Original Article
Tetrandrine down-regulates expression of miRNA-155 to inhibit signal-induced NF-κB activation in a rat model of diabetes mellitus

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Abstract: Aims: This study is to investigate expression of miRNA-155 and the related signaling pathway in a rat model of diabetes mellitus (DM). Methods: Thirty-six SD rats were divided into control, DM, and tetrandrine groups. A rat model of DM was constructed by tail vein injection with alloxan. Levels of related cytokines in serum samples were detected. The mRNA levels of IκBα and TNF-α in pancreatic islet tissues were detected by real-time PCR. Protein expression of IκBα and TNF-α was detected by western blotting. Expression of miRNA-155 in pancreatic islet tissues and serum samples was detected by real-time PCR. Results: Compared with those in the control and the tetrandrine groups, activities of methane dicarboxylic aldehyde and reactive oxygen species in serum samples and pancreatic islet mitochondria tissues in the DM group were increased (P < 0.05), while activity of superoxide dismutase in the DM group was decreased (P < 0.05). Activities of haemoglobin A1c and glucose in serum samples in the DM group were increased, while insulin in the DM group was decreased (P < 0.05). The mRNA and protein levels of IκBα in pancreatic islet tissues in the DM group were decreased (P < 0.05), while the mRNA and protein levels of TNF-α in the DM group were increased (P < 0.05). Expression of miRNA-155 in pancreatic islet tissues and serum samples in the DM group was increased (P < 0.05). Conclusion: Tetrandrine prevented injury in rat pancreatic islet caused by alloxan, which was associated with decreased oxidative stress, down-regulated miRNA-155 and decreased TNF-α in the NF-κB signaling pathway. These results indicate that tetrandrine plays an important role in DM by regulating expression of miRNA-155.

Keywords: miRNA-155, diabetes mellitus, TNF-α, NF-κB, tetrandrine

Introduction
Diabetes mellitus (DM) with a characteristic of hyperglycemia is a common metabolic disease. Hyperglycemia is due to defect in insulin secretion or injury in biological action, which may result in chronic injury and dysfunction to tissues, particularly eyes, kidneys, heart, serum vessels, and nerves. DM is caused by inadequate secretion of insulin and hyposensitivity of target cells to insulin, resulting in metabolic disorders of carbohydrates, protein, and fat. Incidence of DM varies in countries and people [1]. Number of patients with DM in China is the highest in the world [2]. Almost 95% of patients are diagnosed with type 2 DM (T2DM) in China [3]. There are microvascular diseases and macrovascular diseases with progression of DM, followed by systemic injury in the eyes, nerves, cardiovascular system, kidneys, and others, finally resulting in organ defect and failure [4-7]. In process of vasculopathy, vascular endothelial is damaged, followed by abnormal secretion, resulting in imbalance between proliferation and apoptosis of smooth muscle. Moreover, atherosclerosis causes reduction in vasoactive substances, followed by dysfunction in systolic and diastolic functions and various complications [8]. Mechanism of T2DM on vasculopathy is closely related with genetic predisposition, oxidative stress, advanced glycation end products, aldose reductase, and inflammation [9].

Alloxan can quickly produce reactive oxygen species (ROS). Moreover, alloxan damages cytomembrane of pancreatic islet β cell and
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Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lKβα_F</td>
<td>CCTCACCCCTCCCAATAAT</td>
</tr>
<tr>
<td>lKβα_R</td>
<td>GTGTAATGGTGCGGTACGAC</td>
</tr>
<tr>
<td>β-actin_F</td>
<td>CCCATCTAGGGGTACG</td>
</tr>
<tr>
<td>β-actin_R</td>
<td>TTTAAGTGACGGCAGATTC</td>
</tr>
<tr>
<td>TNF-α_F</td>
<td>AGACCCTCAACACAGATCATCCTC</td>
</tr>
<tr>
<td>TNF-α_R</td>
<td>CTCCGGCTGTTTGGTTGCTA</td>
</tr>
<tr>
<td>GAPDH_F</td>
<td>CGAGGGCCACACTAAGG</td>
</tr>
<tr>
<td>GAPDH_R</td>
<td>GCTGTTGAAGTCACAGGACA A</td>
</tr>
<tr>
<td>miRNA-155_F</td>
<td>GAGGTTAATGCTAATTGTGATA</td>
</tr>
<tr>
<td>miRNA-155_R</td>
<td>GTGCAGGGTCCGAGGA</td>
</tr>
<tr>
<td>β-actin_F</td>
<td>CTCTCCAGCCTTCCTCCTC</td>
</tr>
<tr>
<td>β-actin_R</td>
<td>TCATCGTACTCCTCCTGCTG</td>
</tr>
</tbody>
</table>

In the present study, a rat model of DM was established by the use of alloxan. Levels of cytokines in rat pancreatic islet tissues and serum samples were detected by real-time PCR and western blotting. This study is to provide theoretical basis for diagnosis, prevention, and treatment of DM.

