Original Research

Treatment and risk factor analysis of hypoglycemia in diabetic rhesus monkeys

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Abstract

In order to anticipate and promptly treat hypoglycemia in diabetic monkeys treated with insulin or other glucose-lowering drugs, the relationships between the incidence and symptoms of hypoglycemia in these animals, and many factors involved in model development and sustainment were analyzed. Different procedures were performed on 22 monkeys for the induction of diabetes. The monkey models were evaluated by blood glucose, insulin, C-peptide levels and intravenous glucose tolerance tests. A glucose treatment program for the diabetic monkeys was administered and laboratory tests were regularly performed. A standard procedure of hypoglycemia treatment was established and the risk factors of hypoglycemia were analyzed by a logistic regression model. Furthermore, the relationships between the four methods of diabetes induction, renal function, glycemic control and hypoglycemia were studied using one-way analysis of variance and *t*-test. We found that the hypoglycemic conditions of diabetic monkeys were improved rapidly by our treatment. The statistical analysis suggested that the modeling methods, renal function and glycemic control were related to the incidence of hypoglycemia. In detail, the progress of diabetes, effects of glycemic control and, particularly, the severity of the hypoglycemia differed according to the induction strategy used. The models induced by partial pancreatectomy with low-dose streptozotocin were not prone to hypoglycemia and their glycemic controls were stable. However, the models induced by total pancreatectomy were more vulnerable to severe hypoglycemia and their glycemic controls were the most unstable. Moreover, the levels of blood creatinine and triglyceride increased after the development of diabetes, which was related to the occurrence of hypoglycemia. In conclusion, we suggested that total pancreatectomy and renal impairment are two important risk factors for hypoglycemia in diabetic monkeys. More attention should be paid to daily care of diabetic monkeys, particularly monitoring and protecting their renal function.

Keywords: hypoglycemia, diabetic monkey, modeling method, renal function, glycemic control

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Introduction

Diabetes mellitus is associated with significant morbidity and mortality derived from long-term complications of chronic hyperglycemia. The Diabetes Control and Complications Trial[1] and the UK Prospective Diabetes Study[2] have clearly shown the benefits of intensive glycemic control for preventing or delaying the development and progression of long-term complications. However, intensive glycemic control, particularly when accompanied by insulin therapy, is associated with an increased incidence of hypoglycemia, which has been identified as the primary barrier to optimization of glycemic control in diabetes.[3]

Hypoglycemia is the result of the interplay of relative or absolute insulin excess and compromised physiological defenses against falling plasma glucose concentrations in type 1 and advanced type 2 diabetes.[3,4] During mild hypoglycemia, the patient experiences impaired motor function, confusion and inappropriate behavior, but is still aware enough to take action, whereas severe hypoglycemia may lead to disabling neurological impairment, coma, seizure

or even death.[3] Fortunately, >50% of hypoglycemia can be predicted based on risk analysis of self-monitored plasma glucose data over time.[5] Moreover, in the early stage of hypoglycemia, most patients may recover by self-treatment through timely supplements of food, sugar and so on.

Non-human primates (NHPs) are valuable models for preclinical investigation and new drug evaluation for diabetes.[6–8] In our previous studies, methods including pancreatectomy[9] and the systemic administration of various doses of streptozotocin (STZ)[10–12] were able to successfully induce diabetes in rhesus monkeys, and an available insulin strategy has been established for glycemic control. However, controlling the occurrence of hypoglycemia in diabetic monkeys has not been as successful as it has been in human patients. Because monkeys cannot get food apart from feeding time, when the blood glucose (BG) of the diabetic monkey drops slightly, the symptoms cannot be relieved through self-feeding and the condition may eventually develop into serious hypoglycemia or even death. Nevertheless, limited experiences on the prevention and treatment of hypoglycemia in NHP diabetic models have been reported. We hope that our experiences in this study will benefit researches on diabetic monkeys, and reduce their pain and illness according to the rules of animal welfare. We also hope that our study could provide an acceptable NHP model for studies related to the pathology and therapeutics of diabetes.

