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Original Article

The effect and mechanisms of folic acid complement in protecting myocardium and microvascular of type 2 diabetic rats

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Abstract: Purpose: The aim of this study was to investigate the effect and underlying mechanisms of folic acid complement in protecting myocardium and microvascular in rat model of type 2 diabetes mellitus. Methods: The type 2 diabetes mellitus was induced by low dose streptozotocin (20 mg/kg) combined with sustained high-fat diet in Wistar rats. Experimental groups were designed as follows: normal control, diabetes mellitus, diabetes treated with insulin only, diabetes treated with insulin and folic acid. Blood pressure, blood glucose and homocysteine were measured. The index of cardiac diastolic function was evaluated by ultrasonic cardiogram. The protein expression of MMP-2/9, TIMP-1/2 in cardiac tissues was detected by immunohistochemistry and western blotting. The extracellular matrix of myocardial and microvascular was observed by transmission electron microscope. Results: The induction of streptozotocin followed by high-fat diet resulted in hypertension, hyperglycemia, hyperhomocysteine and hemodynamic anomalies, which could be ameliorated by insulin and folic acid treatment. Our results also showed significant increases in collagen IV and TIMP-1/2 protein levels and a marked decrease of MMP-2/9 activity in the diabetic cardiomyopathy. Compared with the diabetes group, the extracellular matrix of myocardial and microvascular were less deposition in treatment group. Conclusion: The insulin combined with folic acid can improve the structure reconstitution and has effective therapeutic potential in the treatment of the diabetic cardiomyopathy.

Keywords: Folic acid, insulin, diabetic cardiomyopathy, myocardium, microvascular

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by high levels of blood glucose, which is a major cause of serious myocardium and microvascular diseases. Diabetic cardiomyopathy (DCM) is one of serious complications in diabetes patients that are associated with myocardial dysfunction resulting in myocardial ischemia and heart failure. Myocardial microvascular lesion, inflammation, myocardial fibrosis are considered possible pathogenesis basis for the progression of diabetic cardiomyopathy [1, 2]. At present, DCM is the major cause of increased morbidity and mortality in diabetes metabolic syndrome, hence the specific prevention and efficient treatment become impending and imperative [3].

Folic acid (vitamin B9) is an essential water-soluble B vitamin for the body growth and cell proliferation, collectively comprised of pteridine and glutamic acid residues. Folic acid is also essential for homocysteine catabolism, DNA and RNA metabolism, and methylation of multiple proteins [4]. Diabetes mellitus is connected with elevated level of homocysteine (Hcy), a condition called hyperhomocysteinemia (HHcy) that may act as an important independent risk factor of diabetic cardiomyopathy [5]. The high Hcy level can be partially normalized by supplying exogenous folic acid, by which the risk of diabetic complications could be decreased [6]. However, the current understanding of mechanisms underlying folic acid mediated cardioprotection with diabetes cardiomyopathy is still obscure.

Table 1. Basic parameters of different groups

	Body weight (g)	Blood glucose (mM)	Blood pressure (kpa)	Hcy (μmol/L)
Control	412±10	6.8±0.3	12.56±1.21	8.62±0.57
Diabetes	295±7*	19.7±1.2*	15.75±1.65	12.45±0.61*
Insulin	327±12*	10.4±0.6*,#	14.01±1.16	11.97±0.70*
Folic acid	335±10*,#	10.7±0.5*,#	12.17±1.02#	7.76±0.69#,&

Data are presented as the mean ± SD. *P < 0.05 versus control group, #P < 0.05 versus diabetes group, &P < 0.05 folic acid group versus insulin group.

Table 2. Basal hemodynamic characteristics of different groups

	HR (beats/min)	EF (%)	LVEDD (mm)	E/A ratio	IVRT (ms)
Control	325±11	65±8	2.86±0.21	1.78±0.12	31. 4±5.3
Diabetes	275±13*	42±6*	3.65±0.29*	2.97±0.22*	47.8±4.7*
Insulin	315±17#	50±6*,#	3.21±0.17#	2.28±0.14*,#	39.2±4.1*,#
Folic acid	341±10#,&	62±5#,&	2.90±0.23#,&	1.81±0.17#,&	32.1±5.1#,&

Data are presented as the mean ± SD. *P < 0.05 versus control group, #P < 0.05 versus diabetes group, &P < 0.05 folic acid group versus insulin group.