Materials and methods

Drugs and reagents

Alloxan (Sigma, USA), tetrandrine injection (30 mg/2 ml, Hainan Pharmaceutical Co., Ltd., H20066570). Insulin ELISA kit (Linco, USA), kits for hemoglobin A1c (HbA1c), glucose, methane dicarboxylic aldehyde (MDA), superoxide dismutase (SOD), ROS, and total protein quantification (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), miRcute miRNA isolation kit (TIANGEN, Beijing), miRcute miRNA cDNA first strand synthesis kit (TIANGEN, Beijing), miRcute miRNA assay kit (TIANGEN, Beijing). Rabbit anti-mouse TNF-α antibody (Abcam, USA), rabbit anti-mouse lKβα antibody (Abcam, USA). NycoCard Reader II (Axis-Shield, Norwegian), iQ5 (BIO-RAD, USA), Image Lab software.

Rat model of DM

A total of 36 SD rats (150-200 g, male) were provided by Chongqing Xin Teng Bill Sales Co., China. These rats were divided into the control, DM and tetrandrine groups. DM model was constructed by tail vein injection with alloxan (50 mg/kg). One and a half hours before alloxan injection, the mice in tetrandrine group were intraperitoneally injected with tetrandrine (100 mg/kg), and the other two groups were treated with the same volume of saline. Levels of HbA1c, glucose, insulin, SOD, MDA, and ROS were detected at 48 hr.

Real-time PCR

RNA was extracted by Trizol, and was reverse transcribed to cDNA. Purity was detected by UV spectrophotometer at absorbance of 260/280. Primers were showed in Table 1.

Conditions of real-time PCR were designed as followed: pre-denaturation for 2 min at 95°C, denaturation for 20 s at 95°C, 45 cycles of annealing for 25 s at 58°C and extension for 30 s at 72°C. Ratio of lKβα/β-actin was calculated with 2^-ΔΔCt, and β-actin was used as a loading control; pre-denaturation for 2 min at 93°C,
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### Table 2. Levels of HbA1c, glucose, insulin, SOD, MDA, and ROS in serum samples in each group (n = 12)

<table>
<thead>
<tr>
<th>Groups</th>
<th>HbA1c (%)</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (μU/l)</th>
<th>SOD (U/l)</th>
<th>MDA (nmol/l)</th>
<th>ROS (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>6.32 ± 1.75</td>
<td>6.37 ± 0.36</td>
<td>12.41 ± 3.15</td>
<td>18.97 ± 3.65</td>
<td>4.94 ± 1.01</td>
<td>20.45 ± 3.52</td>
</tr>
<tr>
<td>DM</td>
<td>8.61 ± 2.15*</td>
<td>24.81 ± 6.29**</td>
<td>7.26 ± 1.70*</td>
<td>12.47 ± 3.21*</td>
<td>8.56 ± 2.32*</td>
<td>38.89 ± 6.39*</td>
</tr>
<tr>
<td>Tet</td>
<td>6.56 ± 1.95*</td>
<td>7.34 ± 1.89*</td>
<td>11.56 ± 2.35*</td>
<td>16.33 ± 2.64*</td>
<td>5.71 ± 1.85*</td>
<td>25.61 ± 4.11*</td>
</tr>
</tbody>
</table>

Note: Significant difference to group control: *P < 0.05, **P < 0.01. Significant difference to group DM: *P < 0.05, **P < 0.01.