Materials and methods

Animals

Young (3–6 y) rhesus monkeys ($n = 22$) were selected for this study from Chengdu Ping'an Experimental Animal Reproduction Center (Sichuan, China). They were in generally good health and remained in individual cages with controlled temperature, humidity and 12 h cycles of light and darkness. All the animals had free access to water supply and were fed a primate diet twice a day. The animals were cared for in accordance with the guidelines of the Experimental Animal Center, Sichuan University, which have been approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Each subject had a history, recording the general condition (body weight, food intake, behavioral activity), a physical examination, a mental health evaluation, laboratory testing and an echo ophthalmoscopy every 1–2 months.

Development of diabetic models in rhesus monkeys

The monkeys were divided into four groups: group 1 ($n = 2$) subjects were given a total pancreatectomy, group 2 ($n = 4$) subjects were given a partial pancreatectomy (75%) with low-dose STZ (20 mg/kg) administration, group 3 ($n = 7$) subjects were given high-dose STZ (80 mg/kg) administration without pancreatectomy,[13] and group 4 ($n = 9$) subjects were given multiple low-dose STZ administration (25 mg/kg for 5 consecutive days, followed by 2 additional boosting injections given 1 week apart) without pancreatectomy. The model was considered to be successfully established if the fasting blood glucose (FBG) values remained >11.1 mmol/L for two consecutive days and the C-peptide (C-P) concentration was <0.5 nmol/L. The four models were evaluated by the following parameters: (1) intravenous glucose tolerance tests (IVGTTs) (9); (2) radioimmunoassay to analyze insulin and C-P levels (Beijing North Institute of Biological Technology, Beijing, China); (3) biopsies of the liver, kidneys and pancreas; and (4) non-contrast-enhanced abdominal spiral computed tomography scans.[9]

FBG-fasting (12 h) and postprandial blood glucose (PBG-after eating 2 h) levels were monitored twice weekly. To maintain the BG at normal levels, the diabetic monkeys were administered with porcine insulin (Wanbang Biopharma Co Ltd, Xuzhou, China) that included a combination of protamine zinc insulin and insulin.[9] Different types and doses of insulin were administered twice daily (at 09:00 and 16:30) before feeding, and the dose of insulin was adjusted according to the FBG and PBG levels. The glycosylated hemoglobin (HbA_{1c}) levels of the diabetic monkeys were tested regularly.

Hypoglycemia management

When certain symptoms of hypoglycemia, such as debilitation, irritability, flush or pallor, weakness, confusion, blurred vision, seizures and coma, were observed, and its BG was less than 2.8 mmol/L, the diabetic monkey was primarily diagnosed with hypoglycemia. If the symptoms improved rapidly after glucose was given, the diagnosis was confirmed.

The following steps outline the hypoglycemia treatment:

(1) *Emergency treatment*: Those who could take food were often managed with an oral dose of 50% glucose (10–30 mL) or other carbohydrate food. For those who could not take medication orally, 50% glucose was given rectally or intravenously (1–4 mL/kg), followed by the continuous intravenous infusion of a 5% glucose-salt solution. Glucagon (1 mg) was sometimes helpful in monkeys who did not respond to intravenous glucose.
(2) *Symptomatic treatment*: A ketamine hydrochloride (15 mg/kg) intramuscular injection, combined with Sumianxinzhusheye II (0.05 mL/kg) (Military Veterinary Institute, Changchun, China), was used to calm the monkeys. In severe conditions, either hydrocortisone or dexamethasone was given intravenously, with timely rehydration and nutritional support.
(3) *Prevention*: controlling and reducing the possible risk factors of hypoglycemia, monitoring the BG more intensively, adjusting the dosage of exogenous insulin according to the BG level, and providing more care when the consumption of fruit is increased, may all possibly aid in the prevention of hypoglycemia.

The hypoglycemia treatments were administered according to standard clinical procedures, with some adjustments based on our own experiences with diabetic monkeys.

