Original Article

Association between collagen cross-linking and cardiac function and remodeling in rats with furazolidone-induced dilated cardiomyopathy

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Abstract: Objective: To explore the association between collagen cross-linking (CCL) and cardiac function in rats with furazolidone induced dilated cardiomyopathy (DCM). Methods and results: Forty 2-week-old Sprague-Dawlay (SD) rats were used, 30 rats were treated with furazolidone (0.15 mg/body weight (g)/day per gavage for 8 weeks, FZ group), 10 rats were treated by equal volume water daily per gavage for 8 weeks (Control group, CON). After 8 weeks, survival rate was 100% in CON group and 73.33% in FZ group (22/30, \( P < 0.05 \)). Echocardiography revealed that both left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were significantly reduced, while left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) were significantly increased (both \( P < 0.001 \)). Body weight was significantly decreased while heart weight/body weight ratio was significantly increased in FZ group (both \( P < 0.05 \)). Collagen volume fraction (16.39±2.06% versus 2.78±1.10%, \( P < 0.001 \)) and the degree of CCL (6.13±0.96 versus 5.18±0.12, \( P < 0.05 \)) were significantly increased in FZ group compared with CON group. mRNA expression of myocardial collagen I, collagen III, N2B and LOX were significantly upregulated, while mRNA expression of titin and N2BA were significantly downregulated in FZ group compared with CON group (all \( P < 0.05 \)). Moreover, CCL was negatively correlated with LVEF and LVFS while positively correlated with LVEDD and LVESD in FZ group. Conclusion: Increased collagen cross-linking is linked with reduced left ventricular dysfunction and aggravated left ventricular remodeling in this FZ-induced DCM rat model.

Keywords: Dilated cardiomyopathy, furazolidone, collagen remodeling, animal model, rat

Introduction

Non-ischemic dilated cardiomyopathy (DCM), a severe and progressive cardiomyopathy, is characterized by myocardial cell necrosis, interstitial fibrosis, collagen proliferation as the main pathological features, cavity expansion, arrhythmias and congestive heart failure or even sudden death are the main clinical manifestations [1, 2]. Despite modern therapy strategies, the general outcome of DCM patients remains poor and the 5 year mortality remains as high as 20% [3]. Animal models of dilated cardiomyopathy for translational research. Animal models of cardiovascular disease have proved critically important for the discovery of pathophysiological mechanisms and for the advancement of diagnosis and therapy. They offer a number of advantages; principally the availability of adequate healthy controls and the absence of confounding factors such as marked differences in age, concomitant pathologies and pharmacological treatments. Over the past thirty years, investigators have developed numerous small and large animal models to study this very complex syndrome [4]. DCM could be induced in rats either by immunization with porcine cardiac myosin [5, 6] or by oral treatment with doxorubicin [7, 8]. Recently, several reports described the enhanced distribution of calreticulin at cardiomyocyte mitochondria [9, 10], and found that calreticulin-STAT3 signaling pathway modulates mitochondrial function in a rat model of furazolidone-induced...
DCM [11], studies from our group demonstrated the beneficial effects of intramyocardial mesenchymal stem cells and VEGF165 plasmid injection in this furazolidone-induced DCM rat model [12]. Recent clinical study demonstrated that myocardial fibrosis (detected by late gadolinium enhancement cardiovascular magnetic resonance [LGE-CMR] imaging) is an independent and incremental predictor of mortality and sudden cardiac death (SCD) in non-ischemic dilated cardiomyopathy [13]. Previous study also demonstrated that collagen cross-linking (CCL) is associated with elevated filling pressures in hypertensive patients with stage C heart failure, moreover, CCL is positively correlated with insoluble stiff collagen and myocardial expression of lysyl oxidase (LOX) and negatively correlated with ejection fraction (EF), suggesting that LOX-mediated excessive CCL plays an important role in the pathogenesis of heart failure [14]. Present study thus evaluated the association between the quality of collagen (CCL) and cardiac function in the furazolidone-induced DCM rat model.

Materials and methods

Reagents

Furazolidone (C8H7N3O5, MW 225.16, 99%) was purchased from Mongxin pharmaceutical of Chifeng Co, Ltd. (Chifeng city, Inner Mongolia Autonomous Region, China).

