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Brief communication (Original)

Immune injury in rat models of type 2 diabetes mellitus

Xiu-lan Zou, Jun Yang, Jian-ming Yang, Yan-qun Liu, Wen Wang, Jun Zheng, Bao-hua Ma, Fang-yuan Chen
People's Hospital of Three Gorges University, Department of endocrinology, the First People's Hospital of Yichang, Yichang 443000, China

Background: Diabetic cardiomyopathy is an important complication of type 2 diabetics. The role of immuno-inflammation, immunity, and diet has not been adequately clarified.
Objective: We investigated the relationships between diabetic cardiomyopathy and immuno-inflammation, as well as immunity and diets.
Methods: Sixty Sprague-Dawley (SD) male rats were included in this study, from which 12 were randomly selected as the normal control group (group A) and of which the remaining 48 were considered as the type 2 diabetes mellitus (T2DM) model group. Group A was fed with common diets and the T2DM model group, with high-glucose diets (by adding 20% cane sugar, 10% lard, and 2.5% cholesterol into a 67.5% common diet). After 4-week feeding, the T2DM model group was randomly allocated into three groups according to the diet, high-fat diet group (group B), common diet group (group C), and low-fat diet group (group D). All the three groups were then fed for another 10 weeks. At the end of the experiment, body weight, random blood glucose levels, and cardiac weight were measured. Left ventricular tissue was obtained for light microscopy and electron microscopy. Deposits of immunoglobulin G (IgG) in myocardium were identified by immunohistochemistry. Serum levels of high-sensitivity C-reactive protein (hs-CRP) were determined using the enzyme-linked immunosorbent assay (ELISA). All data were statistically analyzed.
Results: The serum level of hs-CRP was significantly higher in groups B, C, and D, than in the control group. Therefore, IgG deposits among cardiac muscle cells were observed in all the model groups, significant deviations were noted in group A (p <0.01) and IgG deposits were less in group D than in groups B and C (p <0.01).
Conclusion: Immuno-inflammation participates in the development of T2DM and diabetic cardimyopathy. Immune injury can be alleviated following dietary interference.

Keywords: Diabetic cardiomyopathy, diet, high-sensitivity C-reactive protein, immunity, type 2 diabetes mellitus

Diabetic cardiopathy is a major serious and chronic complication of type 2 diabetes mellitus (T2DM), including cardiac macroangiopathy, microangiopathy, cardiomyopathy, and vegetative nerve functional disturbance. Diabetic cardiomyopathy (DCM) is an independent and specific myocardial disease closely related to the high incidence and mortality of heart failure in patients with diabetes. Numerous studies have shown that the incidence of T2DM and DCM is related to multiple factors. In this study, the role of immunity in the occurrence and development of T2DM and DCM was investigated.

Correspondence to: Xiu-lan Zou**,** People's Hospital of Three Gorges University, Department of endocrinology, the First People's Hospital of Yichang, Yichang 443000, China. E-mail: zouxl61@126.com

Methods

Animals

This study was approved by the Animal Ethical Review Board of Tongji Medical University, China. Sixty male Sprague-Dawley (SD) rats, of clean grade, weighing 180 to 200 g (purchased from the Experimental Animal Center of Tongji Medical University) were raised in a clean laboratory animal room at room temperature (18 to 24°C) and given 12 hours illumination daily in a diurnal cycle of 12 hours of light alternating with 12 hours of darkness (12L:12D). Rats were free to eat or drink during the experiment.

Reproduction of animal models

Twelve rats were randomly selected from 60 rats as the normal control group (group A) and the remaining 48 rats were considered as the T2DM model group. Group A was fed with common diets

and the T2DM model group, with high-glucose and high-fat diets (by adding 2.5% cholesterol, 20% cane sugar, and 10% lard into a common diet). After 4-week feeding, rats in the model group were given intraperitoneal injection of 30 mg/kg streptozotocin (STZ, prepared with 0.1 mol/L citric acid-sodium citrate buffer, pH = 4.2) and one week after that, rats with random blood glucose levels greater than 16.7 mol/L and accompanied by reduced insulin sensitivity were selected as animal models of T2DM.

