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Effects of Folic Acid on Cardiac Myocyte Apoptosis in Rats with Streptozotocin-induced Diabetes Mellitus

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Abstract

Backgrounds: The effect of folic acid on cardiac myocyte apoptosis secondary to diabetes is unknown.
Methods: Diabetic rats were divided into diabetic control (DC, n=11), low-dose (LDF, 0.4mg/kg/day, n=12) and high-dose (HDF, 1.2mg/kg/day, n=12) folic acid groups. Non-diabetic rats (n=11) were used as the normal control (NC).

Results: After 11 weeks of treatment, compared with the NC group, the DC group showed a reduced blood levels of reactive oxygen species (ROS, \( P<0.01 \)). The rate of cardiac myocyte apoptosis in the diabetic control group was also greater than in the non-diabetic control group (\( P<0.01 \)). In folic acid-treated rats, the blood levels of ROS was higher than in the diabetic control group (\( P<0.05 \)). There was a dose-dependent reduction in the rate of cardiac myocyte apoptosis in the folic acid groups (\( P<0.01 \)), and this was accompanied by an increased level of anti-apoptotic protein Bcl-2 and decreased level of pro-apoptotic protein Bax and Fas (\( P<0.01 \)).

Conclusions: Dietary folic acid supplementation diminishes the cardiac myocyte apoptosis in streptozotocin-induced diabetes. The apoptosis suppression is accompanied by an increase in the expression of Bcl-2 and a decrease in Bax and Fas.

Key words: diabetes, streptozotocin, apoptosis, folic acid, oxidative stress
Introduction

In patients with diabetes, there is an increased risk of symptomatic heart failure largely due to the presence of hypertension or ischemic heart disease [1]. Diabetic cardiomyopathy refers to the heart failure that occurs in the absence of ischemic heart disease or hypertension [1]. Diabetic cardiomyopathy, which is related directly to hyperglycemia, encompasses the spectrum from subclinical disease to the full-blown syndrome of heart failure [1, 2].

Diabetic cardiomyopathy involves several cellular and molecular mechanisms, such as upregulation of renin-angiotensin system, cardiac myocyte apoptosis and cell necrosis [2]. Recent experimental and clinical studies have demonstrated that high blood glucose levels significantly increase the rate of cardiac myocyte apoptosis and cell necrosis, and may lead to a myocyte loss of up to 30% [3-5].

Folic acid supplementation offers several clinical benefits to patients with diabetes. Short-term oral folic acid supplementation enhances endothelial function [6]. Combined supplementation of folic acid and vitamin E to pregnant diabetic rats diminished diabetes-induced malformations, concomitant with normalization of apoptotic protein levels [7]. On the other hand, folic acid deficiency during late gestation decreases progenitor cell proliferation and increases apoptosis in fetal mouse brain [8]. Furthermore, in vitro folate deficiency is associated with S phase accumulation and apoptosis in peripheral blood lymphocytes [9], or human hepatoma Hep G2 cells [10].

The primary aim of the study is to investigate whether a short-term supplementation of folic acid would reduce cardiac myocyte apoptosis in streptozotocin (STZ)-induced diabetes. The effect of folic acid supplementation on nitric oxide, superoxide dismutase and malondialdehyde, a biomarker of free radical production, was also investigated.

Materials and Methods

Preparation of diabetes model
This study was approved by the institution review board for animal research at Taishan Medical College. A total of 62 male Wistar rats (10 weeks old) were used for the study, of which 11 were used as the normal control (NC group). Fifty-one animals had free access to the high calorie diet (20.1 KJ/g) in chow form for six weeks. These rats were subsequently injected with streptozotocin (STZ) (30mg/kg) via the tail vein. In a pilot study, STZ of 40, 50 and 60 mg/kg was administered to four rats each. All animals in the 60 mg/kg group and one animal in the 40 mg/kg and one in the 50 mg/kg group died during the observation period of four weeks. Therefore, a conservative dose of 30 mg/kg was chosen for this study.

The 11 rats in the NC group were on a normal calorie diet (15.4 KJ/g). They were also injected with the same volume of vehicle for STZ (sodium citrate-citric acid buffer solution, 0.1mol/L, pH 4.2) through the tail veins.

