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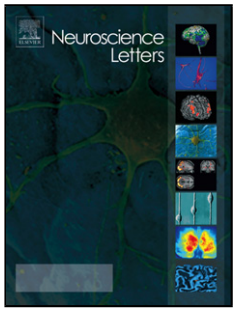

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Pancreatic-islet microvascular vasomotion dysfunction in mice with spinal cord injury

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Highlights

- Blood glucose was abnormal, glucose tolerance was impaired in injury-induced mice.
- Pancreatic-islet microvascular vasomotion was impaired following injury.
- VEGF-A level was correlated with the functional parameters of vasomotion.

Abstract

Patients with spinal cord injury (SCI) have an increased risk for developing type 2 diabetes. It is unknown whether the pancreatic-islet microvascular vasomotion is involved. We used female C57BL/6 mice and a 100-kilodyne T10 Infinite Horizons contusion SCI (or T10 laminectomy) to detect blood glucose and pancreatic-islet microvascular vasomotion. Blood glucose obtained from tail vein was detected using one Touch UltraEasy glucometer. Glucose tolerance test was performed by D-glucose administration intraperitoneally. Functional status of pancreatic-islet microvascular vasomotion was determined by laser Doppler monitoring. Expressions of insulin and glucagon were determined by immunohistochemistry. Expression of VEGF-A was determined by immunohistochemistry and Western blotting. Our result demonstrated that blood glucose was significantly increased at 4 h postinjury compared to that in sham group, with continuous higher blood glucose until 4 days postinjury ($p < 0.05$). SCI mice at day 7 and day 14 had significantly impaired glucose tolerance following glucose administration ($p < 0.01$). Average blood perfusion, amplitude, frequency, and relative velocity of vasomotion were significantly lower at 6 h postinjury than those in the sham group ($p < 0.05$), which were gradually upregulated over time. The expression of insulin was

decreased, while the expression of glucagon was increased at 6 h postinjury. Similarly, the expression of VEGF-A was significantly decreased at 6 h postinjury, compared to that in sham group ($p < 0.05$), with slight increases by 14 days postinjury. Our study suggests that the functional status of pancreatic-islet microvascular vasomotion is impaired after injury, which may have implications for developing effective therapeutic interventions for SCI.

Keywords: spinal cord injury; blood glucose; microvascular vasomotion; VEGF-A

1 INTRODUCTION

Traumatic spinal cord injury (SCI) is a catastrophic and disabling event that results in severe motor and sensory dysfunction. Disruption of neural and vascular structures, apparent immediately followed contusion and defined as primary injury, is followed by a natural evolution of secondary pathology, which determines the extent of functional recovery[1]. The mechanisms of tissue damage during and after SCI are complex and have not been fully elucidated yet. With advanced in acute care and in the management of multiple organ dysfunction, chronic conditions are becoming a focus.

Besides sensory-motor loss, SCI triggers alterations in body composition and physical activity that contribute to abnormalities of carbohydrate and lipid metabolism, which are more common among people in the SCI population than in the able-bodied population [2].These occur as a result of the loss of descending control. Several studies have suggested an increased risk for developing type 2 diabetes among individuals with both traumatic and non-traumatic SCI [3-5]. Bauman and Spungen [6] reported that 62% of individuals with tetraplegia and 50% with paraplegia had abnormal oral glucose tolerance test, compared to only 18% in the able-

bodied-control group. Duckworth et al.[7] previously reported that approximately 50% of patients with chronic SCI had diabetes mellitus (DM) despite having normal fasting glucose levels. Those with SCI who were 45–59 years of age had a higher prevalence of DM than other age-matched veterans [5]. Additionally, Cragg et al. observed an increased likelihood of type 2 diabetes after appropriate adjustment for potential confounders in individuals with SCI [8]. A recent development of diabetes suggests that microcirculation may play an important role in the pathogenesis of diabetes [9].

Pancreatic-islet microcirculation is the basis of structure and function which makes a rapid response to glucose variations. Recent research has shown that pancreatic-islet microvascular vasomotion might be involved in the pathogenesis of diabetes [10]. During spinal shock, as for other types of shock regardless of the etiology, blood flow is redistributed [11]. However, microvascular vasomotion is the oscillation of vascular tone in vascular beds, regulating blood flow distribution [12]. Analyzing pathological changes of the functional status of pancreatic-islet microvascular vasomotion may be a feasible strategy leading to unveiling of the contribution that pancreatic-islet microcirculation makes to SCI. Therefore, the present study was aimed at evaluating the changes of pancreatic-islet microvascular vasomotion and the change of blood flow perfusion in SCI mice.

