Protective effect of trimetazidine on myocardial ischemia reperfusion injury in ovariectomized rats and the effect on Fas and FasL gene expression

Bo Zhao, Wei Zhang
Cardiovascular Medicine Department, Yan’an University Affiliated Hospital, Yan’an 716000, China

ARTICLE INFO
Article history:
Received 7 Nov 2016
Received in revised form 17 Nov 2016
Accepted 12 Nov 2016
Available online 24 Nov 2016

Keywords:
Ischemia reperfusion injury
Trimetazidine
Fas
FasL
Autophagy

ABSTRACT
Objective: To study the protective effect of trimetazidine on myocardial ischemia reperfusion injury in ovariectomized rats and the effect on Fas and FasL gene expression. Methods: Female Wistar rats were selected and divided into ovariectomized model group (OVX group), ovariectomized + ischemia reperfusion model group (OVX+I/R group) and ovariectomized + ischemia reperfusion + trimetazidine intervention group (OVX+I/R+TMZ group) (n=8), and the content of serum myocardial enzymes and structural proteins, hemodynamic indexes and the expression levels of apoptotic molecules and autophagy genes in myocardial tissue were determined. Results: Serum creatine kinase (CK) and isoenzyme (CK-MB), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (α-HBDH) content, troponin I (cTnI) and troponin T (cTnT) content as well as LVEDP level of OVX+I/R group were significantly higher than those of OVX group (P<0.05) while LVSP, +dp/dtmax and −dp/dtmax levels were significantly lower than those of OVX group (P<0.05), and serum CK, CK-MB, LDH, α-HBDH, cTnI and cTnT content as well as LVEDP level of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group (P<0.05) while LVSP, +dp/dtmax and −dp/dtmax levels were significantly higher than those of OVX+I/R group (P<0.05). Fas, FasL, Beclin-1 and P62 expression levels as well as LC3II/LC3I ratio in myocardial tissue of OVX+I/R group were significantly higher than those of OVX group (P<0.05), and Fas, FasL, Beclin-1 and P62 expression levels as well as LC3II/LC3I ratio in myocardial tissue of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group (P<0.05). Conclusions: Trimetazidine has protective effect on myocardial ischemia reperfusion injury in ovariectomized rats, and inhibiting cell apoptosis and cell autophagy mediated by Fas/FasL is the molecular mechanism for trimetazidine to play the protective role.

1. Introduction
Postmenopausal women are the high risk group of coronary atherosclerotic heart disease, postmenopausal ovarian function declines and estrogen levels decrease significantly in the body, which weaken the myocardial protective effect and gluco lipid metabolism regulation effect of estrogen. Existing epidemiological data have shown that postmenopausal women are with significantly increased risk of myocardial infarction[1,2]. The pathological characteristics of myocardial infarction are coronary artery lumen occlusion, blood flow interruption and ischemic-hypoxic myocyte injury, and although the timely recovery of coronary perfusion could alleviate the myocardial injury caused by hypoxia, coronary artery reperfusion will increase myocardial injury and affect disease outcome by ischemia-reperfusion injury (IRI)[3,4]. Therefore, preventing IRI during restoring coronary perfusion is an important link to protect the myocardial cells and improve the prognosis of disease. Trimetazidine is the drug with anti-ischemic, anti-inflammatory and antioxidant effect, it can alleviate myocardial ischemia-reperfusion injury[5], but there is no clear report about the effect of trimetazidine in the treatment of ischemia-reperfusion...
injury in postmenopausal women. In the following study, the protective effect of trimetazidine on myocardial ischemia reperfusion injury in ovariectomized rats and the effect on Fas and FasL gene expression were analyzed.

2. Materials and methods

2.1. Experimental animals

Experimental animals were 24 adult female Wistar rats with the body mass of 200–250 g, they were purchased and raised by laboratory animal center of Yan’an University, and the animal license was SYXK (Shaan) 2012-0081. After approved by the ethics committee of Yan'an University affiliated hospital, the Wistar rats were randomly divided into ovariectomized model group (OVX group), ovariectomized + ischemia reperfusion model group (OVX+I/R group) and ovariectomized + ischemia reperfusion + trimetazidine intervention group (OVX+I/R+TMZ group), 8 animals in each group.

2.2. Experimental materials

Trimetazidine was purchased from Sigma Company, animal tissue RNA extraction, cDNA synthesis and fluorescence quantitative PCR kits were bought from Promega Company, and Fas, FasL, LC3, Beclin-1 and P62 monoclonal antibody were purchased from Santa-cruz Company. Small animal ventilator and multi-channel biological signal acquisition system were from Chengdu Techman Technology Company, and both fluorescence quantitative PCR apparatus and Image-Pro Plus image analysis system were from Bio-rad Company.