One-week after the injection of STZ, blood was collected (4 hours after the last feeding) from the tail vein to determine blood glucose, using a glucose meter (Roche Diagnostics, Shanghai, China). Rats whose blood glucose level was greater than 300 mg/dL were defined as being diabetic.

Diabetes was established in 39 (77%) of the 51 rats. The diabetic rats were randomly divided into diabetic control group (DMC group), low-dose folic acid group (LDF group) and high-dose folic acid group (HDF group). The LDF and HDF groups were fed with folic acid (Feiying Pharmacy Ltd, Tianjin, China) at 0.4 and 1.2 mg/kg/day, respectively, by oral gavage with feeding needle for 11 weeks. Animals in the DMC group were fed with a placebo through oral gavage for the same period as the treatment group.

During the entire period of study, all treatment and DMC group animals had free access to the higher calorie diet.

Blood glucose and serum insulin level was measured at the end of the 11-week treatment. On the day of measurement, feed was removed from the cage early in the morning and four
hours later blood was drawn from the tail vein. Insulin levels were measured by radioimmunoassay, following the instructions in the measurement kits (North Institute of Biological Technology, Beijing, China).

Measurement of serum nitric oxide, superoxide dismutase and malondialdehyde

After 11 weeks of folic acid administration, 1ml of blood was collected from the tail vein to determine the levels of nitric oxide using nitrate reductase method [11]. Superoxide dismutase activity was determined according to the method of Durak et al. [12]. The principle of the method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine/xanthine oxidase system as a superoxide generator. Malondialdehyde levels were estimated by the double-heating method of Draper and Hadley [13]. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid with malondialdehyde. All measurements were performed using commercially available reagent kits (Jiancheng Bioengineering Institute, Nanjing, China).

Analysis of cardiac myocyte apoptosis

At the end of the study, rats were euthanized with intravenous injection of sodium pentobarbital (50mg/kg). The heart was quickly harvested and myocardial tissues on the left ventricular anterior wall were collected to make olefin sections.

Ten rats were selected from each group and the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) method was used to detect cardiac myocyte apoptosis, according to the instructions in the reagent kits (Boshi Microbiological Engineering Ltd, Wuhan, China)

An Olympus visible light microscope was used to visualize these sections at 400 times magnification. Three different visual fields were randomly chosen from each section. In each
visual field, the number of positive cell nuclei was calculated among 200 myocardial cell nuclei. The rate of apoptotic cell nuclei is defined as apoptotic positive cell nuclei/total cell nuclei in the field ×100.

_Immunohistochemical detection of Bcl-2, Bax and Fas protein in myocardium_

Myocardial tissue slides were processed with poly-L-lysine solution before being made into olefin samples. The streptavidin-biotin Complex (SABC) method was used for immunohistochemical detection, using reagent kits (Boshi Microbiological Engineering Ltd, Wuhan, China). Microwave-stimulated antigen retrieval was conducted. Diluted Bcl-2, Bax and Fas primary antibody (rabbit anti-mouse, rabbit anti-rat and rabbit anti-human polyclonal antibody) drops were added. After the olefin sections were washed, biotinylated goat anti-rabbit IgG drops were added and the reaction was allowed for 20 minutes at 20°C. The tissue sections were washed once more and SABC was added. In the experiment, PBS solution was used to replace primary antibody for negative control slides. An Olympus B x 51 visible light microscope was used to obtain images at 400 times magnification. Three different visual fields were randomly chosen to measure optical density values of Bcl-2, Bax and Fas protein. The average optical density value from three measurements was used to reflect the positive expression of these proteins.

_Statistical analysis_

All data were processed with SPPS10.0 statistical software. Data were expressed as mean ± standard deviation where appropriate. Comparison between two groups was performed with student \( t \) test, while comparison among multi-groups was conducted by one-way ANOVA. \( P<0.05 \) was considered statistically significant.
Results

Effects of folic acid on the levels of blood glucose and insulin

As shown in Table 1, there was no significant difference in the average body weight between the four groups of animals ($P>0.05$). The levels of fasting blood glucose in the three diabetic groups were higher than the normal control ($P<0.01$). There was no significant difference in the serum insulin levels between the four groups ($P>0.05$).

