number of insulin secretion granules, presumably containing insulin, in the β -cells from experimental diabetic rats was markedly lower compared with the normal control rats. Enlargement of mitochondria, destruction of mitochondrial cristae and development of the Golgi apparatus were observed in the electron microscopic images of the pancreatic β -cells from the experimental diabetic rats, compared with the normal control rats. In the diabetic rats treated with EPO, ultrastructural analysis demonstrated that the number and distribution of mitochondria were similar to the control and only a few mitochondria exhibited signs of fragmentation (Fig. 3). The majority of the secretory granules contained a dense core, with a few exhibiting less dense cores and very few being empty.

Effect of EPO on the serum levels of MDA, SOD and GSH-PX. As shown in Fig. 4A-C, the MDA concentration, which is a marker for injury induced by free radicals, was determined. The antioxidant enzyme activities, including SOD and GSH-PX were simultaneously measured. The serum MDA level in the model group was markedly increased (20.70 ± 1.38 nmol/ml), compared with the control group (11.49 ± 1.01 nmol/ml; $P<0.001$). However, the activities of SOD (220.3 ± 8.0 U/ml) and GSH-PX (81.7 ± 2.3 $\mu\text{mol/l}$) were markedly reduced in the model group, compared with the control group (SOD, 267.0 ± 4.6 U/ml, $P<0.01$; GSH-PX, 94.8 ± 2.8 $\mu\text{mol/l}$, $P<0.01$). This increase in the levels of MDA was significantly attenuated by treatment with EPO (14.54 ± 0.68 nmol/ml), compared with the model group ($P<0.001$). Additionally, EPO administration increased the activities of SOD (259.6 ± 4.3 U/ml, $P<0.05$) and GSH-PX (86.8 ± 1.2 $\mu\text{mol/l}$, $P<0.05$).

EPO increases blood selenium in diabetic rats. Numerous clinical assays have demonstrated significantly lower plasma and serum concentrations of selenium in patients with cardiovascular diseases, compared with those measured in healthy individuals (18). Selenium is a trace element, which exerts certain insulin-like actions on the glucose homeostasis of diabetic rats. To observe whether EPO affected the selenium concentration at the end of investigation, blood samples were collected from the abdominal aorta following overnight fasting. The selenium content in whole blood was determined using an atomic fluorescence spectrometry technique in all groups (Fig. 4D). The result demonstrated that the selenium concentration in the diabetic model rats (511.7 ± 15.2 $\mu\text{g/l}$) was significantly lower than that in the control rats (611.7 ± 14.5 $\mu\text{g/l}$; $P<0.001$). Treatment with EPO significantly increased the selenium concentration (562.3 ± 21.2 $\mu\text{g/l}$; $P<0.05$), compared with the model.