2.3. Experimental methods

2.3.1. Modeling and intervention methods

OVX group, OVX+I/R group and OVX+I/R+TMZ group all received ovariectomized surgery, and laparotomy was conducted after intraperitoneal anesthesia to separate bilateral ovaries and completely remove the ovaries and surrounding adipose tissue. Ischemia reperfusion models were established after 10 d, and the ischemia reperfusion models of OVX+I/R+TMZ group was established according to the following method: after intraperitoneal anesthesia, endotracheal intubation was conducted and small animal ventilator was connected for mechanical ventilation, then the thoracotomy was conducted between the left 3rd and 4th rib to reveal the heart, then separate left anterior descending coronary artery, ligature the coronary artery with No. 0 thread in 2–3 mm under the left auricle and tie a slipknot, ischemia time was 30 min and then the reperfusion time was 120 min; OVX group only received left anterior descending coronary artery separation and threading without ligation. OVX+I/R+TMZ group received intraperitoneal injection of trimetazidine 10 mg/kg before thoracotomy, and the OVX group and OVX+I/R group received intraperitoneal injection of same dosage of saline.

2.3.2. Hemodynamics evaluation methods

After 120 min of reperfusion, the right common carotid artery was separated, the catheter with inner diameter of 1 mm was inserted into the left ventricle through common carotid artery, the multi-channel biometric signal acquisition system was connected, and then the left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum left ventricular pressure-rising rate (+dp/dtmax) and maximum left ventricular pressure-dropping rate (−dp/dtmax) were measured.

2.3.3. Serum index evaluation methods

Rats were executed after hemodynamic indexes were determined, 10ml of peripheral blood was collected and centrifuged to separate serum, then automatic biochemical analyzer was used to determine creatine kinase (CK) and isoenzyme (CK-MB), lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) content, and enzyme-linked immunosorbent assay kit was used to detect troponin I (cTnI) and troponin T (cTnT).

2.3.4. Detection methods of molecule expression in myocardium

After the rats were executed, proper amount of myocardial tissue was collected, frozen with liquid nitrogen and then divided into two. One was added in Trizol lysis buffer to extract total RNA, cDNA synthesis kit was used to reverse-transcribe the RNA into cNDA, then fluorescence quantitative PCR amplification was conducted, the amplified genes included Fas, FasL, Beclin-1 and P62, reference gene was β-actin, and the target gene mRNA expression levels were calculated according to the amplification curve; the other was added in RIPA protein lysis buffer to extract total protein, western-blot method was used to obtain the Fas, FasL, Beclin-1 and P62, protein bands, and the Fas, FasL, Beclin-1, P62, LC3II and LC3I protein content as well as LC3II/LC3I ratio were calculated.

2.4. Statistical analysis

SPSS20.0 software was used to input and analyze data, measurement data analysis was by variance analysis and P<0.05 indicated statistical significance in differences.

3. Results

3.1. Serum myocardial enzymes and myocardial structural proteins of rats
Analysis of serum myocardial enzymes CK, CK-MB, LDH and $\alpha$-HBDH of three groups of rats is as follows: serum CK, CK-MB, LDH and $\alpha$-HBDH content of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and serum CK, CK-MB, LDH and $\alpha$-HBDH content of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$); analysis of serum myocardial structural proteins cTnI and cTnT content of three groups of rats is as follows: serum cTnI and cTnT content of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and serum cTnI and cTnT content of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$) (Table 1).

### 3.2. Hemodynamic indexes of rats

Analysis of hemodynamic parameters LVSP, LVEDP, $+dp/dt_{\text{max}}$ and $-dp/dt_{\text{max}}$ of three groups of rats is as follows: LVSP, $+dp/dt_{\text{max}}$ and $-dp/dt_{\text{max}}$ of OVX+I/R group were significantly lower than those of OVX group ($P<0.05$) while LVEDP was significantly higher than that of OVX group ($P<0.05$); LVSP, $+dp/dt_{\text{max}}$ and $-dp/dt_{\text{max}}$ of OVX+I/R+TMZ group were significantly higher than those of OVX+I/R group ($P<0.05$) while LVEDP was significantly lower than that of OVX+I/R group ($P<0.05$) (Table 2).

### 3.3. Fas and FasL expression levels in myocardial tissue of rats

Analysis of Fas and FasL mRNA expression levels in myocardial tissue of three groups of rats is as follows: Fas and FasL mRNA expression levels in myocardial tissue of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and Fas and FasL mRNA expression levels in myocardial tissue of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$); analysis of Fas and FasL protein expression levels in myocardial tissue of three groups of rats is as follows: Fas and FasL protein expression levels in myocardial tissue of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and Fas and FasL protein expression levels in myocardial tissue of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$) (Table 3).