Effects of folic acid on the levels of serum nitric oxide, superoxide dismutase and malondialdehyde

As shown in Table 2, the levels of nitric oxide and superoxide dismutase in the diabetic control group were significantly lower than in the normal control (Table 2, $P<0.01$), while the level of malondialdehyde in the diabetic control group was higher ($P<0.01$). Compared with the diabetic control group, the levels of nitric oxide and superoxide dismutase in the treatment groups (LDF and HDF) were higher, and the levels of malondialdehyde were lower (Table 2, $P<0.01$).

Changes in cardiac myocyte apoptosis

Apoptotic cardiac myocytes in rats in the treatment groups and the diabetic control group showed aggregated distribution, while apoptotic cardiac myocytes in rats in the normal control group appeared to be isolated (Fig 1).

As shown in Table 3, the rate of apoptosis in all diabetic groups were greater than the normal control group ($P<0.05$). The rate of apoptosis in the treatment groups was lower than in the diabetic control group ($P<0.01$). The high dose folic acid group had a lower rate of apoptosis than in the low dose group ($P<0.01$).
Changes in the expression of Bcl-2, Bax and Fas proteins

Compared with the normal control group, all diabetic groups had elevated optical density of Bcl-2, Bax and Fas proteins ($P<0.05$, Table 3). The optical density of Bcl-2 in the treatment groups was higher than in the diabetic control group, with the high dose folic acid group being the highest ($P<0.01$, Table 3). The optical density of Bax and Fas in the treatment groups was lower than in the diabetic control group ($P<0.05$, Table 3). The low dose folic acid group had a higher optical density of Bax and Fas protein than in the high dose group ($P<0.05$, Table 3).

Discussion

Oxidative stress and cardiac myocyte apoptosis in diabetics

The mechanisms of cardiac myocyte apoptosis in diabetic animals or in patients have been under some investigation. Studies in patients and animals have demonstrated a direct correlation between hyperglycemia and the production of reactive oxygen species [14, 15]. Moreover, the activated renin-angiotensin system, in particular angiotensin II, leads to an oxidative stress response through stimulation of NADH/NADPH oxidase [16]. NADH/NADPH oxidase is a major source of superoxide via the transfer of electrons from NADH or NADPH to reactive oxygen species [16]. Pre-treatment with angiotensin-converting enzyme inhibitors reduced apoptosis and oxidative stress in the diabetic rats [17].

Nitric oxide also plays an important role in cardiac myocyte apoptosis. Under physiological conditions, endothelium-derived nitric oxide synthase (eNOS) generates nitric oxide which helps to protect myocardial cells and resists apoptosis. However, local nitric oxide generated from the induced nitric oxide synthase (iNOS) in the ischemic myocardium may damage myocardium and induce myocardial apoptosis [18, 19].
The present study has clearly demonstrated that the rate of cardiac myocyte apoptosis in diabetic rats is substantially higher than in the normal control group. In the diabetic control rats, the blood levels of anti-apoptotic nitric oxide and oxygen free radical scavenger, superoxide dismutase, are decreased, while the levels of free radical biomarker, malondialdehyde, are elevated. These changes may have contributed to the increased cardiac myocyte apoptosis in the diabetic control rats.

**Bcl-2, Bax, Fax and cardiac myocyte apoptosis in diabetics**

Apoptosis is a highly controlled program that relies on the interplay between pro-apoptotic and pro-survival proteins. Recent evidence shows that the Bcl-2 (pro-survival)-to-Bax (pro-apoptotic) ratio in the cell determines to a large extent whether the cell initiates apoptosis or alternatively, re-enters the cell cycle [20]. In addition, FasL/Fas/Fas-associated death domain exemplifies the activation of the death receptor apoptosis [21].