2 METHODS

2.1 Animals

Specific pathogen-free female C57BL/6 mice were purchased from the Center of Experimental Animals, Capital Medical University (Beijing, China). Mice were maintained in

an air-conditioned room with 12:12 light/dark cycles at 22 ± 2°C and 55 ± 10% relative humidity. Food and water were available ad libitum. Animal protocols followed guidelines established by the NIH, and were approved by the Animal Care and Use Committee of Capital Medical University.

2.2 Experimental design

Mice were randomized into the following four groups: sham group; 6H group (6 hours postinjury); 7D group (7 days postinjury); 14D group (14 days postinjury). Blood glucose was detected by one Touch UltraEasy glucometer at different time points of sham group and 14D group (n = 5). Glucose tolerance test was performed by D-glucose administration at sham group and 7D group (n = 5). Glucose tolerance test was performed by D-glucose administration at sham group and 14D group (n = 5). Functional status of pancreatic-islet microvascular vasomotion among four different groups was determined by laser Doppler monitoring (n = 5). Expressions of VEGF-A among four different groups was determined by immunohistochemistry (n = 3) and Western blotting (n = 3). Expressions of insulin and glucagon among four different groups were determined by immunohistochemistry (n = 3).

2.3 Induction of SCI

Mice were anesthetized with an intraperitoneal injection of phenobarbital sodium (40 mg/kg) and placed prone on a heating pad to maintain a constant body temperature. Mice received a moderate 100-kilodyne spinal contusion injury using the Infinite Horizons injury device. Briefly, a laminectomy was performed at the T10 level. The spinous processes of T8 and T11 were then clamped to stabilize the spine. After injury, bladders were voided manually at least

twice daily for the duration of the study. All surgical interventions and postoperative animal care were performed in accordance with the guidelines and policies for rodent survival surgery provided by the Experimental Animal Committee of Capital Medical University.

2.4 Blood glucose and intraperitoneal glucose tolerance test

Mice were fasted overnight for approximately 12 h. Blood glucose obtained from tail vein was detected by One Touch UltraEasy® glucometer (Lifescan, Johnson and Johnson, CA, USA) at 7 and 14 day post injury. To evaluate the function of islet β cells and their ability to regulate the glucose level, the intraperitoneal injection of D-glucose solution (2 g/kg body mass) was performed. Blood samples were collected from the tail vain before and immediately after glucose aiministration at 15, 30, 60 and 120 min for measurement of blood glucose. Area under curve (AUC) for glucose was calculated by the trapezoidal method using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA).

2.5 Assessment of pancreatic-islet microvascular vasomotion

Pancreatic-islet microvascular vasomotion was assessed by dual channel laser Doppler monitor (Moor - VMS - LDF2) instrument (Moor Instrument, Ltd., Axminster, UK) and Moor software for Windows version (Moor VMS - PCV3.1, Moor Instrument) as previously described [13]. Briefly, mice were examined after a 10 min acclimatization. After anesthetizing with 3 % pentobarbital sodium injected intraperitoneally, mice were placed in a supine position and incised around the upper abdomen to expose pancreas. The electrode was approached to the pancreas within 1 mm to collect data of functional status of pancreatic-islet microcirculation. The probes were repositioned after each run to avoid additive effects and

localized exhaustion of contractive and relaxative capacity. Changes in perfusion unit (PU) of parameters on pancreatic-islet microvascular vasomotion were evaluated. The frequency was defined as numbers occurred in microvascular vasomotion wave per minute. The amplitude was calculate as the difference between maximum PU and minimum PU (∆PU).

2.6 Immunohistochemistry

Immunostaining for insulin, glucagon and VEGF-A was performed using Polink-2 plus polymer HRP detection system (Zhongshan Golden Bridge Biotechnology, Beijing, China). Pancreas tissue was immediately fixed in 4 % paraformaldehyde, infiltrated and embedded in paraffin. Deparaffinated sections were treated with 3 % hydrogen peroxide to inhibit endogenous peroxidase, and then blocked with 4 % bovine serum albumin (BSA, TBD science technology, Co., Tianjin, China). After blocking, the sections were incubated overnight at 4 °C with primary antibodies against glucagon, VEGF-A and insulin (1:100, Abcam, Cambridge, MA, USA). After washing with PBS containing 0.1 % Tween-20 for three times, the sections were incubated with anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer respectively (Zhongshan Golden Bridge). Quantitative analysis was accomplished with Image Pro Plus 7.0 (Media Cybernetics, Silver Spring, MD, USA).