Statistical analysis

Statistical analyses were conducted by SPSS. Primary analyses were designed to filter out the risk factors of hypoglycemia. Logistic regression analysis, adopting forward (conditional) methods, was carried out with gender, body weight, the methods of modeling, diabetes duration, exercise, mental health, and the amount of exogenous insulin, HbA1c, blood creatine (Crea) and triglyceride as independent variables. With regard to C-P, FBG, exogenous insulin dose and HbA1c, one-way analyses of variance (ANOVAs) were performed among the four groups. Furthermore, the *t*-test was used to identify the differences between those with hypoglycemia and those without hypoglycemia within the group. The different levels of Crea and triglyceride at six time points (0, 1, 3, 5, 7 and 9 months after developing diabetes) were compared using the descriptive statistics of the one-way ANOVAs.

Results

Evaluations of the diabetic models

All the monkeys in this study were successfully induced to diabetes, and their insulin treatment was able to maintain their BG near normal levels. There was no significant difference among the four groups in terms of FBG, exogenous insulin dosage and HbA1c ($P > 0.05$), but the C-P levels were different (Figure 1a). The C-P levels in groups 1 and 3 were much lower than those in the other two groups ($P < 0.001$). The IVGTT results showed that groups 2 and 4 had a slight response for glucose stimulation, and the insulin secretion function of the islet β-cells of group 2 was more effective than that of groups 3 and 4 (Figure 1b).

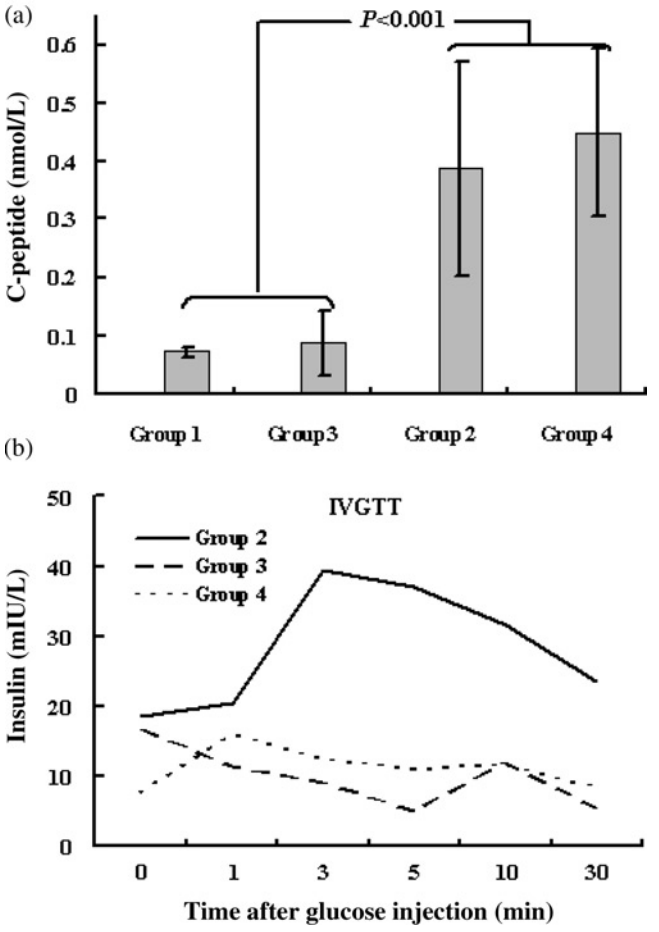

Figure 1 Evaluation of beta-cell function in diabetic monkeys. (a) The average level of serous C-peptide (mean [SD]). (b) The average insulin secretion levels in the IVGTT. The monkeys of group 1 were unstable after surgery; thus, there were no data for IVGTT. IVGTT, intravenous glucose tolerance test