In the present study, considering the potential therapeutic properties of folic acid, the aim was to investigate the protective effects and mechanisms of folic acid complement in the alterations of myocardium and microvascular function in a rat model of DM.

Materials and methods

Animals and experimental protocol

All animals were performed according to the ethical guidelines for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication, Revised 1996). The study protocol was reviewed and approved by the Research Ethics Committee of Shaoxing people's Hospital.

Four-week-old male Wistar rats, weighing between 120 to 160 g, were purchased from the Experimental Animal Center of the Zhejiang University in Hangzhou, China. The rats were randomly divided into four groups: normal control, diabetes mellitus, diabetes treated with insulin only, diabetes treated with insulin and folic acid (n=10 for each group). All of the 40 rats, 10 rats were treated with common diet (normal control group) and the other groups were treated with the high fat diet, followed by single intraperitoneal injection streptozotocin (20 mg/kg) for the induction of type 2 diabetes. After the type 2 diabetes model establishing, the insulin group (diabetes treated with insulin only) was given intraperitoneal injections of 0.2 IU/kg zinc protamine insulin and in addition, the folic acid group (diabetes treated with insulin and folic acid) was simultaneously feed with 2 mg/kg folic acid for four weeks.

Basic parameters evaluations

Body weight changes of each animal were observed and blood glucose levels were measured using a glucometer (Shanghai Sinocare Inc, China) following tail vein puncture blood sampling, and blood pressure was determined by the Softrum-BP-98A pressure gauge (Softron, Japan). Serum samples of venous blood were collected immediately for homocysteine by using available ELISA kits (Biosource Inc, China).

Echocardiographic assessment

The rats were anesthetized by the intraperitoneal injection of pentobarbital (30 mg/kg). There is the echocardiography probe which transmits and receives ultrasonic wave to measure the heart rate (HR), ejection fraction (EF), left ventricular end-diastolic dimension (LVEDD), E/A ratio (E/A), isovolume relaxation time (IVRT).

Immunohistochemistry

In brief, the left ventricular tissues from the heart of each animal were dissected out and fixed in paraformaldehyde (4%, w/v) for 2 hours, and then the specimens were embedded in paraffin. After paraffin removal, sections of 5 μm thickness were rehydrated in graded alcohol. The sections were immersed into citrate buffer (0.01 M, pH 6) in order to retrieve antigens. Then endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 and methanol. After being washed in solution, the sections were incubated with the primary monoclonal antibody (anti-MMP-2/9, TIMP-1/2 antibody, collagen IV antibody, sigma, USA)