Animal model and study protocol

Forty of Sprague-Dawley rats (2 weeks old and 20~30 g), provided by Experimental Animal Center of Dalian Medical University, were randomly divided into two groups. Thirty rats were treated with FZ [43 mg/ml solution, 0.15 mg/BW (g) by gavage] for 8 weeks. Ten rats received equal volume water daily per gavage for 8 weeks (control group, CON). After 8 weeks, survived rats underwent echocardiography examination under light anesthesia (intramuscular ketamine hydrochloride injection, 100 mg/kg), hearts were excised and weighed. Atria and right ventricle were separated from left ventricle, left ventricle was cut into three sections (each 3 mm in thickness) along long axis and processed for histological (middle section), immunohistological (basal section), biochemical analysis and soluble collagen determination (apical section). For biochemical tests, sample was quick-frozen and for histological analysis the sample was fixed in 4% paraformaldehyde, embedded in paraffin. Basic myocardial histology was evaluated with hematoxylin and eosin (H&E) staining, Masson staining and Sirius Red staining, For histomorphometric examinations, single slide was taken and the total field area for ten randomly selected fields were analyzed in a blinded fashion using Image Analysis System (Media Cybernetics, Inc. (Rockville, MD USA). The collagen volume fraction (CVF = area of the collagen/area of field of vision ×100%) was measured and ten separate areas of high power fields (100×) in each section were visualized under light microscope. Ten sections from each rat were observed and the results were averaged.

Real-time PCR analysis

Total RNA was isolated from 100 mg left ventricular tissue using the High Pure RNA Isolation Kit according to the manufacturer’s instructions. (Takara Bio Inc., Dalian City, China). All
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Table 1. Body and heart weight and echocardiographic measurements

<table>
<thead>
<tr>
<th></th>
<th>CON group</th>
<th>FZ group</th>
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<tbody>
<tr>
<td>BW (g)</td>
<td>228.60±30.78</td>
<td>161.81±17.65*</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>906.00±150.27</td>
<td>756.36±83.93*</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.98±0.58</td>
<td>4.71±0.63*</td>
</tr>
<tr>
<td>LVEDD (cm)</td>
<td>0.50±0.03</td>
<td>0.59±0.08†</td>
</tr>
<tr>
<td>LVESD (cm)</td>
<td>0.23±0.03</td>
<td>0.47±0.09†</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>88.51±3.91</td>
<td>65.69±8.45†</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>53.51±5.92</td>
<td>32.09±5.68†</td>
</tr>
</tbody>
</table>

CVF, collagen volume fraction; CCL, collagen cross-linking. *P<0.001 vs. CON group, †P<0.05 vs. CON group.

Table 2. Histomorphological parameters

<table>
<thead>
<tr>
<th></th>
<th>CON group</th>
<th>FZ group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVF (%)</td>
<td>2.78±1.10</td>
<td>16.39±2.06*</td>
</tr>
<tr>
<td>Insoluble collagen (μg/mg)</td>
<td>1.28±0.10</td>
<td>2.32±0.26*</td>
</tr>
<tr>
<td>Soluble collagen (μg/mg)</td>
<td>0.26±0.07</td>
<td>0.38±0.03*</td>
</tr>
<tr>
<td>CCL (%)</td>
<td>5.19±0.12</td>
<td>6.13±0.96†</td>
</tr>
</tbody>
</table>

CVF, collagen volume fraction; CCL, collagen cross-linking. *P<0.001 vs. CON group, †P<0.05 vs. CON group.

The primer sequence designed and synthesized by (Beijing AuGCT Biotechnology Co. Ltd. Beijing City, China) as follow, primers specific for rat collagen type I (5'-TGC CGT GAC CTC ATG TG-3' and 5'-CAC AAG CGT GTA GGT GA-3'), collagen type III (5'-CTGGAC CAA AAG GTG ATG CGT-3' and 5'-TGC CAG GGA ATC CTC GATGTC-3'), Titin (5'-AAA CCA GAA GAG CCA CCA CC-3' and 5'-TTT GGC TTT GGT TCA GGT CC-3'), N2BA (5'-GTC GTT GAA GAG CCA GT-3' and 5'-TCT GCC TCC CT TTC TAC AAC GGG-3'), N2B (5'-GAA GTG GAC AAA GAG CAA GT-3' and 5'-GGG TTT TGC CTG-3'), Titin (5'-AAA CCA GAA GAG CCA CCA CC-3' and 5'-TTT GGC TTT GGT TCA GGT CC-3'), collagen I and III primary antibodies (GeneTex, USA; Santa Cruz, USA) were added and incubated in a humidified chamber at 4°C overnight, the slides were treated with monohydrated citrate buffer (pH 6.0) in a water bath for 3 min and cooled at room temperature for 30 min for the antigen retrieval, then incubated with inactivated enzyme reagent for 15 min at room temperature, sealing fluid in a humidified chamber at room temperature for 30 min. Each slice was dried at room temperature for 40 min, and then incubated with HRP secondary antibody at room temperature for 30 min, washed three times; brown chromogenic granules were visualized with DAB. Finally, sections were counterstained with hematoxylin for 5 min followed by dehydration and coverslip mounting. Ten separate areas of high power fields (×200) in each section were visualized under light microscopy, the average optical density value of positive zone was determined.