2.7 Western blotting analysis

To detect the expressions of VEGF-A, pancreas tissue was suspended in lysis buffer containing

a protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). All samples were sonicated for seconds. The protein content of the supernatant was determined using a protein assay kit (BCA, Pierce, Rockford, IN, USA). Proteins were heated at 95°C for 10 min in loading buffer. Equal amounts of total protein (50 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat skim milk in Tris-buffered saline solution with 0.05% Tween-20 (TBST) for 1 hour, and then incubated with antibodies against VEGF-A (1:100, Abcam) at 4°C overnight. After washing 3 times with TBST, the primary antibodies were detected with the appropriate horseradish peroxidase-conjugated secondary antibodies, and β-actin (1:1000, Cell Signaling Technology) was used as an internal control. The bands were visualized using enhanced chemiluminescence, and images were acquired with ChemiDoc MP System (Bio-Rad, Hercules, CA, USA). The relative band intensities were quantified using Quantity One (Bio-Rad, Hercules, CA, USA).

2.8 Statistical analysis

Statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) statistical package. Measurements were presented as means ± standard deviation. Comparisons were carried out by independent-sample *t*-test and pearson's correlation. A P value under 0.05 was considered statistically significant.

3 RESULTS

3.1 Body weight, blood glucose and intraperitoneal glucose tolerance

Body weight was measured and compared according to the time course in sham group (n = 5) and SCI group (n = 5). The mean body weight of the sham group was higher than that of the SCI group, and there was significant difference in body weight at all time points after injury ($p < 0.05$) (Figure 1A). Basso mouse scale (BMS) scores were performed to assess hind limb locomotor function, the mean score for the SCI group at 14 day postinjury was 2.63 ± 0.13, which was significantly higher than that for the sham group (8.80 ± 0.20) (data not shown). Blood glucose level of mice increased at 1 h after laminectomy in the sham group, but decreased to normal levels at 4 h postinjury. Blood glucose levels of mice were significantly higher from 4 h to day 4 following SCI as compared to level of sham group ($p < 0.05$) (Figure 1B) (n = 5). Compared to the sham group (n = 5), SCI mice at day 7 and day 14 had significantly impaired glucose tolerance following glucose administration (Figure 1C and 1E). SCI mice had higher area under curve (AUC) values compared with those of the sham group (Figure 1D and 1F). The data showed that the pancreatic islet function of regulating glucose level was impaired after SCI.

3.2 Pancreatic-islet microvascular vasomotion dysfunction in SCI mice

Figure 2A showed the time-domain LD signal in which periodicity variation was observed over time in different groups (n = 5). Several characteristics parameters of pancreatic-islet microvascular vasomotion were evaluated between groups. In mice subjected to SCI, average blood perfusion of pancreatic-islet microcirculation was significantly decreased compared with that of the sham group (Figure 2B). The decrease was most prominent at early time points, 6 h to day 14 after injury ($p < 0.001$). Then, the blood flow was markedly up-regulated

at day 7 and day 14 when compared with the 6 hour group ($p < 0.001$, $p < 0.001$). Meanwhile, the amplitude (Figure 2C) between contraction and relaxation and frequency (Figure 2D) of pancreatic-islet micro-vessels in SCI mice were significantly decreased. The trend of amplitude and frequency at 7 day and 14 day following injury was similar to that of blood flow. Relative velocity of SCI 6 hour group showed a 46.33 % decrease compared with that of the sham group (Figure 2E). Compared with that of the 6 hour group, relative velocity at 7 day and 14 day following injury respectively increased 12.23% and 27.47% ($p > 0.05$, $p < 0.01$). The distribution pattern was compared using microvascular blood perfusion data derived from laser Doppler monitoring. Sham group and 14D group showed a higher blood perfusion, whereas 6H group and 7D group displayed a relative lower scale blood perfusion pattern (Figure 2F).This suggests that the function of pancreatic-islet microcirculation was impaired, rendering the regulation of blood glucose ineffective.