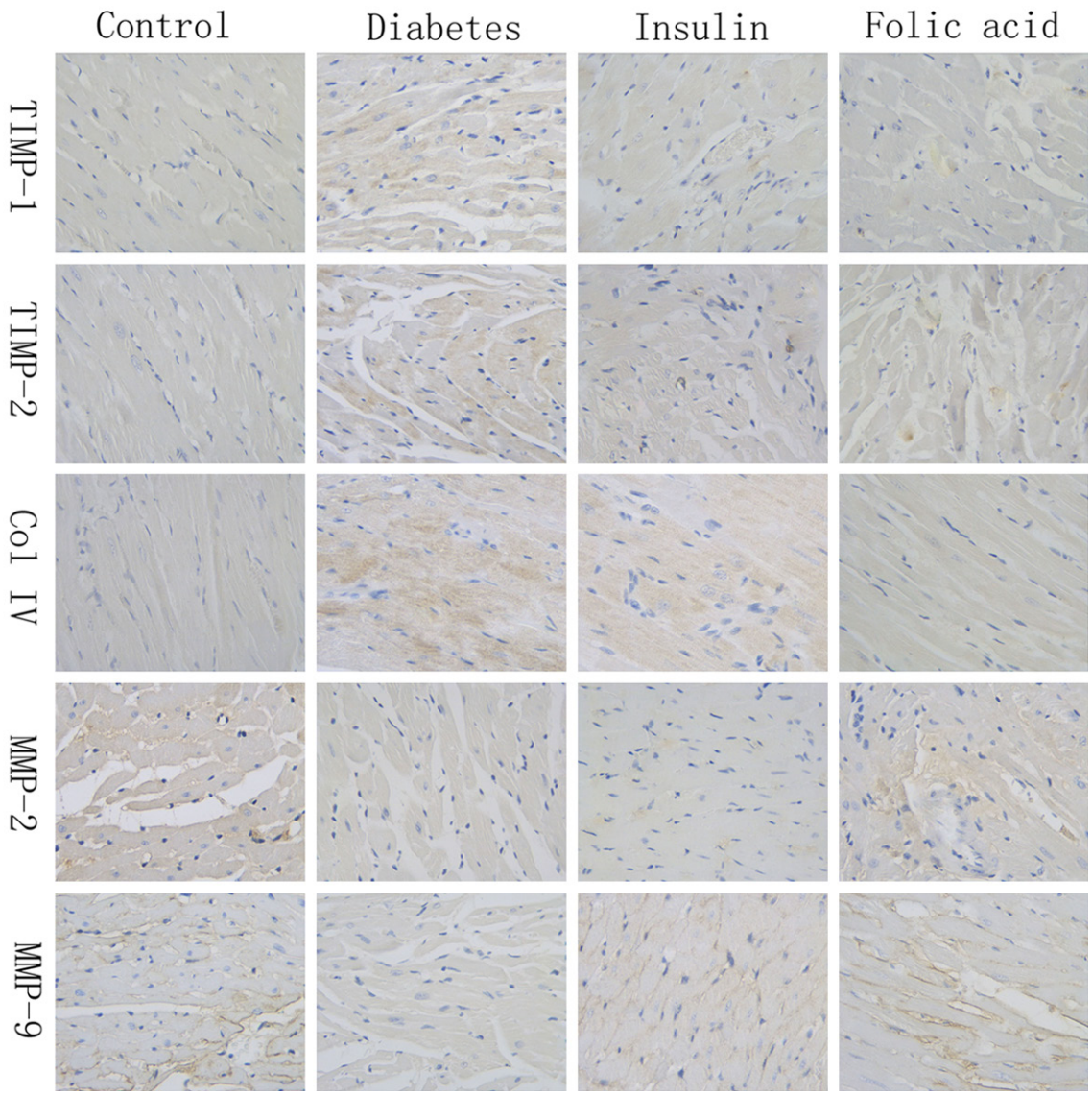

Figure 1. Immunohistochemical staining showed that left ventricular TIMP-1/2, collagen IV protein expression were enhanced inordinately in the diabetes group. However MMP-2/9 protein levels were markedly decreased compared with control group and administered group. (Magnification: ×200).

overnight at 4°C. After rinsing in PBS, the sections were incubated with goat anti-mouse antibody followed by avidin-biotin peroxidase complex. At last, the diaminobenzidine Kit (DAB, Genemed Biotechnologies, CA, USA) was applied for 15 min at room temperature. Semi-quantitative evaluation was carried out using image analysis software (Image J, NIH, USA).

Western blotting analysis

Frozen left ventricular samples were homogenized in ice-cold lysis buffer containing: 20 mM Tris·HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1 mM NaF, and 1 mM orthovanadate. The protein concentrations were determined with the bicinchoninic acid (BCA) Protein Assay Kit (Biotechnology, USA) according to the manufacturer's instructions. Equal amounts of membrane protein (20 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were incubated with anti-MMP-2/9, TIMP-1/2, collagen IV (Abcam, Cambridge, UK) antibodies for 2