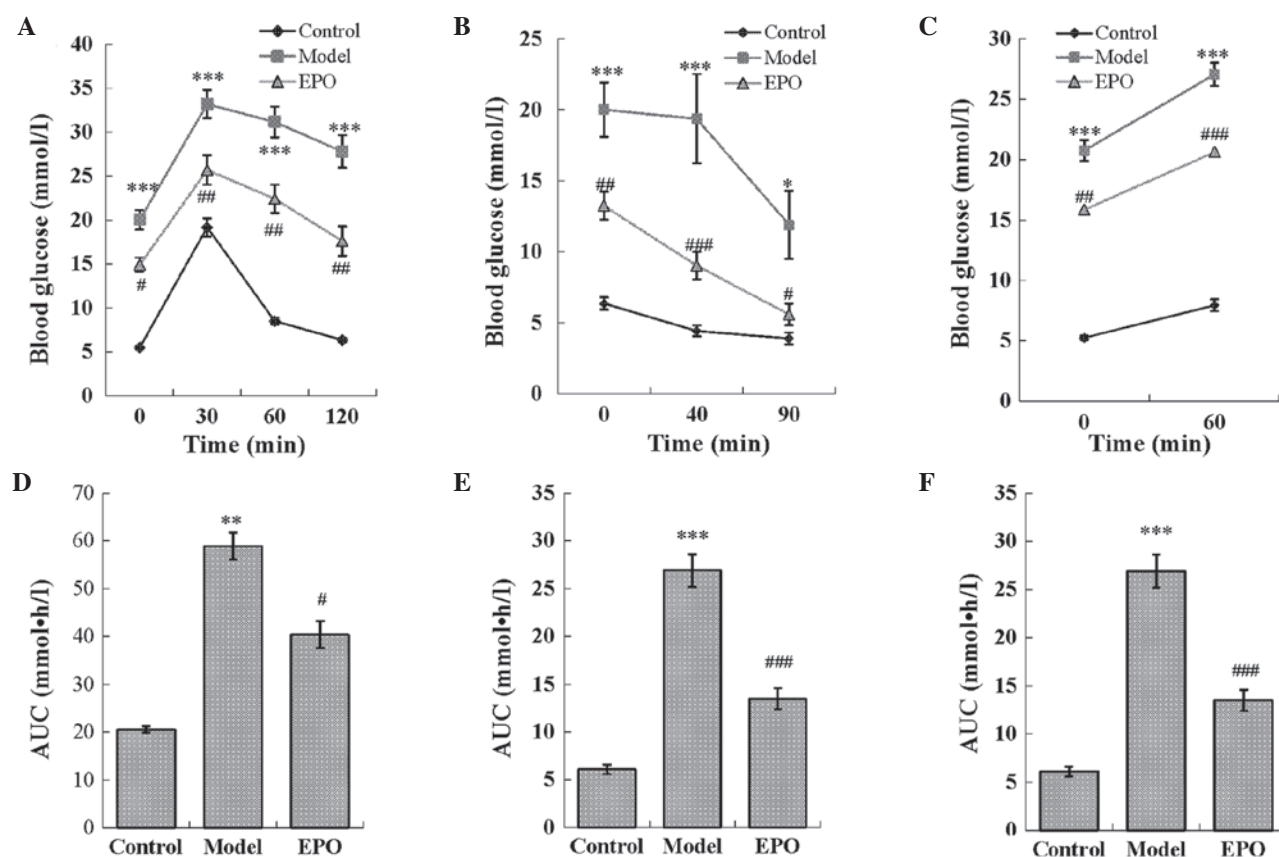


Figure 1. EPO mediates the improvement of glucose metabolism. The blood glucose concentration was measured at different time points during (A) IPGTT, (B) ITT and (C) gluconeogenesis. The values of the AUC were determined for (D) IPGTT, (E) ITT and (F) gluconeogenesis. The results are expressed as the mean \pm standard error of the mean ($n=8$ rats/group). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$, compared with the control. # $P<0.05$, ## $P<0.01$ and ### $P<0.001$, compared with the model. AUC, area under curve; EPO, erythropoietin.

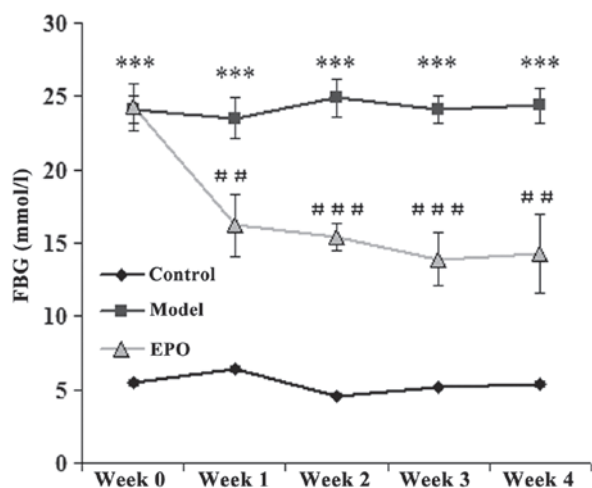


Figure 2. Effects of treatment with EPO on FBG. FBG was detected in all groups each week following fasting for 16 h. The results are expressed as the mean \pm standard error of the mean ($n=8$ rats/group). *** $P<0.001$, compared with the control. ## $P<0.01$ and ### $P<0.001$, compared with the model. FBG, fast blood glucose; EPO, erythropoietin.