3.3 Alteration of Insulin, glucagon and VEGF-A expression following SCI

Insulin and glucagon are two important pancreatic hormones involved in glucose homeostasis. We detected the expressions of insulin and glucagon by applying immunostaining analysis. Figure 3A and 3B showed that the level of insulin was significantly decreased, whereas the level of glucagon was markedly increased at 6 h following SCI when compared with sham groups. Subsequently, the level of insulin gradually increased and the level of glucagon gradually decreased at day 7 and day 14 post-injury. Dysfunction of pancreatic islet microvascular vasomotion may result from the imbalance of insulin and glucagon secretion ($n = 3$). The level of VEGF-A expressed by islet β cells is critical for islet

vascularization during development and postnatally [14]. To explore whether VEGF-A was related to dysfunction of microvascular vasomotion, we detected the expression of VEGF-A by immunostaining analysis (n = 3) and western blotting (n = 3). Figure 3C and 3D showed that the level of VEGF-A was significantly decreased at 6 h following injury when compared with sham group ($p < 0.001$), then the level of VEGF-A was gradually increased at day 7 and day 14 post-injury when compared with 6h group ($p > 0.05$, $p < 0.01$). Next, the result of western blotting indicated that the expression of VEGF-A was remarkably decreased at 6h following injury when compared with sham group, and the expression of VEGF-A was increased at 7 day and 14 day post-injury when compared with 6 h group ($p > 0.05$, $p < 0.05$ (Figure 3E, 3F), which was consistent with the result of immunostaining analysis. Furthermore, significant positive correlations were found between the expression level of VEGF-A and pancreatic-islet microvascular vasomotion parameters (VEGF-A and blood perfusion, $R = 0.918$, $P < 0.01$; VEGF-A and amplitude, $R = 0.812$, $P < 0.01$; VEGF-A and frequency, $R = 0.843$, $P < 0.01$; VEGF-A and relative velocity, $R = 0.830$, $P < 0.01$) (Figure 3G-J). Significant negative correlations were found between the expression level of blood glucose and pancreatic-islet microvascular vasomotion parameters (blood glucose and blood perfusion, $R = -0.536$, $P < 0.05$; blood glucose and amplitude, $R = -0.539$, $P < 0.05$; blood glucose and frequency, $R = -0.682$, $P < 0.01$; blood glucose and relative velocity, $R = -0.248$, $P < 0.05$) (Figure 3K-N). Taken together, these data highlighted that VEGF-A level and blood glucose are related with pancreatic-islet microvascular vasomotion.

4 DISCUSSION

In the present study, our data demonstrated that blood glucose was abnormal, and glucose tolerance was impaired in SCI-induced mice. Simultaneously, the functional status of pancreatic-islet microvascular vasomotion was also impaired following injury. The expression level of blood glucose was negatively correlated with pancreatic-islet microvascular vasomotion. Therefore, impairment of pancreatic-islet microcirculation may be a crucial played in the secondary injury of hyperglycemia complication.

SCI disrupts the autonomic nervous system and results in a state of stress, which affects the homeostasis of blood glucose. Individuals with SCI are at high risk of developing glucose intolerance or insulin resistance compared to the able-bodied population due to the associated changes in body composition and lower physical activity levels after paralysis [15, 16]. Increased fat accumulation in the liver increases insulin resistance and allows increased gluconeogenesis and glucose export out of the liver adding to hyperglycemia which is a precursor of type II diabetes mellitus (DM) [17, 18]. Physical inactivity due to bed rest for as little as 7 days results in a significant reduction in insulin sensitivity in inactive muscles [19]. It has been proven that insulin is capable of regulating vasodilation of precapillary arterioles, which may lead to increase microvascular blood perfusion [20, 21]. Decreased frequency, amplitude, and relative velocity of pancreatic islet microvascular vasomotion may result in a deficiency of blood perfusion, which may be associated with microvascular insulin level. In a model of diabetic mice model, insulin administration revealed an improvement of pancreatic islet microvascular vasomotion, which may be associated with avoiding blood glucose fluctuations and persistent hyperglycemia [10]. In the current article, hyperglycemia may be one of the reasons leading pancreatic islet microvascular vasomotion dysfunction.