In the present study, the positive expression of apoptotic proteins, Bax and Fas, in the diabetic control group were higher than the normal control group, indicating an activation of these protein synthesis systems in the presence of diabetes. It is also interesting to note that the expression of anti-apototic protein, Bcl-2, was also increased in the diabetic control group. Whether this increase is a self-protection mechanism triggered by diabetes, or is due to technical limitations of the present study remains to be determined.

**Folic acid and cardiac myocyte apoptosis in diabetics**

The existing preventive measures for diabetic cardiomyopathy include controlling blood glucose, restraining the generation of oxygen radicals in cells, clearing oxygen radicals, and suppressing the activity of renin-angiotensin system in the myocardium [19]. As a new antioxidant, folic acid seems to be able to diminish apoptosis in several types of non-cardiac
cells. An in vitro study found that high dose of folic acid weakened death signal transduction and cell apoptosis and reduced the levels of reactive oxygen species [22]. The present study in rats shows that the rate of cardiac myocyte apoptosis in both low- and high-dose folic acid supplementation groups were significantly lower than in the diabetic control group. The apoptosis rate and the average optical density values of positive expression of Bax and Fas protein in the high-dose group were lower than in the low-dose group. In addition, the levels of nitric oxide and scavenger-superoxide dismutase in the folic acid groups were markedly higher than in the diabetic control group, while the level of malondialdehyde was significantly lower. These results indicate that, short-term supplementation of folic acid can dose-dependently diminish cardiac myocyte apoptosis in diabetic rats. The suppression of cardiac myocyte apoptosis is likely due to the enhanced expression of apoptosis suppressor protein, Bcl-2, and the reduced expression of apoptosis stimulant protein, Bax and Fas.

Compared with the low-dose folic acid group, the high-dose group did not show further increase in blood levels of nitric oxide or superoxide dismutase (Table 3). Therefore, the increased anti-apoptotic effect of high-dose folic acid was not accompanied by the similar alterations in blood biomarkers of oxidative stress. The possible explanations of this are that first, apart from reduction in oxidative stress, other signaling pathways may be involved in the anti-apoptotic effects of folic acid. Second, blood levels of nitric oxide or superoxide dismutase may not accurately reflect the changes in the myocardium.

In the present study, folic acid supplementation did not reduce blood glucose levels but it did reduce global oxidative stress and apoptosis. These results suggest that folic acid’s anti-oxidative stress and anti-apoptotic effects in the diabetic rats may involve other mechanisms. Diabetes is associated with high levels of plasma free fatty acid [23], which is known to induce apoptosis in pancreatic beta-cells and human vascular endothelia cells [24, 25]. Future studies are warranted to assess whether free fatty acids in this diabetic model are
associated with the increased global oxidative stress and cardiac myocyte apoptosis, and more importantly, whether folic acid supplementation would diminish oxidative stress and cardiac myocyte apoptosis by suppressing the blood levels of the free fatty acids.

Limitations of the study

Previous study showed that fasting blood glucose measured 4-5 days after STZ injection was 3-4 times higher than the normal control group, whereas the fasting insulin levels was 3 to 4 times lower [26]. In the present study, although the blood glucose levels in the diabetic control group were significantly higher than the normal control group, the blood insulin levels between the two groups were similar. The differences in the blood insulin profile between our and other studies may be due to the fact that a smaller dosage of STZ was used to induce diabetes in the present study [26].

This study shows that in the STZ-induced diabetic rats there was a global oxidative stress (e.g. increase in serum levels of malondialdehyde), which was diminished by folic acid treatment. However, the levels of oxidative stress in the myocardium and the impact of folic acid treatment on the local oxidative stress are yet to be determined. In addition, further studies are required to assess whether the degree of cardiac myocyte apoptosis in the diabetic group would have any detrimental effect on the left ventricular function, and whether pre-treatment with folic acid would prevent or diminish the apoptosis-induced ventricular dysfunction.