### Table 3. Levels of SOD, MDA, and ROS in pancreas islet mitochondria tissues in each group (n = 12)

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg)</th>
<th>MDA (μmol/g)</th>
<th>ROS (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.21 ± 0.09</td>
<td>1.54 ± 0.19</td>
<td>31.94 ± 6.19</td>
</tr>
<tr>
<td>DM</td>
<td>0.07 ± 0.02**</td>
<td>4.62 ± 0.25**</td>
<td>58.54 ± 8.31**</td>
</tr>
<tr>
<td>Tet</td>
<td>0.13 ± 0.06***</td>
<td>2.77 ± 0.31***</td>
<td>46.33 ± 6.31***</td>
</tr>
</tbody>
</table>

Note: Significant difference to group control: *P < 0.05, **P < 0.01. Significant difference to group DM: *P < 0.05, **P < 0.01.

Denaturation for 60 s at 93°C, 40 cycles of annealing for 60 s at 55°C and extension for 1 min at 71°C. Ratio of TNF-α/GAPDH was calculated with 2\(^{-\Delta\Delta Ct}\), and GAPDH was used as a loading control; pre-denaturation for 10 min at 95°C, denaturation for 15 s at 95°C, 40 cycles of extension for 1 min at 60°C. Ratio of miRNA-155/β-actin was calculated with 2\(^{-\Delta\Delta Ct}\), and β-actin was used as a loading control.

**Western blotting**

Proteins were extracted and protein concentration was detected by BCA assay kit. SDS-PAGE sample buffer was added, and boiled for 5 min. Then 20 μg of samples were detected by 10% SDS-polyacrylamide gel, followed by electrophoretic transfer for 2 hr at 100 V, and then blocked with 5% skim milk for 1 hr at room temperature. Rabbit anti-mouse antibodies (kβα 1:1000, TNF-α 1:200, β-actin or GAPDH 1:5000) were added and incubated overnight at 4°C, then the secondary antibody (1:3000) was added and incubated for 1 hr at room temperature. Images were obtained and analyzed by Image Lab. The relative expression of target gene was the ratio of gray scale value of target gene to β-actin or GAPDH.

**Statistical analysis**

All data were analyzed using SPSS18.0. Results were given as X ± s. One-way ANOVA was performed to compare difference between groups. LSD and SNK comparisons were used in the homogeneous variance, and Tamhane’s T2 or Dunnett’s T3 comparisons were used in the heterogeneity variance. P value less than 0.05 was considered statistically significant.

**Results**

**Levels of HbA1C, glucose and insulin in serum**

To measure the levels of HbA1C, glucose and insulin, serum samples were collected and analyzed. As shown in Table 2, insulin level in serum in the DM group was significantly lower than those in the control and the tetrandrine groups, but the levels of HbA1c and glucose in the DM group were significantly higher than those in the control and the tetrandrine groups (P < 0.05). This data suggests that the rat model with DM is successfully established and that tetrandrine could alleviate the symptoms of DM.

**Oxidative stress and anti-oxidant capacity in serum and pancreatic islet mitochondria**

To detect levels of SOD, MDA, and ROS, serum samples and pancreatic islet mitochondria were collected and analyzed. As shown in Table 2, activities of SOD in serum in the DM group were significantly lower than those in the control and the tetrandrine groups, but the levels of MDA and ROS in the DM group were significantly higher than those in the control and the tetrandrine groups (P < 0.05). As shown in Table 3, activity of SOD in pancreatic islet mitochondria in the DM group was significantly lower than that in the control and the tetrandrine groups, but MDA and ROS in the DM group were significantly higher than those in the control and the tetrandrine groups (P < 0.05). These results indicate that SOD level is down-regulated in DM, while MDA and ROS levels are up-regulated in DM.

**Tetrandrine elevates kβα while suppresses TNF-α mRNA and protein expression in pancreatic islet**

To detect the mRNA levels of kβα and TNF-α in pancreatic islet tissues, real-time PCR was per-
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![Graphs showing mRNA and protein levels](image)

**Figure 1.** The mRNA levels of IκBα and TNF-α in pancreatic islet tissues. After 48 hr treated with tetrandrine, RNA was extracted from pancreatic islet tissues. Real-time RT-PCR was performed to detect the mRNA levels of IκBα and TNF-α. β-actin and GAPDH were used as loading controls, respectively. Experiments were repeated more than 3 times. A. The relative mRNA level of IκBα in pancreatic islet tissues. B. The relative mRNA level of TNF-α in pancreatic islet tissues. Significant difference to group control: *P < 0.01. Significant difference to group DM: *P < 0.05, **P < 0.01.