Our data demonstrated that injury induced the increase of blood glucose from 4 h to day 4. In order to respond to hyperglycemia, islet needs sufficient blood flow perfusion to avoid large fluctuations. Pancreatic-islet microcirculation is extremely important for maintaining the physiological function of pancreatic-islet. Microvascular vasomotion can be measured as an important parameter of microcirculation. The assessment of spontaneous oscillations is influenced by the tissue microarchitecture and spatial distribution of capillaries, arterioles and venules [22]. However, microvasculature of the pancreas is interconnected and homogeneous as an organic integrity, which regulates pancreatic-islet blood flow perfusion [23, 24]. Blood flow of pancreatic-islet significantly decreased at 6 h after injury, afterwards, blood flow showed a rise during the observation period of day 14. The deficiency of blood perfusion was accompanied with the decreased of frequency, amplitude and relative velocity of pancreatic-islet microvasculature. Subsequently, the functional parameters of microvascular vasomotion were gradually upregulated followed by progression of SCI, which was basically consistent with the change of blood flow of pancreatic-islet microvasculature. Collectively, alterations of pancreatic-islet microcirculation especially in the acute period of SCI may be a pathogenic mechanism underlying transient hyperglycemia.

The expression levels of VEGF-A were reduced during the acute period of SCI, then the reduction of VEGF-A levels were partially reversed followed secondary injury. VEGF-A produced by islet endocrine cells is a principal regulator of islet vascular development and vascular homeostasis [25, 26]. Then, VEGF-A signaling could modulate the formation of highly vascularized islets and the islet microenvironment [27]. The data of VEGF-A expression and pancreatic islet microcirculation suggested that the declined expression of

VEGF-A might have contributed to the loss of microcirculation. Correlation analysis showed that the expression of VEGF-A correlated with the functional parameters of pancreatic-islet microvascular vasomotion. Moreover, the lower level of VEGF-A in SCI-induced mice might be one of the possible explanations of microvascular vasomotion dysfunction.

It is well known that hyperglycemia is a common complication after brain or spinal cord injury [28, 29]. We demonstrated that blood glucose was increased and functional status of pancreatic-islet microvascular vasomotion was impaired in mice following injury. Failure of pancreatic-islet microvascular vasomotion might be involved in secondary injury. Therapeutic intervention targeting on pancreatic-islet microvascular vasomotion might be a new approach in SCI treatment.

CONFLICT OF INTEREST STATEMENT:

The authors have no conflict of interest to disclose.

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AUTHOR CONTRIBUTIONS:

DGY, YLJ and MML conceived the study, designed the experiments, analyzed the data and wrote the manuscript. YLJ and FB carried out the animal experiments and obtained the data. FB critically revised the manuscript for important intellectual content. DL performed

immunohistochemistry experiment. All authors have read and approved the final version of the manuscript.

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Figure legends

Figure 1 Comparison of glucose tolerance in mice of SCI group and sham group. (A) Body weight and (B) blood glucose in mice at different time post-injury in SCI group and sham group (n = 5). (C) In mice subjected to SCI at day 7, glucose tolerance was measured

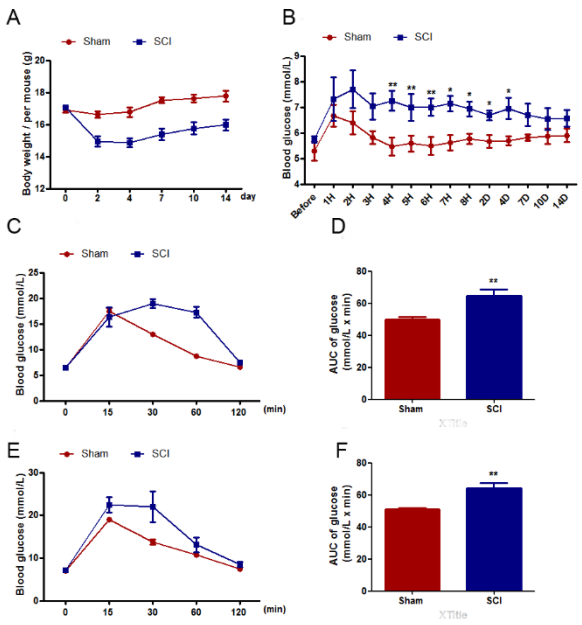
following glucose administration, and (D) area under the curve (AUC) was calculated to evaluate the glucose tolerance (n = 5). (E) In mice subjected to SCI at day 14, glucose tolerance was measured following glucose administration, and (F) AUC was calculated to evaluate the glucose tolerance (n = 5). The ***P<0.001 compared with sham group, **P<0.01 compared with sham group, *P<0.001 compared with sham group.