Dietary intervention

Rats with T2DM were randomly assigned into three groups based on the diet: high-fat group (group B), normal diet group (group C), and low-fat diet group (group D). Group A: fed with common diets and free to drink water during the experiment; group B: fed with high-fat diets and free to drink water during the experiment; group C: fed with common diets and free to drink water during the experiment; group D: fed with low-fat diets and free to drink water during the experiment.

Collection of specimens

At the end of the fourth week of the experiment, body weight of rats was measured orbital venous sampling was performed to determine fasting blood glucose (FBG), fasting insulin (FINS) and blood lipid levels. At the end of the fifteenth week, blood specimens were obtained via the cardiac aorta under anesthesia induced by intraperitoneal injection of 2.5% sodium pentobarbital (2.5 ml/kg) to determine serum indices. Then open the chest cavity, rapidly obtain the heart, wash it with brine ice, and dry and weigh the heart. Obtain a portion of left ventricular tissue and fix it in 10% neutral formaldehyde for observation under a light microscope and detection by immunohistochemistry; obtain another portion and fix it in 2.5% glutaraldehyde for observation under an electron microscope.

Contents of observation and detection of indices

Statuses of observation models

Statuses of observation models included the mental state, water intake, urine output, hair, and hair quality, etc.

Determination of biochemical indicators

- Determination of FBG: FBG was determined by the glucose oxidase method.

- Determination of the cardiac index: Rapidly remove the heart, wash it with brine ice and weigh the heart; measure the heart weight index, namely, cardiac index. (Cardiac index = heart weight mg/body weight).

- Determination of FINS: FINS was determined by the double antibody radioimmunoassay.

- Insulin sensitivity index (ISI): ISI was calculated using the formula: ISI = 1/ (FBG × FINS).

- Determination of serum triglyceride (TG) and total cholesterol (TC): Serum TG and TC were determined using the automatic biochemical analyzer.

Determination of serum indices

Serum levels of high-sensitivity C-reactive protein (hs-CRP) were determined using the enzyme-linked immunosorbent assay (ELISA). Reagents were purchased from the Shanghai Westang Bio-tech Co., Ltd.

Statistical analysis all data were analyzed using SPSS statistical software and expressed as mean ± standard deviation ($\bar{x} \pm s$). Inter-group differences were compared by one-way analysis of variance (SNK method) and the correlation between two variables was determined by bivariate correlation analysis (Pearson method), $p < 0.05$ considered statistically significant.

Results

General conditions of laboratory animals

Models of T2DM were established in the 48 laboratory rats. One rat died of hyperglycemia during the process of modeling and two rats in group D died of hyperglycemia. At the end of the experiment, the number of rats was 12, 16, 15, and 14 respectively in groups A, B, C, and D. Rats in the model group developed obviously increased urine output and food intake three days after injection of STZ, which were about three of four times compared to those prior to injection, in addition to significantly increased appetite and gradual body weight loss. Rats in the control groups had smooth hair and those in the model groups developed brick red hair following injection of STZ, which spontaneously recovered, but their hair quality was drier and more yellow after spontaneous recovery, compared to hair quality of rats in group A.

Changes of different indices during the process of modeling

After 4-week feeding with different diets, rats in

the T2DM groups had greater body weight and FBG levels than group A (p <0.05) and higher levels of FINS, TG, and TC than group A (p <0.01). One week after injection of STZ (namely, the fifth week since the initiation of the experiment), the level of random blood glucose (20.18 ± 3.88) was still higher than that in group A (3.98 ± 0.65), suggesting that the models of T2DM were successfully reproduced as shown in **Table 1**.

Changes in serum levels of hs-CRP in rats of different groups

At the end of the experiment 15 weeks later, the serum levels of hs-CRP in rats of the model group (groups B and C) were significantly higher than that of rats in group A (p <0.01). The serum level of hs-CRP in rats of group C was lower than that in rats of group B, but the difference had no statistical significance. The serum level of hs-CRP was significantly decreased in group D (p <0.01) (**Table 2**).