Conclusions

In this STZ-induced diabetic rat model, folic acid supplementation to the diet for 11 weeks reduces the cardiac myocyte apoptosis. The reduction in apoptosis is accompanied by increased serum levels of nitric oxide and superoxide dismutase, as well as decreased
malondialdehyde. Folic acid treatment is also associated with a dose-dependent increase in expression of the anti-apoptotic protein Bcl-2, and a decrease in the pro-apoptotic proteins Bax and Fas. These results indicate that oxidative stress may be an important signaling pathway of cardiac myocyte apoptosis in this rat model. Further studies are required to investigate whether the diminished cardiac myocyte apoptosis following folic acid treatment can be translated into reduced prevalence of diabetic cardiomyopathy and improved left ventricular function.
References


9. Lin HL, Chen CJ, Tsai WC, Yen JH, Liu HW. In vitro folate deficiency induces


Table 1: The levels of blood glucose and insulin after 11 weeks of folic acid treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/L)</th>
<th>Insulin (μIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>11</td>
<td>424.6±53.4</td>
<td>5.0±0.5</td>
<td>21.8±3.3</td>
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<tr>
<td>DMC</td>
<td>12</td>
<td>415.1±64.9</td>
<td>19.4±1.4*</td>
<td>20.2±4.1</td>
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<tr>
<td>LDF</td>
<td>12</td>
<td>421.4±57.8</td>
<td>20.8±1.0*</td>
<td>20.8±3.6</td>
</tr>
<tr>
<td>HDF</td>
<td>13</td>
<td>405.6±62.7</td>
<td>20.3±1.3*</td>
<td>22.4±3.9</td>
</tr>
</tbody>
</table>

*Compared with NC (normal control) group, *P* < 0.01. DMC: diabetic control, LDF and HDF: low and high dose folic acid group.
Table 2. The levels of nitric oxide (NO), superoxide dismutase (SOD) and malondialdehyde (MDA)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>NO</th>
<th>SOD</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>11</td>
<td>32.8±7.1</td>
<td>411.0±40.8</td>
<td>8.5±2.8</td>
</tr>
<tr>
<td>DMC</td>
<td>12</td>
<td>23.0±5.2**</td>
<td>315.9±56.3**</td>
<td>19.0±4.6**</td>
</tr>
<tr>
<td>LDF</td>
<td>12</td>
<td>28.8±6.7△</td>
<td>368.8±38.3*△△</td>
<td>11.2±3.5△△</td>
</tr>
<tr>
<td>HDF</td>
<td>13</td>
<td>29.6±6.6△</td>
<td>358.2±33.6**△△</td>
<td>11.0±4.6△△</td>
</tr>
</tbody>
</table>

Compared with NC group, *P<0.05. ** P<0.01. Compared with DMC group, △ P<0.05. △△ P<0.01. NC: normal control group, DMC: diabetic control, LDF and HDF: low and high dose folic acid group.
Table 3: The rate of apoptosis and the average optical density (ODs) of Bcl-2, Bax and Fas protein.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Rate of Apoptosis</th>
<th>ODs of Bcl-2</th>
<th>ODs of Bax</th>
<th>ODs of Fas</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>11</td>
<td>0.7±0.4</td>
<td>224.5±3.0</td>
<td>214.4±5.2</td>
<td>226.3±4.7</td>
</tr>
<tr>
<td>DMC</td>
<td>12</td>
<td>8.3±1.1**</td>
<td>230.2±4.5*</td>
<td>234.8±5.7**</td>
<td>247.3±6.3**</td>
</tr>
<tr>
<td>LDF</td>
<td>12</td>
<td>4.3±0.6***△△</td>
<td>236.2±7.8***△</td>
<td>226.7±7.2***△△</td>
<td>239.1±6.1***△△</td>
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<tr>
<td>HDF</td>
<td>13</td>
<td>2.3±0.9***△△#</td>
<td>241.6±5.8***△△#</td>
<td>220.2±5.4***△△#</td>
<td>233.4±7.4***△△#</td>
</tr>
</tbody>
</table>

Compared with NC (normal control) group, *P<0.05. **P<0.01. Compared with DMC (diabetic control) group, △P<0.05. △△P<0.01. Compared with LDF (low dose folic acid) group, #P<0.05. HDF: high dose folic acid.
Figure legends

Figure 1: Myocardial apoptosis (TUNEL×400)

A: normal control group, B: diabetic control group, C: low dosage folic acid group, D: high-dosage folic acid group.