### 3.4. Expression levels of autophagy genes in myocardial tissue of rats

Analysis of autophagy gene mRNA expression levels in myocardial tissue of three groups of rats is as follows: Beclin-1 and P62 mRNA expression levels in myocardial tissue of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and Beclin-1 and P62 mRNA expression levels in myocardial tissue of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$) (Table 3).

---

### Table 1
Comparison of serum myocardial enzyme and myocardial structural protein content among three groups of rats ($n=8$, $\overline{x} \pm s$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Myocardial enzymes (kU/L)</th>
<th>Structural proteins (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK</td>
<td>CK-MB</td>
</tr>
<tr>
<td>OVX</td>
<td>0.43±0.06</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>OVX+I/R</td>
<td>3.75±0.61</td>
<td>1.89±0.23</td>
</tr>
<tr>
<td>OVX+I/R+TMZ</td>
<td>1.24±0.15</td>
<td>0.68±0.07</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

### Table 2
Comparison of hemodynamic parameters among three groups of rats ($n=8$, $\overline{x} \pm s$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>$+dp/dt_{\text{max}}$ (mmHg/s)</th>
<th>$-dp/dt_{\text{max}}$ (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>214.5±29.5</td>
<td>17.4±2.5</td>
<td>11.453±2±1425.6</td>
<td>9.465±7±1103.5</td>
</tr>
<tr>
<td>OVX+I/R</td>
<td>139.8±17.4</td>
<td>38.6±5.7</td>
<td>7.135±6±889.5</td>
<td>6.013±5±768.9</td>
</tr>
<tr>
<td>OVX+I/R+TMZ</td>
<td>179.3±22.1</td>
<td>25.2±3.6</td>
<td>9.586±4±1104.8</td>
<td>7.871±4±935.6</td>
</tr>
<tr>
<td>$F$</td>
<td>7.598</td>
<td>11.384</td>
<td>6.698</td>
<td>7.793</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

### Table 3
Comparison of Fas and FasL expression levels in myocardial tissue among three groups of rats ($n=8$, $\overline{x} \pm s$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>mRNA expression levels</th>
<th>Protein expression levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fas</td>
<td>FasL</td>
</tr>
<tr>
<td>OVX</td>
<td>1.04±0.16</td>
<td>1.09±0.13</td>
</tr>
<tr>
<td>OVX+I/R</td>
<td>2.89±0.36</td>
<td>3.42±0.48</td>
</tr>
<tr>
<td>OVX+I/R+TMZ</td>
<td>1.56±0.22</td>
<td>1.44±0.18</td>
</tr>
<tr>
<td>$F$</td>
<td>9.393</td>
<td>13.857</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$); analysis of autophagy gene protein expression levels in myocardial tissue of three groups of rats is as follows: Beclin-1 and P62 protein expression levels as well as LC3II/LC3I ratio in myocardial tissue of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and Beclin-1 and P62 protein expression levels as well as LC3II/LC3I ratio in myocardial tissue of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$) (Table 4).

4. Discussion

Estrogen has a clear myocardial protective effect, the ovarian function declines and the estrogen levels decrease rapidly in postmenopausal women. As the myocardium loses the protection from the estrogen, the incidence of a variety of cardiovascular events rises significantly. Epidemiological data have shown that the incidence of cardiovascular disease among postmenopausal women is 7 times of the premenopausal, and the risk of myocardial infarction, in particular, increases by 40 times[6,7]. Although thrombolytic and interventional therapy can restore coronary perfusion in time, ischemia reperfusion injury produced after coronary artery reperfusion can aggravate myocardial injury and affect the disease outcome[8,9]. Therefore, preventing IRI during restoring coronary perfusion is an important link to protect the myocardial cells and improve the prognosis of disease. Trimetazidine is a drug that can inhibit inflammation and oxidative stress reaction, and improve the energy metabolism. The drug can inhibit the activity of mitochondrial enzyme-long chain 3-ketoacyl-CoA thiolase to inhibit fatty acid β oxidation, which further increases the glucose oxidation function and improves myocardial ischemia; meantime, trimetazidine can also reduce the uncoupling of glucose glycolysis and oxidation and reduce the production of oxygen free radicals and inflammatory mediators, which protect the ischemic myocardium and ischemia-reperfusion myocardium[10,11]. Animal research report has shown that trimetazidine can alleviate myocardial ischemia-reperfusion injury[12], but there is no clear report about the protective effect of trimetazidine on myocardial ischemia-reperfusion among postmenopausal women.