Changes in myocardial histopathology of rats in different groups

Light microscopy: rats in group A had a regular arrangement of cardiac muscle fibers, evenly colored cytoplasm and normal nuclear morphology, without widening or narrowing of intercellular spaces; rats in group B had an irregular arrangement of cardiac muscle fibers and intercellular fat tissue; rats in group C had a loose arrangement of cardiac muscle fibers and widening of intercellular spaces; rats in group D had a basically normal arrangement of cardiac muscle cells (**Figure 1**).

Electron microscopy: Rats in group A had a regular distribution for myotome of cardiac muscle cells, regular mitochondrial structure and clear cristae; rats in group B had degenerative necrosis mainly in cardiac muscle fibers, loss and an irregular arrangement of myofilament fibers, and mitochondrial swelling and dilation; rats in group C had a loose structure of cardiac muscle fibers and unobvious mitochondrial swelling; rats in group D had a compact structure and a regular arrangement of cardiac muscle fibers and a normal structure of mitochondria (**Figure 2**).

Immunohistochemical detection of immunoglobulin G (IgG) deposits

There were no immunoglobulin deposits among cardiac muscle cells in rats of group A. IgG deposits were observed among cardiac muscle cells in rats of the T2DM group, which were significantly different from those in rats of group A (p <0.01); compared with groups B, C, and D had less IgG deposition and the differences were significant (p <0.01); compared with groups C and D had less IgG deposition and the difference was significant (p <0.01) as can be seen in **Table 3** and **Figure 3**.

Table 1. Changes in rats' body weight, FBG, FINS and serum lipid levels after four weeks ($\bar{x}\pm s$)

Group/ Index	Quantity (n)	Body weight (g)	FBS (mmol/l)	FINS (mIU/L)	TG (mmol/l)	TC (mmol/l)
Group A	12	326.33 ± 20.97	5.24 ± 0.67	16.48 ± 5.16	0.73 ± 0.15	1.56 ± 0.27
T2DM	48	343.79 ± 27.49*	5.75 ± 1.26*	32.70 ± 8.10**	1.07 ± 0.21**	2.25 ± 0.46**

*p <0.05, **p <0.01

Table 2. Changes in serum levels of hs-CRP ($\bar{x}\pm s$)

Group/Index	Quantity (n)	hs-CRP (ng/ml)
Group A	12	708.18 ± 54.06
Group B	16	2264.32 ± 66.65**
Group C	15	2210.09 ± 51.74**
Group D	14	2194.17 ± 62.95**++

Compared with group A, ** p <0.01, compared with group B, ++ p <0.01

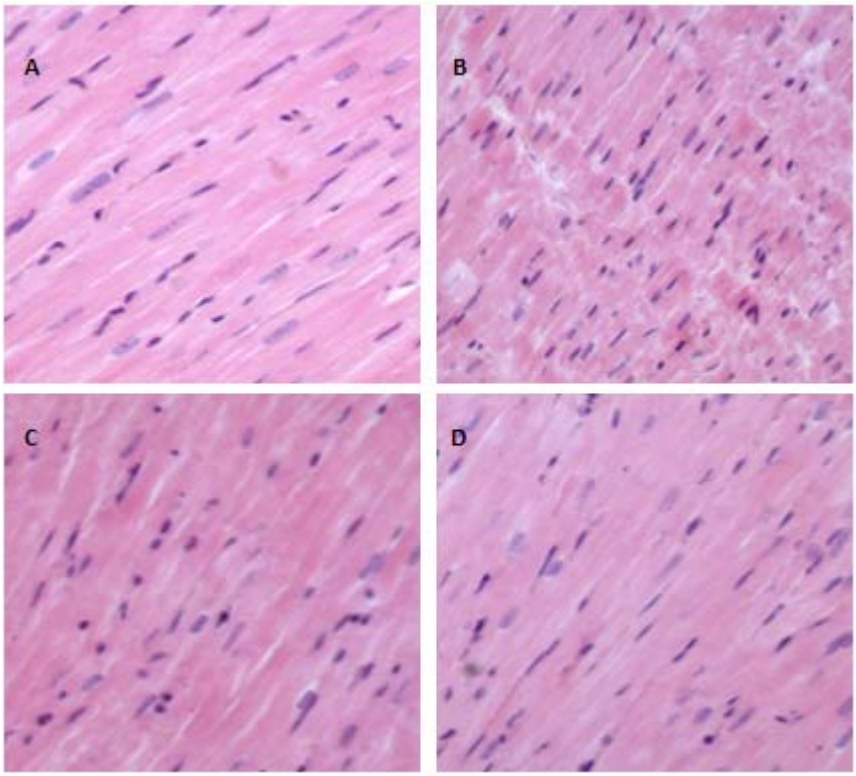

Figure 1. Myocardial HE staining of rats in four groups (A, B, C, and D) (200 ×).