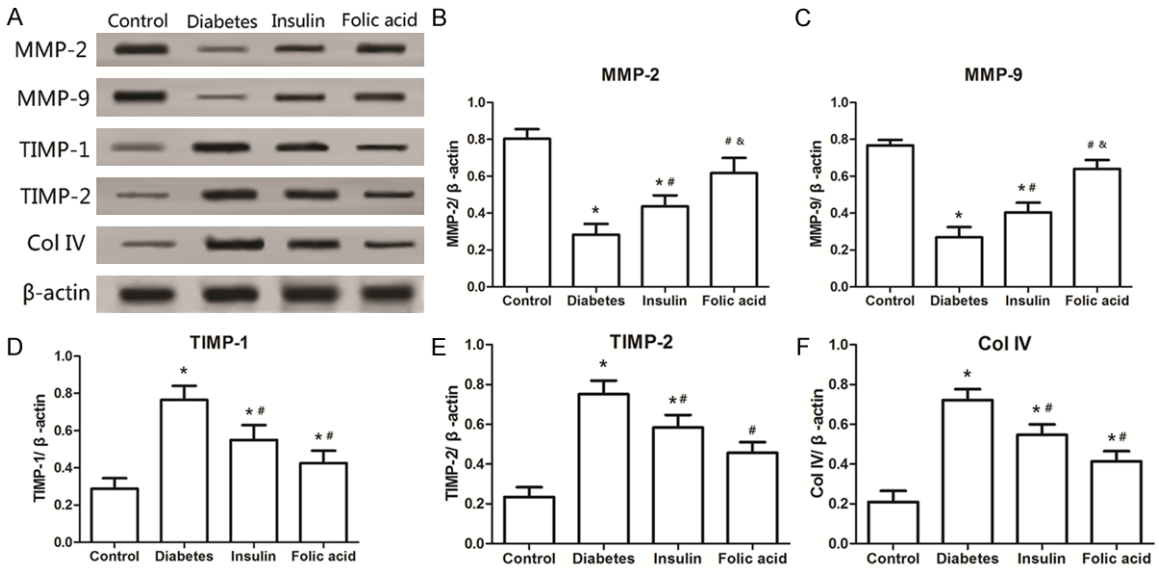

Figure 2. Protein expression by western blotting showed the levels of MMP-2/9, TIMP-1/2, collagen IV in different groups. A. Representative results of MMP-2/9, TIMP-1/2 and β-actin abundances in rat cardiac tissues; B and C. The levels of MMP-2/9 protein expression; D and E. The levels of TIMP-1/2 protein expression; F. The levels of collagen IV protein expression. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus diabetes group, $^{\&}P < 0.05$ folic acid group versus insulin group.

hours at room temperature and then incubated with a horseradish peroxidase-conjugated second antibody (sigma, USA) for 1 hour at 37°C. Immunoreactive bands were analyzed by an enhanced chemiluminescence detection kit (Biotechnology, USA).

Ultrastructural examination

Left ventricular tissues were cut into approximately 1 mm^3 pieces, and fixed in 2.5% glutaraldehyde for 3 hours at 4°C and 1% osmium tetroxide for 1 hour at 4°C. After dehydration with graded ethanol, the samples were embedded in ethoxyline resin and sectioned to 60 nm ultrathin slices. Sections were observed and photographed by the Transmission Electron Microscopy (TEM) of HITACH500 (HITACHI, Japan).

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 and data were expressed as x±s. Analyses of variance (ANOVA) were conducted to compare trends among different groups, q test was conducted to compare trends among multiple groups, with the level of significance set at $P < 0.05$.

Results

Basic parameters of different groups

Diabetes rats had significantly lower final body weights and higher blood glucoses compared with the matched control rats and administered rats ($P < 0.05$). There was significant difference of the blood pressure between folic acid treated rats and diabetic rats ($P < 0.05$). Compared with the insulin group, the levels of serum homocysteine concentration were markedly decreased in folic acid treated group ($P < 0.05$) (**Table 1**).