Symptoms and treatments of hypoglycemia in diabetic monkeys

For diabetic monkeys with hypoglycemia, mild hypoglycemia was often accompanied by autonomic nervous system symptoms (flush, debilitation, irritability, lack of coordination and blurred vision), and the BG levels were 1.0–2.8 mmol/L. Severe hypoglycemia was often characterized by severe autonomic nervous system symptoms and neurophysiological dysfunction (disturbance of consciousness, convulsions and coma), and the BG levels were <1.0 mmol/L. After our treatment, a satisfactory effect was soon observed in mild hypoglycemia. The symptoms disappeared after 5–10 min and the BG returned to normal. The treatment also had some effect on severe hypoglycemia. In most cases, the symptoms improved after one hour and the monkey was nearly returned to normal after 24 h. For hypoglycemia that occurred at night, the results were worse, because the condition was hard to discover and diagnose in time. Two monkeys appeared with blurred vision, coma and other serious symptoms, but their BG levels were too low to be measured by a glucometer. We tried our best to rescue them, but they eventually died.

Analysis of the risk factors of hypoglycemia in diabetic monkeys

A logistic regression analysis was applied to 10 factors that were possibly relative to hypoglycemia. *B* and OR represent the regression coefficient and odds ratio, respectively. It has been suggested that the modeling methods and blood creatinine level (Crea) had significant relationships with hypoglycemia: the method ($B = 1.891$, OR = 6.629, $P = 0.026$) and Crea ($B = 0.053$, OR = 1.054, $P = 0.036$). When we further relaxed the criteria, HbA1c ($B = -0.998$, OR = 0.369, $P = 0.144$) was identified as another important risk factor. The differences in seven other factors showed no statistical significance ($P > 0.2$).

Relationship between modeling method and hypoglycemia

In the statistical analysis, we considered one day's observation of one monkey as a unit (1 monkey-day). In 4851 monkey-days, hypoglycemia was observed 27 times, and the average probability of hypoglycemia occurrence was 0.56 (standardized to per 100 monkey-days). Differences were observed in hypoglycemia severity and probability among the four groups with different diabetes induction

Table 1 Hypoglycemic (hypo) status of the four groups

Groups (monkey-days)	Group 1 (303)	Group 2 (827)		Group 3 (1726)		Group 4 (1995)	
	Hypo	Hypo	No-hypo	Hypo	No-hypo	Hypo	No-hypo
Number (*n*)	2	1	3	4	3	6	3
Mild hypo (time)	0	1	\	6	\	12	\
Severe hypo (time)	3	0	\	1	\	4	\
Hypo-probability* (per 100 monkey-days)	0.99	0.12		0.41		0.80	

*The hypo-probability is equal to the total hypo-times/cumulative time (100 monkey-days). The observed cumulative time was calculated from the day when the diabetes mellitus model was successfully developed

strategies (Table 1). In the accumulated 303 monkey-days, the animals of group 1 appeared three times with severe hypoglycemia, and eventually died. In contrast, severe hypoglycemia did not occur in group 2, and its probability ratio was the lowest (0.12) of the four groups. Meanwhile, in groups 3 and 4, the probability ratios were close to the average level (0.41 and 0.8, respectively), and severe hypoglycemia had occurred in both groups with no deaths.

Relationship between creatinine with hypoglycemia

Crea values, tested every two months, were divided into four classes (<40, 40–80, 80–120 and >120 μmol/L) to compare the hypoglycemia probabilities under different Crea levels (Table 2). Table 2 shows that the hypoglycemic probability was increased by increasing the Crea level (Wilcoxon test, $P = 0.014$). In each two-month monitoring period, when the Crea level was lower than 80 μmol/L, the hypoglycemic probability was less than 0.2. However, when the Crea level was above 120 μmol/L, the hypoglycemic probability was more than 0.4. When the Crea levels were compared among the groups, we found that the Crea levels in the four groups all constantly increased (Figure 2a). Compared with the initial few months, the Crea levels of the monkeys were significantly higher after 5–9 months of being diabetic (Figure 2b). However, there was no significant difference between before induction and 1–3 months after being diabetic ($P > 0.05$). Moreover, a high triglyceride level is an independent predictive factor of renal complications in patients with type 1 diabetes.[14] In this study, the concentrations of triglyceride in all monkeys maintained high levels after the monkeys became diabetic, significantly exceeding the normal level (Figure 2b).