EPO regulates hematopoietic function in diabetic rats. To determine whether EPO (300 U/kg, three times a week for 4 weeks) affected the hematopoietic function, routine examination of the blood from each rat was performed on a Coulter JT automated hematology analyzer. As shown in Fig. 5, a

significant decrease in RBC, HB and HCT levels were observed in the diabetic model group, compared with the control group ($P<0.01$). In addition, statistically significant increases in the RBC, HB and HCT levels was observed in the EPO treatment group, compared with the model group ($P<0.05$). No difference in the MCV and MCH levels were observed in any group ($P>0.05$).

Discussion

Previous studies have reported that there may be an association between EPO levels and hypoglycemia, which suggests a potential protective effect of EPO in the treatment of diabetes (1-3,19,20). The present study demonstrated that treatment with EPO (300 U/kg, three times a week for 4 weeks) was effective in reducing FBG and improving insulin resistance in high-fat diet/low dose STZ-induced experimental diabetic rats. Ultrastructurally, EPO prevented the dysfunction of pancreatic β -cells, ameliorated cytoplasmic vacuolation and fragmentation of mitochondria, and increased the number of secretory granules. Administration of EPO increased the activities of SOD and GSH-PX, and decreased the level of MDA. In addition, EPO increased blood selenium in diabetic rats and produced a hematopoietic effect.

Several previous studies have demonstrated that treatment with EPO improves insulin sensitivity and reduces insulin resistance in dialysis patients, obesity and metabolic