Figure 2 The functional status of pancreatic-islet microvascular vasomotion was assessed using laser Doppler perfusion monitoring. (A) A 15-s sample of the raw blood flow outputs from the combined probe among different groups (n = 5). (a: sham group; b: 6H group (6 hours postinjury); c: 7D group (7 days postinjury); d: 14D group (14 days postinjury)). Compared with sham group, SCI mice showed decrease functional parameters of hemodynamics including (B) average blood perfusion, (C) amplitude, (D) relative velocity and (E) frequency of pancreatic-islet microvascular vasomotion. (F) Distribution patterns of pancreatic islet microvascular blood perfusion among four groups. (Red dots: blood perfusion of Sham; Green dots: blood perfusion of 6H; Blue dots: blood perfusion of 7D; Purple dots: blood perfusion of 14D) ###P<0.001 compared with sham group, ##P<0.01 compared with sham group, #P<0.05 compared with sham group, ***P<0.001 compared with 6H group, **P<0.01 compared with 6H group, *P<0.05 compared with 6H group.

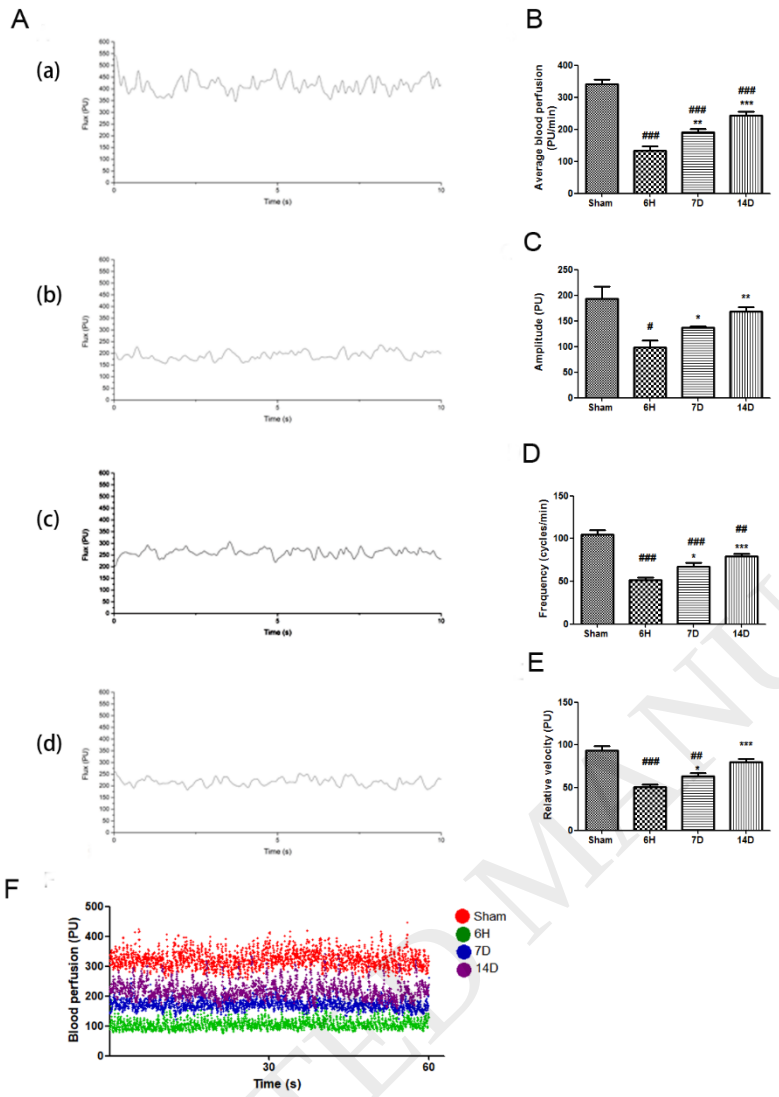
Figure 3 The expression of insulin, glucagon and VEGF-A in pancreatic islet was detected. (A-B) Insulin and glucagon were detected in pancreatic islet by immunostaining among four different groups. (n = 3/group) Scale bar = 50 μm. (C) VEGF-A was visualized by anti-VEGF-A immunohistochemistry in pancreatic-islet and was presented for each of the