Other risk factors of hypoglycemia

Since the fluctuations of BG in the four groups were quite different, we introduced the coefficient of variation (CV) to do further statistical analysis. Among the four groups, the CV of group 1 was the highest and the CV of group 2 was the lowest (Figure 3), which is consistent with the probability of hypoglycemia in these groups. Consistently, in the same group, the CV of BG in monkeys with hypoglycemia was higher than that in monkeys without hypoglycemia (Figure 3).

Table 2 Hypoglycemic probability under different blood creatinine levels in each two-month monitoring period

Crea (μmol/L)	<40	40–80	80–120	>120
Total number of monkeys (*n*1)	14	40	18	10
Number of hypo-Crea monkeys (*n*2)	1	6	6	4
Hypoglycemic probability (*n*2/*n*1)	0.071	0.150	0.333	0.400

Crea values, tested once every two months, were divided into four classes (<40, 40–80, 80–120 and >120). The number of monkeys who have had hypoglycemic events in the monitoring period is indicated as *n*2. *n*2/*n*1 is calculated to observe the hypoglycemic probability under different Crea in each two-month monitoring period

Discussion

The present study successfully developed diabetic models in rhesus monkeys through four methods. They are acceptable NHP models for studies related to progression of diabetes and its control. The results suggested that the model induced by partial pancreatectomy with low-dose STZ treatment, with low probabilities of hypoglycemia and stable glycemic controls, was better than others. Focusing on hypoglycemia, we first described the features of hypoglycemia in diabetic monkeys, and then reported a practical strategy for its treatment and prevention. Moreover, we analyzed the important risk factors of hypoglycemia in diabetic monkeys, suggesting that modeling methods and blood creatine level had some relationship with the probability of hypoglycemia, to which more attention should be paid in the research on diabetic monkeys.

Hypoglycemia management

In cases of mild hypoglycemia, most patients may soon recover through self-treatment. For younger and older diabetic patients, however, with a lack of sufficient awareness and capability of self-care, the symptoms will become very serious if they are not promptly treated, which is similar in diabetic monkeys. The condition of monkeys suffering from hypoglycemia is generally more serious, as it is often accompanied by severe convulsions. So, intensive care is needed in tending to these animals. Hypoglycemia is usually treated with oral or intravenous glucose in clinical practice. In cases of severe hypoglycemia, rapid treatment is required. If monkeys did not respond to intravenous 50% glucose, glucagons (1 mg) should be given immediately. At the same time as sugar is being administered, other complications, especially convulsions, should be treated to avoid new damage. In contrast with human patients, the prompt discovery and treatment of diabetic monkeys at the early stage of hypoglycemia are more difficult, so more attention needs to be paid to daily care. For example, housing such diabetic monkeys in a room with round the clock video monitoring system may be an