Insoluble collagen and soluble collage analyses

The degree of CCL was calculated as the ratio between the insoluble collagen (insCol) and soluble collagen (solCol) = total collagen-soluble collagen) and soluble collagen. Briefly, LV tissue (middle section) was embedded paraffin and cut into 5 μm sections. Then the total collagen was detected with Sirius Red/Fast Green Collagen Staining Kit (Chondrex, Inc, NE Redmond, WA) according to the manufacturer's instructions. Then, the eluted dye solution was collected and 200 μl of which was injected into 96 orifice plate, and OD val-

Collagen I and III immunostaining analyses

Myocardial expression of collagen I and III was examined with Instant Immunohistochemistry Kit I (Sangon Biotech, Shanghai, China). LV (basal section) were fixed with formalin, embedded with paraffin and cut into 5 μm sections. After incubation the sections for 5 min with ddH$_2$O$_2$, collagen I and III primary antibodies were added and incubated in a humidified chamber at 4°C overnight, the slides were treated with monohydrated citrate buffer (pH 6.0) in a water bath for 3 min and cooled at room temperature for 30 min for the antigen retrieval, then incubated with inactivated enzyme reagent for 15 min at room temperature, sealing fluid in a humidified chamber at room temperature for 30 min. Each slice was dried at room temperature for 40 min, and then incubated with HRP secondary antibody at room temperature for 30 min, washed three times; brown chromogenic granules were visualized with DAB. Finally, sections were counterstained with hematoxylin for 5 min followed by dehydration and coverslip mounting. Ten separate areas of high power fields (×200) in each section were visualized under light microscopy, the average optical density value of positive zone was determined.
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Figure 1. Representative examples of HE staining (magnification ×100) in CON group (A), FZ group (B). Representative examples of Masson staining (magnification ×100) in CON group (C), FZ group (D). Representative examples of Sirius red staining (magnification ×100) in CON group (E), FZ group (F).

Figure 2. Representative examples of I collagen protein expression (magnification ×100) in CON group (A), FZ group (B). Representative examples of III collagen protein expression (magnification ×100) in CON group (C), FZ group (D).
Collagen cross-linking and cardiac function in DCM rats


ues at 540 nm and 605 nm was obtained with a spectrophotometer. Total collagen (μg/section) = [OD 540 value - (OD 605 value × 0.291)]/0.0378. Soluble collagen was detected with Sircol Soluble Collagen Assay (Biocolor Ltd, Northern Ireland, U.K.) according to the manufacturer's instructions. For tissue samples, the Acid-Pepsin (0.1 mg Pepsin: 1 ml 0.5M Acid) Extraction procedure was performed and samples were incubated overnight at 4°C. 200 μl sample was transferred to individual wells of a 96 micro well plate, and microplate reader was set to 555 nm, the sircol soluble collagen value was calculated according to standard curve.

Statistical analysis

Data (mean ± SD) were analyzed by one-way or two-way ANOVA followed by Bonferroni's post-hoc comparisons with the SPSS statistical program (Statistical Product and Service Solutions); IBM (International Business Machines Corporation), (Armonk, NY, USA). A p value < 0.05 was considered as statistically significant.

Results

Survival rate and echocardiographic results on survived rats

Twenty-two out of thirty rats (73.3%) survived at 8 weeks in FZ group, and 10 rats survived (100%) at 8 weeks in CON group.

Table 1 shown body weight, heart weight and echocardiographic parameters assessed after 8 weeks of the 2 groups. LVEDD and LVESD were significantly higher while LVEF and LVFS were significantly lower in FZ group than that in CON group. Heart weights were lower and HW/BW ratios were higher in FZ group than in CON group.

Histological analysis

As shown in Table 2, myocardial CVF, insoluble and soluble collagen as well as CCL were all significantly increased in FZ group compared to CON group.