Echocardiographic data

HR was significantly decreased in diabetes rats compared to administered rats, which is a characteristic of diabetes-associated cardiac dysfunction ($P < 0.05$). The values of EF in diabetes rats were the lowest in all groups ($P < 0.05$), although there were no significant differences between the other three groups. The LVEDD was higher in the diabetes group than the control and administered group ($P < 0.05$). Compared with administered groups, the E/A ratio and IVRT in diabetes group were significantly higher ($P < 0.05$) (**Table 2**).

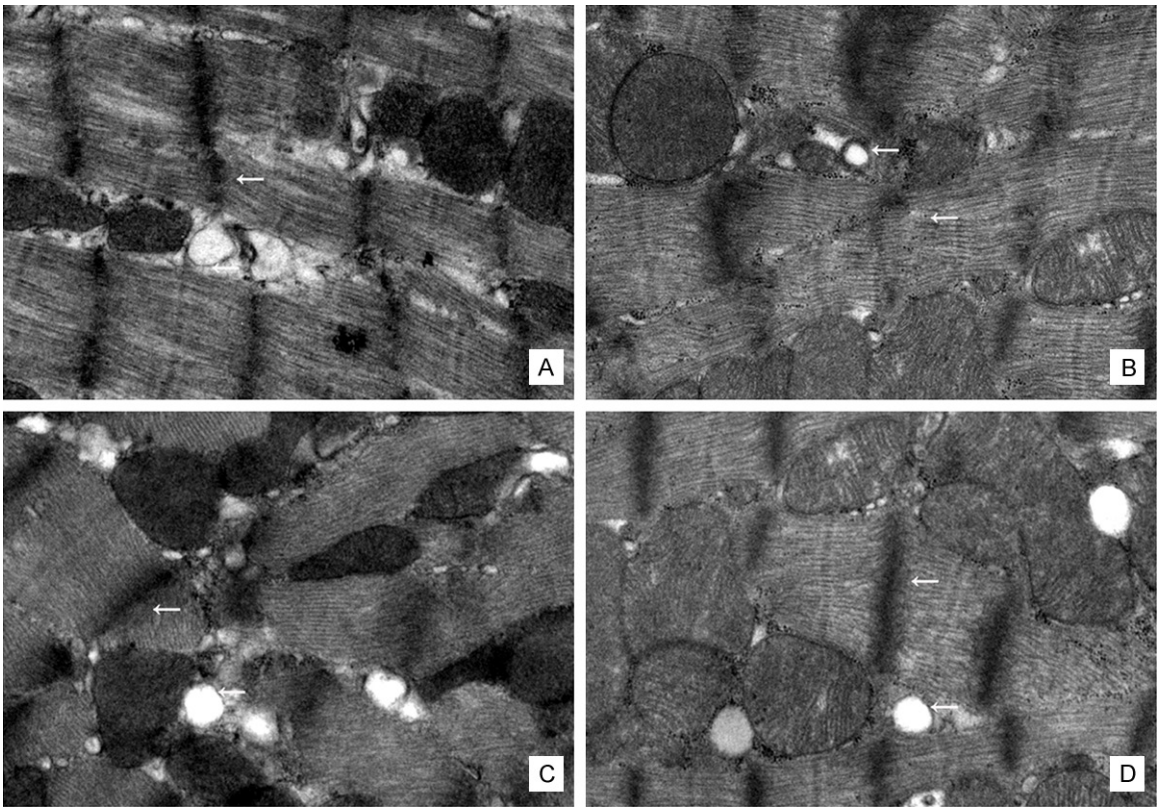

Figure 3. The cardiac tissues were detected by TEM from the left ventricle of the rats. A. Normal control group; B. Diabetes group; C. Insulin- treated group; D. Insulin and folic acid treated group. (Magnification: ×12000).

Values of protein expression by immunohistochemistry

Left ventricular TIMP-1/2, collagen IV, MMP-2/9 protein expression were detected by immunohistochemical staining, which revealed decreased staining for MMP-2/9 and increased staining for collagen IV and TIMP-1/2 in the diabetes group when compared with the control group. However, there was opposite tendency of the administered group compared to the diabetes group that indicated the protective effect of folic acid against the diabetic cardiomyopathy (**Figure 1**).