![Blot images](image)

**Figure 2.** The protein levels of IκBα and TNF-α in pancreatic islet tissues. DM rats were treated with tetrandrine, and the total proteins were extracted from pancreatic islet tissues. Western blotting was performed to detect the protein levels of IκBα and TNF-α. β-actin and GAPDH were used as loading controls, respectively. Experiments were repeated more than 3 times. A. The relative protein level of IκBα in pancreatic islet tissues. B. The relative protein level of TNF-α in pancreatic islet tissues. Significant difference to group control: *P < 0.05, **P < 0.01. Significant difference to group DM: #P < 0.05.

formed. As shown in **Figure 1A**, the mRNA level of IκBα in pancreatic tissues in the DM group was significantly decreased compared with that in the control group, but the mRNA level of IκBα in the tetrandrine group was significantly increased compared with that in the DM group.
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To further determine the protein levels of IκBα and TNF-α in pancreatic islet tissues, western blotting was performed. As shown in Figure 2A, the protein expression of IκBα in pancreatic islet tissues in the DM group protein was significantly decreased compared with that in the control group, but the protein expression of IκBα in the tetrandrine group protein was significantly increased compared with that in the DM group (P < 0.05). As shown in Figure 2B, the protein expression of TNF-α in pancreatic islet tissues in the DM group protein was significantly increased compared with that in the control group, but the protein level of TNF-α in the tetrandrine group was significantly decreased compared with that in the DM group (P < 0.01). These results indicate that tetrandrine increases the protein level of IκBα in pancreatic islet tissues in DM, while decreases the protein level of TNF-α in pancreatic islet tissues in DM.

Discussion

Pancreatic disease is one of the important features in DM. Pancreatic islet β cells in DM fail to produce enough insulin to reduce the concentration of serum glucose and result in a high incidence of hyperglycemia. In the present study, our results showed that the activities of insulin and SOD in serum samples in the DM group were significantly lower than those in the control.

Tetrandrine inhibits miRNA-155 expression in both pancreatic islet and serum

To investigate levels of miRNA-155 in pancreatic islet tissues and serum samples, real-time PCR was performed. As shown in Figure 3A, level of miRNA-155 in pancreatic islet tissues in the DM group was significantly increased compared with that in the control group, but level of miRNA-155 in the tetrandrine group was significantly decreased compared with that in the DM group (P < 0.01). As shown in Figure 3B, level of miRNA-155 in serum samples in the DM group was significantly increased compared with that in the control group, but level of miRNA-155 in serum samples in the tetrandrine group was significantly decreased compared with that in the DM group (P < 0.01) (Figure 3B). These results indicate that expression of miRNA-155 is increased in DM, while tetrandrine decreases expression of miRNA-155.
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control and the tetrandrine groups, but activities of HbA1c, glucose, MDA, and ROS in the DM group were significantly higher than those in the control and the tetrandrine groups. Activity of SOD in pancreatic islet mitochondria tissues in the DM group was significantly lower than that in the control and the tetrandrine groups, but activities of MDA and ROS in the DM group were significantly higher than those in the control and the tetrandrine group. All these results indicate that alloxan inhibits secretion of insulin in rat pancreatic islet β cells.

In this study, alloxan decreased the mRNA and protein levels of IkBα in pancreatic islet tissues, but increased the mRNA and protein levels of TNF-α in pancreatic islet tissues, indicating that mechanism underlying the damaging effect of alloxan on pancreatic islet tissue may be related with activation of NF-κB signaling pathway.

As one target gene of miR-155, TNF-α involves in activation of NF-κB signaling pathway. In the present study, expression of TNF-α in pancreatic islet tissues was increased with the up-regulation of miRNA-155. Expression of miRNA-155 in serum samples was increased with progression of DM, indicating that miRNA-155 may have potential significance in diagnosis of DM.

Tetrandrine scavenges ROS and protects cyto-membrane of pancreatic islet β cells. Moreover, tetrandrine reduces Ca2+ overload, prevents accumulation of superoxide, and suppresses apoptosis of pancreatic islet cells [19, 20]. In the present study, results showed that tetrandrine down-regulated expression of miRNA-155, and decreased the mRNA and protein levels of TNF-α. Finally, tetrandrine influenced activity of NF-κB signaling pathway to regulate apoptosis of pancreatic islet cells. All these results indicate that there is a relationship between NF-κB, TNF-α and miRNA-155 in DM. Tetrandrine may inhibit activation of NF-κB signaling pathway by regulating expression of miR-155 in DM.

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Disclosure of conflict of interest

None.

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References

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