Figure 1 shown representative HE staining (Figure 1A and 1B), Masson staining (Figure 1C and 1D) and Sirius red staining (Figure 1E and 1F) results in CON group and FZ group. HE staining showed that myocardial fibers were dyed uniform and neat. The nucleus structure of cardiomyocytes was clear, size was uniform, only a small amount of collagen fibers in heart muscle fibers or around the blood vessels was detected in CON group (Figure 1A). In FZ group, the nucleus of cardiomyocytes was enlarged, and myocardial collagen fiber was increased and presented in disordered form (Figure 1B). Masson staining demonstrated that there were visible neat rows of dyed red myocardial fibers and a small amount of collagen fibers around the blood vessels in CON group (Figure 1C), while a large number of collagen fibers could be detected in disordered form in FZ group (Figure 1D). Sirius red staining showed that the arrangement of myocardial fibers was neat, a small amount of scarlet collagen fibers was found around myocardial interstitial or blood vessels in CON group (Figure 1E). The content of collagen fiber increased, which was widely distributed in the myocardial interstitial in disordered arrangement in FZ group (Figure 1F).

Figure 2 showed myocardial Collagen I, Collagen III expression in myocardial tissue of rats

Table 3. Myocardial mRNA expressions

<table>
<thead>
<tr>
<th></th>
<th>Col I</th>
<th>Col III</th>
<th>Titin</th>
<th>N2B</th>
<th>N2BA</th>
<th>LOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON group</td>
<td>1.01±0.17</td>
<td>1.01±0.16</td>
<td>1.01±0.17</td>
<td>1.02±0.24</td>
<td>1.03±0.32</td>
<td>1.00±0.06</td>
</tr>
<tr>
<td>FZ group</td>
<td>2.57±0.63*</td>
<td>1.45±0.34*</td>
<td>0.50±0.13*</td>
<td>2.56±0.57*</td>
<td>0.41±0.05*</td>
<td>1.78±0.79*</td>
</tr>
</tbody>
</table>

*Col I, collagen I; Col III, collagen III; LOX, lysyl oxidase. *P<0.05 vs. CON group.

Table 4. N2BA/N2B isof orm rate

<table>
<thead>
<tr>
<th></th>
<th>N2B</th>
<th>N2BA</th>
<th>N2BA/N2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON group</td>
<td>1.02±0.24</td>
<td>1.03±0.32</td>
<td>0.99±0.02</td>
</tr>
<tr>
<td>FZ group</td>
<td>2.56±0.57*</td>
<td>0.41±0.05*</td>
<td>0.16±0.03*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. CON group.
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in CON group and FZ group. Few brown stained collagen I (Figure 2A) and collagen III (Figure 2C) were detected in the myocardial interstitial cells in CON group. Increased collagen I (Figure 2B) and collagen III (Figure 2D) were detected around the blood vessels and in the myocardial interstitial cells in FZ group. Both collagens I and III expressions were significantly increased in FZ group compared to CON group (Figure 2E).

The mRNA expression of collagen I, collagen III, Titin, N2B, N2BA and LOX was detected by Real-time PCR. 2$^{-\Delta\Delta C_t}$ was applied. Compared with CON group, the mRNA expressions of collagen I, collagen III, N2B, LOX were significantly upregulated while the mRNA expressions of Titin and N2BA were significantly downregulated in FZ group (Table 3). Table 4 and Figure 3 demonstrated reduced N2BA/N2B isofrom rate in FZ group. Thus, the myocardium might be become stiffer in FZ group.

Analysis of associations
Correlations of CCL and myocardial mRNA expressions and echocardiographic parameters in FZ group are presented in Table 5. CCL, collagen I and collagen III, N2B and LOX mRNA expressions were negatively correlated with LVEF and LVFS and positively correlated with LVEDD and LVESD while N2BA and titin mRNA expressions were positively correlated with LVEF and LVFS and negatively correlated with LVEDD and LVESD.

Discussion
The DCM animal model in rat induced by FZ imitated the characteristic performance of DCM in human including enlarged LVEDd and LVESd, reduced LVEF and LVFS, above changes are joined by increased heart weight/body weight ratio and increased myocardial collagen expressions including increased CCL in FZ rats. Moreover, increased CCL is linked with reduced cardiac function and aggravated cardiac remodeling in this DCM model.