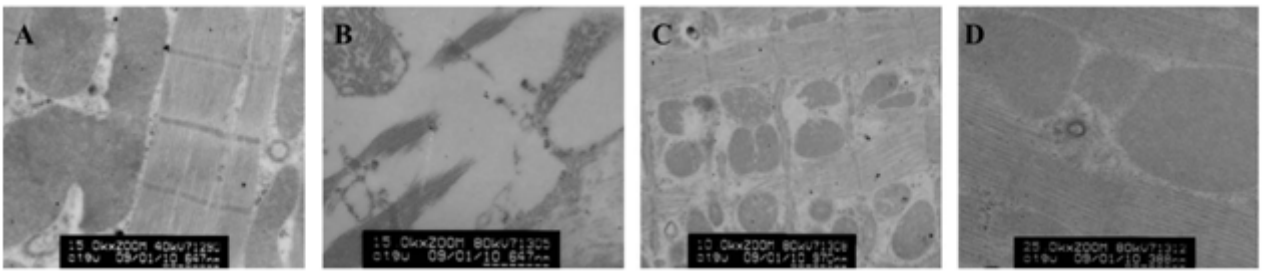

Figure 2. Myocardial electron microscopy of rats in group A (5 × 1000-fold), group B (15 × 1000-fold), group C (25 × 1000-fold), and D (10 × 1000-fold).

Table 3. Comparison of mean optical density for IgG-stained myocardial tissue sections ($\bar{x} \pm s$)

Group/Index	Number of rats	Integrated optical density (IOD)
Group A	12	2097.70 ± 180.75
Group B	16	$3936.45 \pm 353.14^{**}$
Group C	15	$3149.22 \pm 238.51^{**\Delta}$
Group D	14	$2730.04 \pm 280.58^{** \Delta\Delta ++}$

Compared with group A, $^{**}p < 0.01$, compared with group B, $^{\Delta\Delta} = p < 0.01$, compared with group C, $^{++}p < 0.01$

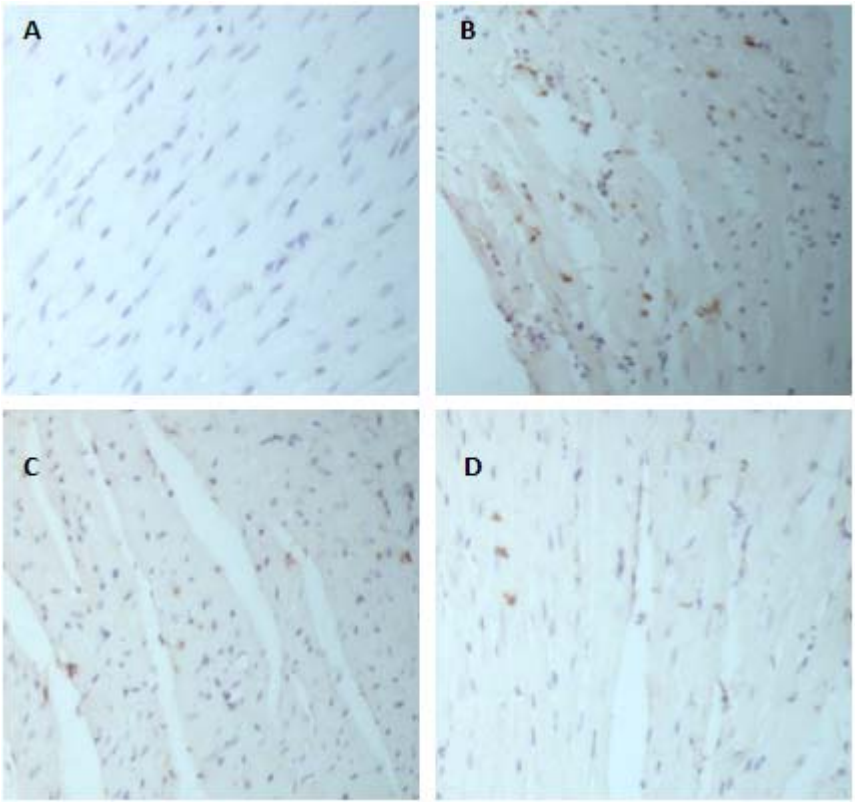

Figure 3. Myocardial IgG staining of rats in four groups (A, B, C, and D)