Values of protein expression by western blotting

The TIMP-1/2, collagen IV protein levels assessed by Western blotting was obviously higher in the cardiac matrix of diabetes rats compared to the other rats, and the folic acid-treated rats had the least protein expression ($P < 0.05$). In comparison, treatment of diabetes rats with insulin and folic acid significantly increased the levels of MMP-2/9 protein ($P < 0.05$), which were similar to the quantity of the control rats (**Figure 2**).

Myocardium and microvascular morphology

Cardiac tissues were used to examine the myocardium and microvascular morphology by TEM. Analysis using TEM indicated the administration of insulin and folic acid reduced the extracellular matrix deposition and myocardial remodeling compared to the diabetes group, which confirmed the less damage to myocardial ultrastructures and the protective effect of folic acid on diabetic cardiomyopathy (**Figure 3**).

Discussion

Diabetic cardiomyopathy was closely associated with dysregulation of extracellular matrix degradation that contributed to the development of cardiac fibrosis and collagen deposition. The myocardial fibrosis of an accumulation of collagen I, IV was characterized by reduced myocardial elasticity, impaired contractility

and cardiac dysfunction [7]. There is a physiologic balance between the synthesis and the decomposition of the collagen within the extracellular matrix, which were under the control of MMPs and specific inhibitors, TIMPs [8, 9]. Previous studies had demonstrated that the weakened MMP-2 activity and expression may result from up-regulation of the endogenous TIMP-2 expression [10]. However the balance was broken in the pathologic process of diabetic cardiomyopathy, which led to the excessive deposition of collagen IV. The remarkable finding of the present study is that MMP-2/9 activity was decreased and TIMP-1/2 activity was enhanced in the cardiac of diabetes rats that resulted in redundancy of collagen IV, whereas insulin and folic acid treatment could significantly ameliorate MMPs and TIMPs unbalance that protected the myocardium structure.

Our study also examined the myocardium and microvascular morphology by TEM, which showed myocardial fibrosis and structure disorder, myocardial remodeling and microvascular basement membrane thickening. By the administrating of insulin and folic acid, our experiment discovered that the cardiac remodeling of diabetes rats was effectively diminished and the cardiac diastolic function of LVEDD, E/A ratio and IVRT were much more regular than diabetes groups. Ventricular EF was the indicators used most commonly in evaluation of the cardiac systolic function [11] and our study found that EF were significantly higher in the folic acid-treated rats than diabetes rats These results indicate that normal diastolic function was impaired following diabetes induction and that folic acid administration had prominent effects against diastolic dysfunction in diabetic cardiomyopathy.

Homocysteine seemed to be a direct consequence of hyperglycemia that may disrupt the endothelium and myocytes, and ultimately contribute to the development of cardiac fibrosis [12, 13]. In diabetes, homocysteine mediated generation of oxidative radicals induces MMPs inhibition and activates their tissue inhibitor-TIMPs that result in extracellular matrix remodeling [14, 15]. Homocysteine was an intermediate of methionine metabolism, and that was managed by serum folic acid concentration. In diabetes mellitus, serum folic acid levels were changed, especially in diabetic cardiomyopathy [13, 16]. HHcy is caused mainly due to the impairment of metabolic enzymes involved in either remethylation pathways that could be partially normalized by supplying exogenous folic acid, which acts well in the remethylation procession [17]. In our context, we observed that folic acid complement could reduce myocardial impairment and improve the myocardial function by mediating the MMPs and TIMPs system, which was similar to those previous studies. Our findings might have implications regarding the efficacy of the administration of folic acid to treat diabetic cardiomyopathy or diabetic cardiovascular complications. There are numerous evidences suggested that folic acid has independent effect and has been tested as therapeutic drug in several cardiac diseases including heart failure [18-20].

Conclusions

In summary, we found that the insulin combined with folic acid was likely to have a cardioprotective effect on myocardium and microvascular structure by regulating MMPs and TIMPs system that has effective therapeutic potential in the treatment of the diabetic cardiomyopathy.

Disclosure of conflict of interest

None.

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