In the study, ovariectomized animal models were used to simulate the condition of postmenopausal ovarian function decline, and then the coronary artery was ligatured and opened to simulate the pathological process of ischemic reperfusion injury after myocardial infarction. In order to define the protective effect of trimetazidine on myocardial ischemia reperfusion injury in ovariectomized rats, the myocardial enzymes and myocardial structural proteins released into the blood circulation were analyzed in the study at first, and the results showed that serum CK, CK-MB, LDH, α-HBDH, cTnI and cTnT content of OVX+I/R group were significantly higher than those of OVX group ($P<0.05$), and serum CK, CK-MB, LDH, α-HBDH, cTnI and cTnT content of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group ($P<0.05$). It means that ischemia-reperfusion injury can cause the myocardial cell rupture in ovariectomized rats as well as the release of myocardial enzymes and myocardial structural proteins in the cytoplasm into the blood circulation; trimetazidine intervention could alleviate ischemia-reperfusion damage to the myocardial cells in ovariectomized rats. The hemodynamic indexes were further analyzed to reflect the cardiac systolic and diastolic function as well as pumping function, and it showed that LVSP, +dp/dtmax and -dp/dtmax of OVX+I/R group were significantly lower than those of OVX group ($P<0.05$) while LVEDP was significantly higher than that of OVX group ($P<0.05$); LVSP, +dp/dtmax and -dp/dtmax of OVX+I/R+TMZ group were significantly higher than those of OVX+I/R group ($P<0.05$) while LVEDP was significantly lower than that of OVX+I/R group ($P<0.05$). It means that ischemia-reperfusion injury will affect the myocardial diastolic and systolic function as well as cardiac pumping function of ovariectomized rats, and trimetazidine intervention could alleviate ischemia-reperfusion damage to the cardiac function of ovariectomized rats.

Cell apoptosis is an important part of the myocardial cell injury caused by ischemia reperfusion, postmenopausal women have lost the cellular protective effects of estrogen, and ischemia-reperfusion injury can aggravate the cell apoptosis. Fas is a member of the tumor necrosis factor receptor superfamily, and the combination with its ligand FasL can activate the apoptosis of death receptor pathway, transduce apoptotic signal via multiple molecules in downstream caspase family and ultimately cause cell apoptosis[13,14]. In the study, analysis of Fas and FasL expression levels in myocardial tissue of ovariectomized rats proved that Fas and FasL expression levels in myocardial tissue of OVX+I/R group were significantly higher

<table>
<thead>
<tr>
<th>Groups</th>
<th>mRNA expression levels</th>
<th>Protein expression levels</th>
<th>LC3II/LC3I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beclin-1</td>
<td>P62</td>
<td>Beclin-1</td>
</tr>
<tr>
<td>OVX</td>
<td>1.09±0.17</td>
<td>1.04±0.13</td>
<td>1.02±0.15</td>
</tr>
<tr>
<td>OVX+I/R</td>
<td>2.89±0.34</td>
<td>3.35±0.51</td>
<td>2.37±0.29</td>
</tr>
<tr>
<td>OVX+I/R+TMZ</td>
<td>1.63±0.20</td>
<td>1.49±0.18</td>
<td>1.52±0.18</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
than those of OVX group (P<0.05), and Fas and FasL expression levels in myocardial tissue of OVX+I/R+TMZ group were significantly lower than those of OVX+I/R group (P<0.05). This means that ischemia-reperfusion injury will cause myocardial cell apoptosis in ovariecetomized rats through Fas/FasL pathways, and trimetazidine intervention can inhibit the myocardial cell apoptosis in ovariecetomized rats caused by ischemia-reperfusion.

Autophagy is a pathological link that is newly discovered in recent years and closely related to I/R injury, and cell autophagy continues in ischemia-reperfusion process. Autophagic vacuole formed by phagocytosis and packaging can only complete the full autophagy flux after mutual combination with lysosomes, and ischemia-reperfusion can destroy the integrity of autophagy flux and cause cellular damage[15]. LC3 is a marker protein of autophagy process, and autophagy activation is characterized by LC3II/LC3I balance shift to LC3II as well as increased LC3II/LC3I ratio. Beclin-1 is the key protein to induce autophagy, and can make P62 protein combined with ubiquitinated protein and then form complexes with LC3-II on autophagosome intima and complete the autophagy flux after mutual combination with lysosomes, and ischemia-reperfusion injury will add to the myocardial cell autophagy in ovariecetomized rats, and trimetazidine intervention can reduce to the myocardial cell autophagy in ovariecetomized rats caused by ischemia-reperfusion.

In conclusion, trimetazidine has protective effect on myocardial ischemia reperfusion injury in ovariecetomized rats, and inhibiting cell apoptosis and cell autophagy mediated by Fas/FasL is the molecular mechanism for trimetazidine to play the protective role.

References