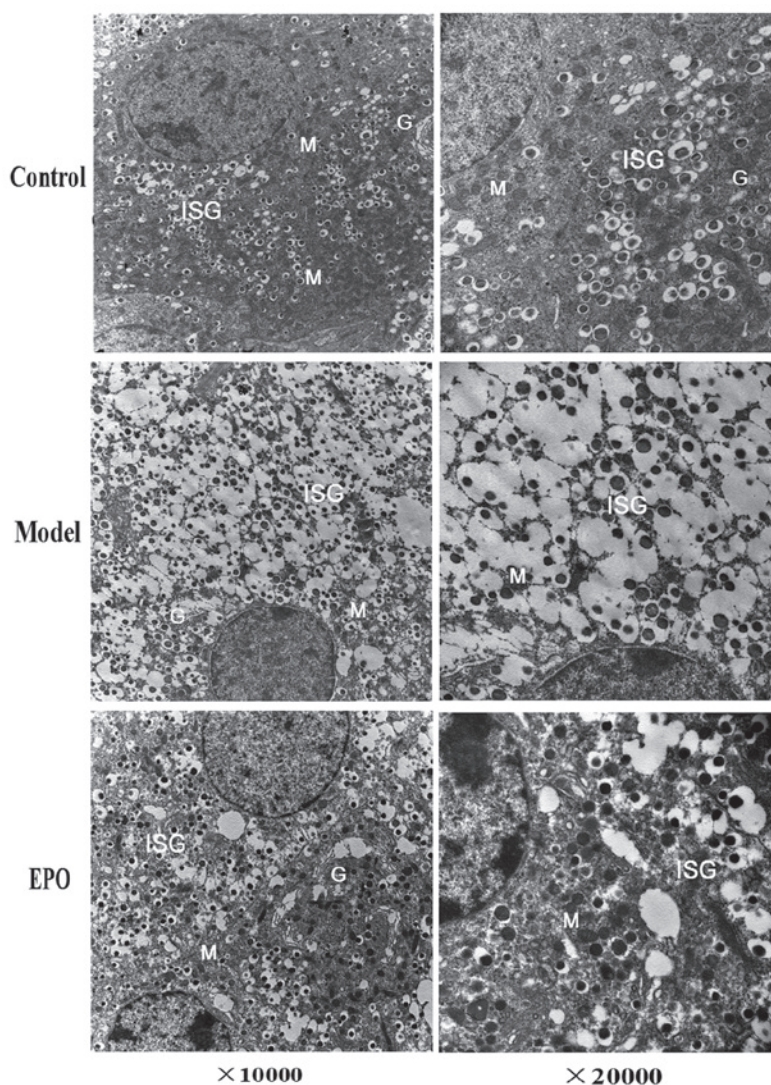


Figure 3. Ultrastructure of the pancreatic islet β -cells in all groups, observed under transmission electron microscopy. The normal β -cells of normal control rats, disordered β -cells of the experimental diabetic model rats (with the cytoplasm vacuolated and degranulated, increased numbers of empty granules, destruction of mitochondrial cristae and golgi development), and improved β -cells of diabetic mice treated with EPO (300 U/kg, three times a week) for 4 weeks were visualized at magnification, $\times 10,000$ (left) and $\times 20,000$ (right). G, Golgi apparatus; M, mitochondria; ISG, insulin secretion granule; EPO, erythropoietin.

syndrome mice and 3T3L1 adipocytes, via EPO receptors in insulin-responsive tissues, including the muscle and liver, or indirectly through the correction of anemia (1-3,21-23). The present study demonstrated that treatment with EPO reduced FBG, ameliorated glucose tolerance and insulin sensitivity, and lowered glyconeogenesis in experimental diabetic rats, which was in agreement with the previous studies. A previous randomized placebo controlled trial (18) demonstrated that insulin sensitivity is not improved following treatment with EPO in healthy humans. This may be due to exogenous EPO normalizing the low EPO levels in these patients.

Abnormal islets induce insulin secretion disorder and hyperglycemia (24,25). These findings are in accordance with the electron microscopy observations in the present study of severe damage to pancreatic β -cells in the experimental diabetic rats, including cytoplasmic vacuolation, decreased insulin secretory granules, enlargement of mitochondria, destruction of mitochondrial cristae and development of the golgi apparatus. EPO normalized the state and the quantity of

the mitochondria and the golgi apparatus, and increased the number of insulin secretion granules in the pancreatic β -cells. These results indicated that EPO exerted a clear protective effect on pancreatic β -cell damage. He *et al* (26) suggested that EPO can protect neonatal islet cells in the porcine model by upregulating B-cell lymphoma-2, and downregulating B-cell-associated X protein and caspase 3. Shuai *et al* (27) suggested that the PI3K/AKT pathway is directly involved in the effect on pancreatic β -cells (27).

Reactive oxygen species are also important in the development of diabetes (2,28), which is consistent with the results from the present study. A significantly higher MDA level, a marker of lipid peroxidation, was observed in the experimental diabetic rats, and the activities of SOD and GSH-PX (markers of antioxidants) were lower. EPO has been revealed to exert anti-oxidation in type 1 and type 2 diabetes (2,28,29). In the present study, administration of EPO decreased the level of MDA and increased the activity of antioxidant enzymes, including SOD and GSH-PX in the serum, compared with