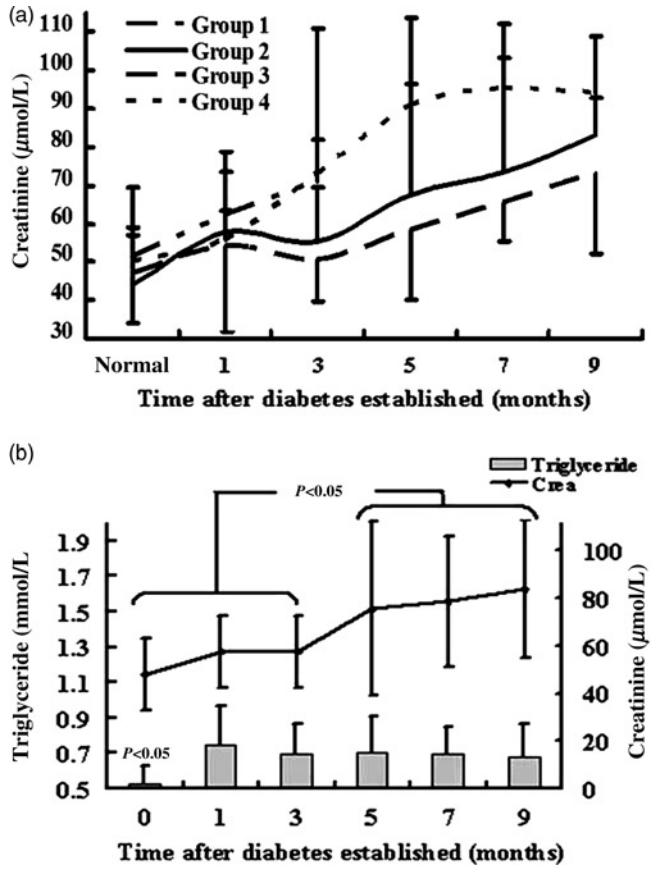

Figure 2 Changes in the biochemical parameters of renal function in diabetic monkeys. (a) The trend graph of creatinine level changes in the four groups. (b) Creatinine and triglyceride levels were changed after diabetes was induced. The data are presented as mean (SD) of all surviving monkeys

acceptable way to prevent acute crisis, particularly in the night. Prevention of the illness' risk factors is another important issue. In our experience, controlling the FBG at 5–10 mmol/L and the HbA1c at 3–6.5% to keep the glycemic control stable, combined with improving the renal function, may be helpful. For the diabetic monkeys with hypoglycemia in our experiment, our treatment programs proved to be effective to reduce permanent damage and mortality. We hope our experiences will not only provide a reference for animal experimenters but will also benefit the prevention and treatment of hypoglycemia in children and aged patients.

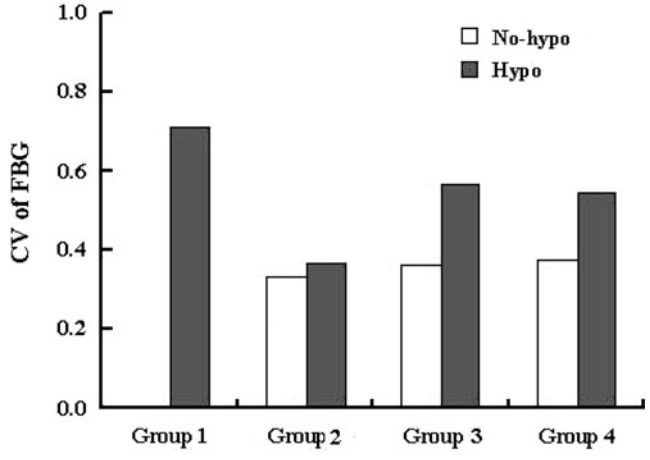

Figure 3 The coefficient of variation (CV) of FBG in the hypoglycemic and non-hypoglycemic diabetes mellitus monkeys. The CV of FBG is equal to SD/mean value. FBG, fasting blood glucose

Risk factors of hypoglycemia

Clinical studies[3,4] have indicated that the risk factors of hypoglycemia include: (1) endogenous insulin deficiency; (2) a history of hypoglycemia, hypoglycemia unawareness or both; (3) aggressive glycemic therapy *per se*, as evidenced by lower glycemic goals, lower HbA1c levels or both; (4) recent moderate or intensive exercise; (5) sleep; and (6) renal failure. Because hypoglycemia is a major cause of death in diabetic monkeys, and there are no reports yet about the prevention and treatment of monkey hypoglycemia, we comprehensively analyzed the risk factors of hypoglycemia in diabetic monkeys. In this study, we found that the modeling method and the blood creatinine level had the closest relationships with the occurrence of hypoglycemia ($P < 0.05$). Moreover, to identify more possible risk factors, we broadened the criteria of the logistic regression analysis ($P < 0.2$) and found that HbA1c was another important factor, with an OR of 0.369 ($P = 0.144$). These results suggest that we should do more work to improve the modeling approach, renal function and glycemic control in diabetic monkeys to avoid hypoglycemia.