different groups. (n = 3/group) Scale bar = 50 μm. (D) The MOD (mean optic density, MOD) of VEGF-A was quantified in all groups. (E-F) The expression of VEGF-A was detected by western blot, and the relative intensity of VEGF-A was analyzed in the different treatment groups. (n = 3) Positive correlations were found between VEGF-A expression and parameters of pancreatic-islet microvascular vasomotion. (G)VEGF-A and blood perfusion; (H) VEGF-A and amplitude; (I) VEGF-A and frequency; (J) VEGF-A and relative velocity. Negative correlations were found between blood glucose and parameters of pancreatic-islet microvascular vasomotion. (K) blood glucose and blood perfusion; (L) blood glucose and amplitude; (M) blood glucose and frequency; (N) blood glucose and relative velocity. ###P<0.001 compared with sham group, ##P<0.01 compared with sham group, #P<0.05 compared with sham group, **P<0.01 compared with 6H group, *P<0.05 compared with 6H group.

Figr-1



Figr-2



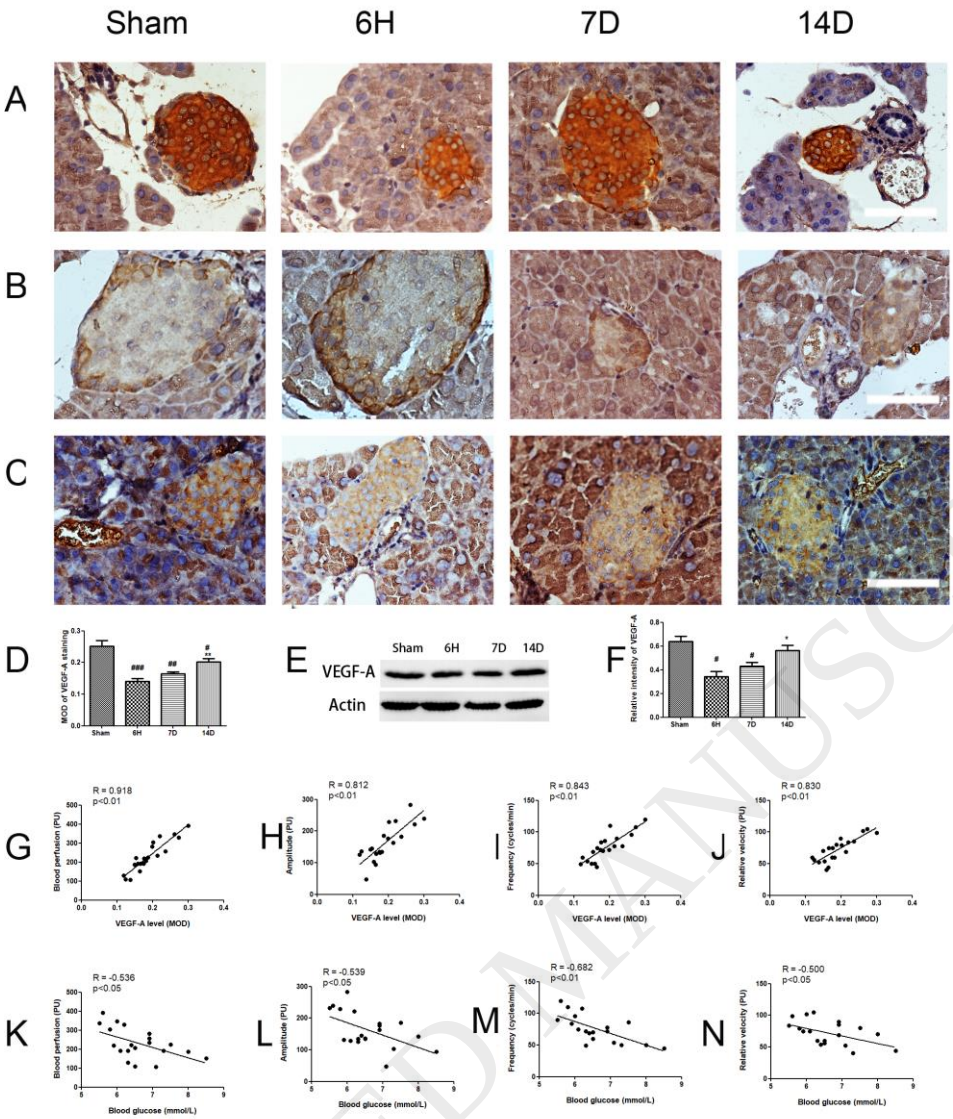


Figure 3