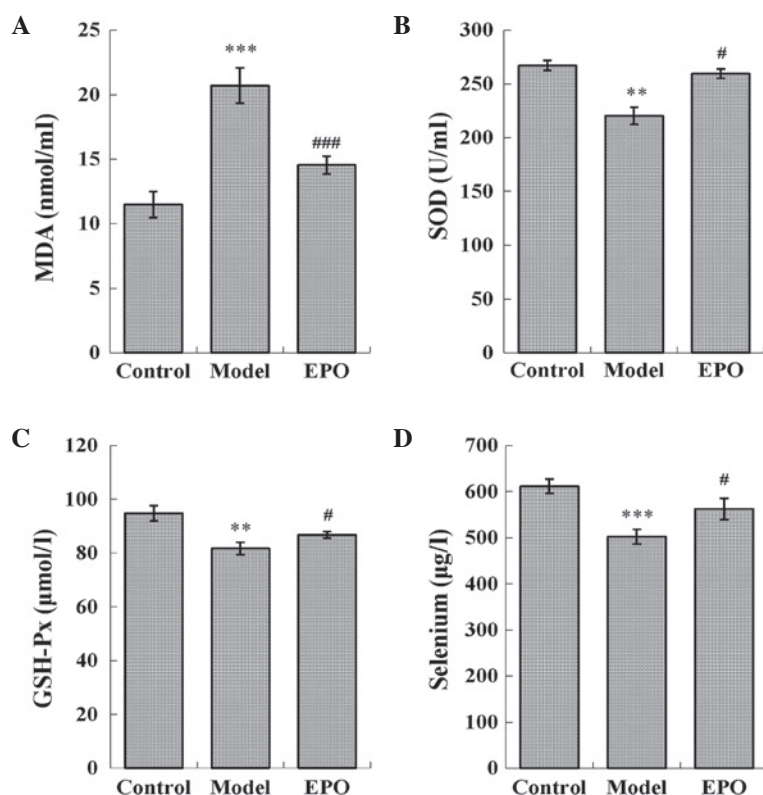


Figure 4. Effects of treatment with EPO on serum levels of (A) MDA, (B) SOD, (C) GSH-Px and (D) blood selenium. The results are expressed as the mean \pm standard error of the mean (n=8 rats/group) ^{**}P<0.01 and ^{***}P<0.001, compared with the control. [#]P<0.05 and ^{###}P<0.001, compared with the model. EPO, erythropoietin; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px; glutathione peroxidase.