Modeling method of diabetes

In previous studies,[9–12] STZ administration has been a classic method to induce type 1 diabetes in rodents, whereas it is still unclear what the best method is for NHPs. In our study, we compared four modeling methods. With regard to total pancreatectomy,[9] more than 90% of the pancreas was resected, resulting in exocrine pancreatic deficiency, which damaged the animal. Partial pancreatectomy[9] prevented injury from important blood vessels and the bile duct system. A single large dose of STZ helped to avoid complicated surgery. However, its damage to the liver and kidneys was shown to be severe.[15] Low doses of STZ, taken intravenously, helped to avoid the severe side-effects caused by large doses. The different modeling methods of diabetes led to different characterizations in the pathological mechanisms, process and complications of the disease. In particular, the severities of metabolic and hormonal abnormalities varied in the individual monkeys and the groups of induction methods, as well as between species.[10] Focusing on hypoglycemia, the four kinds of modeling methods have led to different probabilities: (1) total pancreatectomy resulted in the lowest level of C-P and the most unstable glycemic control, due to the complete deficiency of both the endocrine and exocrine functions of the pancreas. In addition, obvious increases in Crea were found in this group. These risk factors may contribute to the highest probability and the most severe symptoms and results of hypoglycemia; (2) STZ injection after partial pancreatectomy displayed the best islet function, the most stable glycemic control and slow changes in

Crea, so the lowest hypoglycemia probability and mildest symptoms were observed; (3) the diabetes models induced by low-dose STZ, compared with high-dose STZ, have higher levels of C-P and a better islet function, but their highest increase in Crea of the four groups may contribute to their higher frequency and severity of hypoglycemia. Though high-dose STZ resulted in a lower probability of hypoglycemia, the greater liver or kidney toxicity resulted from this treatment can seriously endanger the lives of the animals.[13]

Renal function

In our research, we found that the increase of blood creatinine levels in diabetic monkeys is an important risk factor of hypoglycemia. The levels of creatinine remained normal even after induction of diabetes, but it increased slowly along with the progression of diabetes. STZ is known to be toxic to the kidneys; however, due to its short half-life, it is unlikely to cause long-term renal toxicity. Previous studies have reported that animals given high-dose STZ developed marked steatosis of the liver and renal tubular injury, whereas normal liver and kidney histology were evident in NHPs given a lower dose of STZ.[10,14] Therefore, except for group 3, which was given a high dose of STZ, the increased Crea in the other three groups could not be explained by the STZ administration. On the other hand, continuous hyperglycemia and hyperlipidemia are risk factors for the development and progression of diabetic nephropathy. Moreover, high triglyceride level is an independent predictive factor of renal complications in patients with type 1 diabetes.[16] In our study, we can see elevated Crea accompanied by increased triglyceride levels in every group. Moreover, vascular endothelial function was reduced and abnormal mental symptoms appeared in some of the diabetic monkeys. The above evidences suggest that ongoing diabetes leads to gradually decreased renal function. Therefore, it is reasonable to conclude that the decline in renal function may limit the clearance rate of exogenous insulin, indirectly leading to elevated level of blood insulin, resulting in hypoglycemia in diabetic monkeys. In order to alleviate renal injury in diabetic monkeys and avoid the occurrence of iatrogenic hypoglycemia, we should take certain measures,[17,18] including (1) intensive glycemic control, targeting the HbA1c level at less than 6.5%; (2) restriction of dietary protein and fat intake, and increase of fruit; and (3) when necessary, the use of certain pharmacological therapies.

However, due to the fact that the sample size and observation period were limited, and that individual differences are more apparent in NHPs, our study is merely preliminary. Further investigations are being conducted in an increasing number of diabetic monkeys in order to obtain more knowledge about diabetes in NHPs.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. SH, YC, LW, XJ, LZ and YL conducted the experiments; YR conducted glycemic control; JZ performed pathological analysis; HL and LW supplied critical animals; and SH, YC, YL and JC wrote the manuscript. SH and YC contributed equally to this work and are co-first authors.

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