Discussion

Numerous studies have shown that the major pathological changes of DCM are cardiomyocyte hypertrophy and proliferation, focal necrosis, interstitium remodeling, and myocardial fibrosis [1]. Early clinical manifestations are dominated by left ventricular hypertrophy and diastolic dysfunction. With the gradual progression of disease, patients shall develop ventricular systolic dysfunction until heart failure. Nevertheless, the underlying mechanism remains unclear. Thus, it is of great significance to establish ideal animal models for profound study of the pathogenesis. The models of diabetes established currently are mainly type 1 diabetes models with large consumption of STZ, which can fully destroy islet function, but is still manifested differently from patients with clinically commonly seen type 2 diabetes. Thus, animal models of type 2 diabetes were established in this study firstly by feeding high-glucose and high-fat diets to induce insulin resistance. Then a small dose of STZ was given to destroy partial pancreatic â cell function, followed by feeding of high-fat and high-glucose diets to ultimately construct models of diabetes in rats. Xu Chunsheng et al. [2] observed that in the fourth week of disease, there were no changes in myocardial cells and microvascular basement membrane but myocardial hyperemia, with increased number of mitochondria as well as its degeneration under electron microscopy. In the eighth week of disease, cardiomyocyte hypertrophy developed, with mitochondrial swelling, vacuolar degeneration, crystal rupture, myofibrillar derangement and rupture, and other obvious cardiomyopathies under electron microscopy, indicating that rat models of DCM were successfully established, which is basically consistent with that reported in literatures.

This experiment has validated the impact of humoral immunity on DCM. There is a large number of immunoglobulin among cardiac muscle cells of rats in the experiment group, which has also been confirmed by immunohistochemical results. It is speculated that there might exist one or several kinds of antigens on myocardium of diabetic rats, which might be produced following their own glycosylation or oxidation. The immune system produced antibodies against the antigens and the specific binding of

antibodies to antigens can activate the complement system, so as to form the membrane attack complex (MAC). MAC binding to the cell membrane may on one hand, leads to membrane structure damage, cell swelling, and necrosis, resulting in myocardial injury, and on the other hand, triggers MAC-intratarget cell release of fibroblast growth factors and platelet-derived growth factors, so as to promote fibroblast proliferation and fibrosis [3]. Complement regulatory protein CD59 can be glycosylated during the progression of diabetes, losing its role of inhibiting MAC formation, which further aggravates myocardial damage [4]. Turk Z et al. [5] have also shown that the level of circulating LDL-Ics is a risk factor of macroangiopathy in T2DM, which is more obvious than the direct effect of oxLDL itself. Other studies have shown that autonomic nerve antibodies are related with injury to cardiac function of diabetic patients [6]. These results strongly suggest that immune mechanisms play an important role in the occurrence of DCM.

It was also found in this study that immune complex deposition in myocardial tissue of rats in the low-fat group was eased compared to that in the high-fat group and normal diet group, prompting that immune injury participates in the development and progression of T2DM and its chronic complications and is comparatively related to diets, namely, low-fat diets can alleviate immune response. It can be concluded that immunosuppressive agents can be used for treatment of diabetes and its complications, which also lays the foundation for treatment of diabetes and its chronic complications.

In conclusion, T2DM models and DCM models with similar clinical manifestations can be induced via intraperitoneal injection of low-dose STZ on the basis of insulin resistance, providing a better way to study DCM. The results of this study indicate that immuno-inflammation participates in the development and progression of T2DM and its chronic complications. In addition, the levels of blood glucose and immune function of the body differ among different experimental groups and the immune function decreases with the increasing of blood glucose levels.

The authors declare no conflict of interest to report.

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