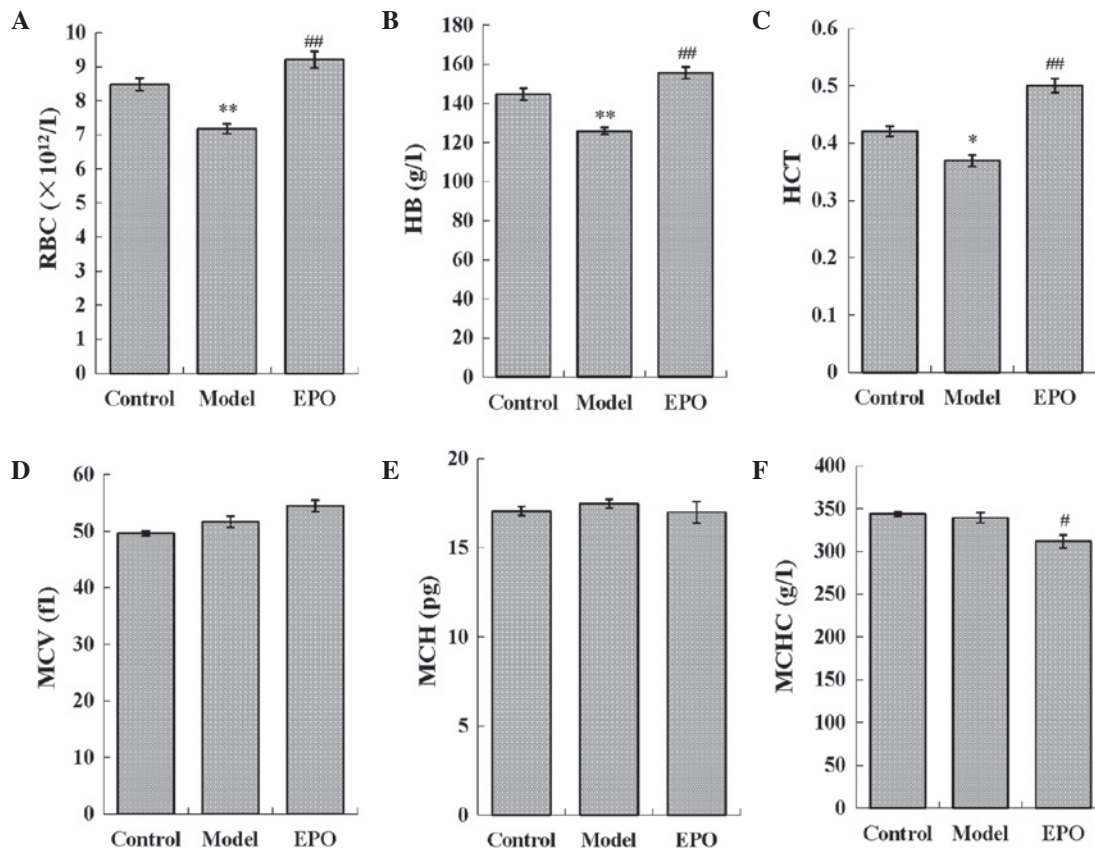


Figure 5. Effect of treatment with EPO on hematopoietic function. The levels of (A) RBCs, (B) HB, (C) HCT, (D) MCV, (E) MCH, (F) MCHC were measured. The results are expressed as the mean \pm standard error of the mean (n=8 rats/group) ^{*}P<0.01 and ^{**}P<0.001, compared with the control. [#]P<0.05 and ^{##}P<0.01, compared with the model. EPO, erythropoietin; RBC, red blood cell; HCT, hematocrit; MCT, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

the diabetic model group. EPO may exert its anti-oxidative effects directly by upregulating hemoglobin oxidase-1 activity (30), and indirectly by inhibiting the activity of induced nitric oxide synthase (31), increasing the number of RBCs in the circulation to reduce cellular oxidative stress, since RBCs are loaded with anti-oxidative enzymes (32).

Selenium is an essential trace element, which scavenges free radicals. Its anti-oxidative function is associated with selenoproteins, which include GSH-PX, thioredoxin reductase, selenoprotein P, selenoprotein T and selenoprotein W (33). Furthermore, selenium has insulin-mimetic properties and can be qualified as a potential antidiabetic agent (33). There are conflicting reports on the link between the micronutrient selenium and the prevalence of diabetes. The use of appropriate selenium supplements may improve glucose metabolism by alleviating hyperglycemia, regulating glycolysis and gluconeogenesis, and activating key components of the insulin signaling cascade (34,35). By contrast, other previous findings (36,37) have revealed that a high selenium status or intake is positively correlated with an increased risk of type 2 diabetes. The present study demonstrated that the selenium concentration in the diabetic model rats was markedly lower than that in the control rats, and that treatment with EPO increased the selenium concentration in the diabetic rats, which may be involved in the protective effect on pancreatic β -cells through maintaining selenium levels and increasing its anti-oxidative and insulin-mimetic effects.

The major side effect of EPO treatment is erythrocytosis, which can lead to increasing thrombogenesis and hypertension (1,3). The prevalence of anemia is higher in patients with diabetes (either type 1 or type 2), compared with the general population (38). The results of the present study revealed that there were significant decreases in the levels of RBCs, HB and HCT in the diabetic model group. By contrast, there were statistically significant increases in the levels of RBCs, HB, HCT in the EPO-treated group (3x300 IU/kg body weight/week for 4 weeks). These results were consistent with the data from Choi *et al* (39), which demonstrated that mice treated with rHuEPO exhibit increased hematocrit (39). However, Wang *et al* (40) demonstrated that EPO (3x256 IU/kg body weight/week) caused no increase in hematocrit in STZ-diabetic Wistar rats for up to 6 months. Therefore, selectively harnessing the favorable metabolic effects of EPO may have therapeutic potential and may be used cautiously in the treatment of diabetes, as the dosage and course of treatment remain to be elucidated.

In conclusion, the results of the present study suggested that EPO modulated glucose metabolism, reduced FBG, ameliorated glucose tolerance and insulin sensitivity, lowered glyconeogenesis, and improved pancreatic β -cells damage by increasing selenium concentration and enhancing the antioxidant effect, which may be associated with extra-hematopoietic and hematopoietic effects. The underlying mechanisms remain to be elucidated.

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