To our knowledge, that is the first report describing the association between CCL and cardiac function and remodeling in this model. Previous study [14] found that it is the degree of CCL and the abundance of insCol but not the amount of total or type I collagen fibers that associate with LV chamber stiffness (i.e., LV chamber stiffness constant) and FPs (i.e., PCWP) in patients with HHD and stage C HF. In line with above report, we found that the increase of CCL is negatively correlated with LVEF and LVFS, suggesting that the qualitative alterations of the myocardial collagen matrix might have a functional impact in FZ-induced DCM rats. It is shown that CCL is positively correlated with the expression of lysyl oxidase (LOX) [14], and expression of LOX is markedly increased in fibrotic tissues, including models of dermal, lung, liver, and arterial fibrosis [15]. Similarly, the myocardial LOX expression was significantly upregulated in FZ rats, supporting the hypothesis that LOX-mediated excessive CCL might

Table 5. Correlations of CCL and myocardial mRNA expressions echocardiographic parameters in FZ group

<table>
<thead>
<tr>
<th></th>
<th>CCL</th>
<th>InsCol</th>
<th>Col I</th>
<th>Col III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>LVEF</td>
<td>-0.76</td>
<td>&lt;0.05</td>
<td>-0.43</td>
<td>NS</td>
</tr>
<tr>
<td>LVEDD</td>
<td>0.66</td>
<td>&lt;0.05</td>
<td>0.18</td>
<td>NS</td>
</tr>
<tr>
<td>LVESD</td>
<td>0.74</td>
<td>&lt;0.05</td>
<td>0.29</td>
<td>NS</td>
</tr>
<tr>
<td>LVFS</td>
<td>-0.77</td>
<td>&lt;0.05</td>
<td>-0.43</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N2B</th>
<th>N2BA</th>
<th>Titin</th>
<th>LOX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>LVEF</td>
<td>-0.74</td>
<td>&lt;0.05</td>
<td>0.71</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVEDD</td>
<td>0.52</td>
<td>&lt;0.05</td>
<td>-0.70</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVESD</td>
<td>0.64</td>
<td>&lt;0.05</td>
<td>-0.75</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVFS</td>
<td>-0.74</td>
<td>&lt;0.05</td>
<td>0.71</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

CCL, collagen cross-linking; InsCol, insoluble collagen; Col I, collagen I; Col III, collagen III; LOX, lysyl oxidase; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVFS, left ventricular fraction shortening. NS, not significant.

Figure 3. LV titin isoforms in CON group and FZ group. Figure 3 shown N2BA/N2B isoform rate was reduced in FZ group. *P=0.046. *P<0.05 vs. CON group.
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Facilitate LV dysfunction and remodeling in this FZ-induced DCM model.

CCL is only one of the determinants of collagen mechanics [16]. Properties such as collagen fiber diameter and the ratio of collagen I to III all contribute to the mechanical behavior at the tissue level [17, 18]. Tissues with predominance of Col I (thick collagen fibers) are characterized by strength and stiffness, whereas tissues containing large amounts of Col III (thin collagen fibers) are characterized by greater elasticity. Because of their different physical properties, the altered Col I/Col III ratio may therefore have a major impact on the diastolic and systolic function of the heart [19]. Our results revealed increased myocardial fibrosis (increased CVF) and myocardial mRNA expressions of Col I and Col III in FZ rats. Since myocardial fibrosis is a known detrimental determinant of LV function and remodeling evolution [20, 21], increased myocardial fibrosis might be responsible for the FZ-induced LV dysfunction and aggravated remodeling in this model [21].

In 1954, Huxley and Hanson found a third type of filament in sarcomere. Since then, people recognized that the sarcomere consists of thick filament, thin filament, and titin. Titin is a giant sarcomeric protein that extends from the Z disk to the M-line and is encoded by a single gene [22]. Its differential splicing leads in the myocardium to the expression of N2B and N2BA isoforms that differ in size [22]. A key difference is that the N2B isoform is stiffer than the N2BA isoform, so that it is not surprising that N2B tends to predominate in stiffer ventricles, whereas N2BA occurs in more compliant hearts [23]. Titin-transcript expression was studied previously in a guinea-pig model of hypertrophic cardiomyopathy, demonstrating increased titin-mRNA transcripts in compensated hypertrophy but declining mRNA-levels in the transition to decompensated CHF [24]. An increased content of the less stiff N2BA isoform has been reported in human dilated cardiomyopathy [25]. By RT-PCR, we found that the total-titin and N2BA-transcript expression were reduced in FZ hearts compared with control hearts, N2B-transcript expression was increased compared with control hearts. Previous studies found that a 47% reduction in total-titin mRNA levels in human DCM compared with control hearts, but no differences in N2B, N2BA transcripts [26]. Future studies are needed to verify the divergent finding in N2B and N2BA between human DCM and FZ-induced rat DCM model.

In conclusion, FZ-induced DCM rat model might be a useful tool to explore the pathological mechanisms and new therapeutic strategies of DCM. Myocardial collagen remodeling, especially increased CCL in this model is significantly correlated with LV dysfunction and remodeling.

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Disclosure of conflict of interest

None.

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